

Editorial

I. Robuffo, E. Toniato, L. Tettamanti, F. Mastrangelo, G. Ronconi, I. Frydas, Al. Caraffa, S.K. Kritas and P. Conti. Mast cell in innate immunity mediated by proinflammatory and antiinflammatory IL-1 family members	837
--	-----

Original articles

L. Jiang, H. Li and N. Zhao. Thymoquinone protects against cobalt chloride-induced neurotoxicity via Nrf2/GCL-regulated glutathione homeostasis	843
M. Dyszkiewicz-Konwińska, A. Bryja, K. Jopek, J. Budna, R. Khozmi, M. Jeseta, D. Bukowska, P. Antosik, M. Bruska, M. Nowicki, M. Zabel and B. Kempisty. Expression of genes responsible for cell morphogenesis involved in differentiation in porcine buccal pouch mucosal cells during long-term primary culture and real-time proliferation <i>in vitro</i>	855
A. Gerges Geagea, M. Rizzo, A. Eid, I. Hajj Hussein, Z. Zgheib, M.N. Zeenny, R. Jurjus, M.L. Uzzo, G.F. Spatola, G. Bonaventura, A. Leone, L. Massaad-Massade and A. Jurjus. Tea catechins induce crosstalk between signaling pathways and stabilize mast cells in ulcerative colitis	865
XH. Wang, YN. Liu, K. Tian, CX. Yao, LM. Li, YQ. Zheng and ZM. Shi. Expression and clinical significance of ARTN and MMP-9 in endometrial carcinoma	879
N.N. Omar, A.H. El-Tawdi, R.F. Tash, Y. Shoukry, N.A. Mahmoud and W. El Bakly. Tumor potential in rat wounds after short- and long-term administration of platelet-rich plasma	889
S. Cantore, A. Ballini, D. De Vito, F.S. Martelli, I. Georgakopoulos, M. Almasri, V. Dibello, V. Altini, G. Farronato, G. Dipalma, D. Farronato and F. Inchingolo. Characterization of human apical papilla-derived stem cells	901
G. Di Spigna, M. Iannone, P. Ladogana, S. Salzano, M. Ventre, B. Covelli, E. De Marinis and L. Postiglione. Human cardiac multipotent adult stem cells in 3D matrix: new approach of tissue engineering in cardiac regeneration post-infarction	911

Letters to the Editor

M. Bednarczyk, M. Muc-Wierżgoń, D. Waniczek, E. Fatyga, K. Klakla, U. Mazurek and J. Wierżgoń. Autophagy-related gene expression in colorectal cancer patients	923
K. Walkiewicz, E. Nowakowska-Zajdel, J. Strzelczyk, S. Dziągiewska-Gęsiak and M. Muc-Wierżgoń. Serum levels of ADAM10, ADAM12, ADAM17 and ADAM28 in colorectal cancer patients	929
S.Y. Liu, L. Zhang, Y. Zhang, Y. Zhen and Y.F. Wu. Bioinformatic analysis of pivotal genes associated with septic shock	935
J. Marti, F. López, I. Gascón and J. Julve. Propolis nasal spray effectively improves recovery from infectious acute rhinitis and common cold symptoms in children: a pilot study	943
M.B. Khawar and N. Sheikh. Alterations in transaminase activity and serum level of leptin and hepcidin induced by high fat diet in albino rats	951
A. Kasperska-Zajac, A. Grzanka, A. Damasiewicz-Bodzek, K. Bieniek, A. Skrzypulec-Frankel, K. Stencel-Gabriel and A. Sikora-Zydek. Elevated plasma IL-8 concentration is related to severity of systemic inflammation in chronic spontaneous urticaria	957
M. Khalid, M. Bilal, D. Hassani, H.M.N. Iqbal and D. Huang. Antimicrobial, antioxidant, cytotoxicity and LC-MS analyses of <i>Aerva javanica</i> : an ethnomedicinally important plant	963
M. Shu, BY. Wang, J. Zhang, CY. Guo and XH. Wang. Analysis of specialized nursing on respiratory functions in thoracotomy patients	971
Q. Wang, J. Wang, D. Gao and J. Li. Inhibition of PAR2 and TRPA1 signals alleviates neuropathic pain evoked by chemotherapeutic bortezomib	977
M.H. Abbasi, S. Fatima, M.B. Khawar, N. Naz, K.A. Mujeib, T. Akhtar and N. Sheikh. Dose-dependent acute phase response of aqueous leaf decoction of <i>Nerium oleander</i> in Wistar rats	985
JK. Li, C. Wang, HD. Gong and HZ. Li. Coagulation in hindbrain membrane meningioma patients treated with different injections using acute hypervolemic hemodilution	991
X. Du, J. Jiao, X. Cheng, L. Wang, K. Li, H. Liu, C. Wang, C. Arena, K. Zuravskva, G. Guglielmi and L. Lo Muzio. Age-related changes of bone mineral density in mandible by quantitative computed tomography	997
Q. Lin, X-Y. Hong, D. Zhang and H-J. Jin. Preoperative evaluation and surgical technique of functional and cosmetic aspects in zygomatic complex fracture patients	1005
A. Goren, J. Shapiro, T. Naccarato, M. Situm, M. Kovacevic, N. Lonky, T. Lotti and J. McCoy. Social selection favours offspring prone to the development of androgenetic alopecia	1013
Y-Z. Deng, L-G. Xu, L. Chen, D. Zhou and Y. Liu. Effectiveness of acupuncture in the management of cervical spondylosis: a meta analysis	1017
I.M. Tahir, T. Iqbal, A. Jamil and M. Saqib. Association of BCL-2 with oxidative stress and total antioxidant status in pediatric acute lymphoblastic leukemia	1023
C.Y. Chen, J. Chen, C.C. Xia, Z.X. Huang and B. Song. T1 mapping combined with Gd-EOB-DTPA-enhanced magnetic resonance imaging in predicting the pathologic grading of hepatocellular carcinoma	1029
T. Jagmag, M. Tirant and T. Lotti. Link between cutaneous infection, stress and depression	1037
H. Mou, L. Yu, X. Zheng, Q. Liao, X. Hou and Y. Wu. p16 gene expression in pancreatic cancer tissue and its importance in diagnosis	1043
A. Goren, T. Naccarato, M. Situm, M. Kovacevic, T. Lotti and J. McCoy. Mechanism of action of minoxidil in the treatment of androgenetic alopecia is likely mediated by mitochondrial adenosine triphosphate synthase-induced stem cell differentiation	1049
ZM. Shi, YN. Li, B. Fu, YF. Shen and LM. Li. Expression profile of eukaryotic translation initiation factor and matrix metalloproteinase 9 in endometrial cancer tissue	1053
T. Akhtar, N. Sheikh, T. Shan and R. Ghazanfar. Tacrolimus induced nephrotoxicity and pulmonary toxicity in Wistar rats	1061
GT. Liu, C. Shen, XH. Ren, L. Yang, YM. Yu, YX. Xiu, RH. Li, L. Jiang, CL. Zhang and YW. Li. Relationship between transmembrane serine protease expression and prognosis of esophageal squamous cell carcinoma	1067
J. Wang, LJ. Ren, XL. Chen, L. Ma, BJ. Chen, SJ. Ran and S. Lu. Quick rehabilitation nursing improves the recovery of colon cancer patients after laparoscopy	1073
G. Vicenti, V. Pesce, D. Bizzoca, V. Nappi, F. Palmiotto, M. Carrozzo and B. Moretti. Perioperative plasmatic presepsin levels in patients undergoing total hip or knee replacement: a preliminary study	1081
V. Musolino, M. Gliozzi, C. Carresi, J. Maiuolo, R. Mollace, F. Bosco, F. Scarano, M. Scicchitano, A. Maretta, E. Palma, M. Iannone, V.M. Morittu, S. Gratteri, C. Muscoli, M. Fini and V. Mollace. Lipid-lowering effect of bergamot polyphenolic fraction: role of pancreatic cholesterol ester hydrolase	1087
G. Varvara, S. Bernardi, T. Cutilli, S. Bianchi, B. Sinjari and M. Piattelli. Anti-inflammatory steroid use in impacted third molar surgery: a systematic review	1095
E. Sinagra, V. Guarnotta, D. Raimondo, F. Mocciano, S. Dolcimascio, C.A. Rizzolo, F. Puccia, N. Maltese, R. Citarrella, M. Messina, M. Spada, G. Tomasello, F. Cappello, A. Leone, F. Rossi, R. Di Mitri, G.C. Morreale, S. Marasà, M. Midiri, F. Midiri and D. Sinagra. Colorectal cancer in patients with type 2 diabetes mellitus: a single-center experience	1101
V. De Iuliis, V. Dadorante, A. Marino, I. Griffo, A. Pennelli, V. Breda, I. Robuffo, S. Ursi, S. Martinotti, S. Caputi and E. Toniato. Cytokine expression profile and blood parameter evaluation of patients undergoing cardiac surgery	1109
P. Ciappetta, S. Taurone, M. Spoleitini and M. Artico. Anteriorly placed tumors to the conus: removal by interradsular window	1115
E. Sinagra, D. Raimondo, G. Pompei, G. Fusco, F. Rossi, G. Tomasello, A. Leone, F. Cappello, G.C. Morreale, F. Midiri, M. Midiri and A.G. Rizzo. Focal active colitis as a predictor of inflammatory bowel disease: results from a single-center experience	1119
R. Celletti, S. Fanali, C.U. Laici, C. Santori, P. Pignatelli and B. Sinjari. Instant loading with intraoral welding technique and Prama implants: a new prosthetic approach	1127
E. Rosato, A. Gigante, M. Liberatori, M. Gasperini, B. Barbano, A. Amoroso, L. Tubani and A. Laviano. Altered diurnal cardiac sympathovagal balance correlates with nutritional status indexes in systemic sclerosis patients	1133
M. Abate and V. Salini. Safety and tolerability of intra-articular hyaluronic acid (Sinovial®/GELSYN-3™) injections in the treatment of knee osteoarthritis	1139
V. Sargentini, D. Collepardo, M. D'Alessandro, G. Petralito, G. Ceccarelli, F. Alessandri, A. Picicchi, A. Angeloni, M. Venditti and A. Bachetoni. Role of biomarkers in adult sepsis and their application for a good laboratory practice: a pilot study	1147

EDITORIAL

MAST CELL IN INNATE IMMUNITY MEDIATED BY PROINFLAMMATORY AND ANTIINFLAMMATORY IL-1 FAMILY MEMBERS

I. ROBUFFO¹, E. TONIATO², L. TETTAMANTI³, F. MASTRANGELO⁴, G. RONCONI⁵,
I. FRYDAS⁶, AL. CARAFFA⁷, S.K. KRITAS⁸ and P. CONTI⁹

¹*Institute of Molecular Genetics, CNR, Sede di Chieti, Italy;* ²*Department of Medical, Oral and Biotechnologic Sciences, "G. D'Annunzio" University, Chieti, Italy;* ³*Department of Medical and Morphological Science, University of Insubria, Varese, Italy;* ⁴*Department of Medical Science and Biotechnology, University of Foggia, Foggia, Italy;* ⁵*UOS Clinica dei Pazienti del Territorio, Policlinico Gemelli, Rome, Italy;* ⁶*Faculty of Parasitology, Aristotle University of Thessaloniki, Macedonia, Greece;* ⁷*Department of Pharmacology, University of Perugia, Perugia, Italy;* ⁸*Department of Microbiology and Infectious Diseases, School of Veterinary Medicine, Aristotle University of Thessaloniki, Macedonia, Greece;* ⁹*Immunology Division, Postgraduate Medical School, University of Chieti, Chieti, Italy*

Received August 25, 2017 - Accepted October 26, 2017

Innate immunity consists of physical and chemical barriers which provide the early defense against infections. Innate immunity orchestrates the defense of the host with cellular and biochemical proteins. Mast cells (MCs) are involved in innate and adaptive immunity and are the first line of defense which generates multiple inflammatory cytokines/chemokines in response to numerous antigens. MC-activated antigen receptor Fc-RI provokes a number of important biochemical pathways with secretion of numerous vasoactive, chemoattractant and inflammatory compounds which participate in allergic and inflammatory diseases. MCs can also be activated by Th1 cytokines and generate *pre-formed* and *de novo* inflammatory mediators, including TNF. IL-37 is an anti-inflammatory cytokine which binds IL-18R-alpha chain and reduces the production of inflammatory IL-1 family members. IL-37 down-regulates innate immunity by inhibiting macrophage response and its accumulation and reduces the cytokines that mediate inflammatory diseases. Here, we discuss the relationship between MCs, innate immunity, and pro-inflammatory and anti-inflammatory cytokines.

Innate and adaptive immunity are important components of the immune system and act differently. The adaptive system is composed of T and B lymphocytes (or T and B cells) which have a very large diversity, elasticity and diverse antigen receptor repertoire. Innate immunity or native immunity is explicated by activated hematopoietic

cells with substantial developmental plasticity and is dedicated to orchestrating the defense of the host even before the infection (1). The innate immune system interacts with and controls adaptive immune responses and consists of cellular and biochemical defense reactions (2). Effector mechanisms of innate immunity include phagocytes, antimicrobial peptides,

Key words: innate immunity, mast cell, macrophages, cytokines, IL-37

Mailing address:

Prof. P. Conti,
Immunology Division,
Postgraduate Medical School,
University of Chieti-Pescara, Chieti, Italy
Tel./Fax: +39 0871 574136
e-mail: pconti@unich.it

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

NK cells, complement system and cytokines which regulate and the innate immunity cells (3).

Macrophages and dendritic cells (DCs) are innate phagocytic cells derived from bone marrow cells, which have many common cell surface receptors, but perform different functions (4). In fact, macrophages express F4/80, CD11b and Fc receptors, have proteolytic and catabolic activities and have the ability to exert phagocytosis by incorporating dead cells and cellular debris. Macrophages and DCs are mediators of the innate and adaptive immune response, exercise a protective activity against pathogens and are involved in remodeling tissues after injury. Macrophages can be distinguished into two subpopulations: M1 which mediate host defense and immunity to tumors; and M2 which are suppressors and regulate wound healing. Inflammatory IL-1 and its inhibitors can mediate macrophage activation syndrome (5).

MCs are derived from hematopoietic stem cells, widely distributed throughout the tissues (6). They interact with environmental antigens and may proliferate after appropriate stimuli. MCs may be protagonists in many innate or acquired immune responses including those mediated by TH1 and TH2 lymphocytes. MCs share many functions with macrophages such as damage-associated molecular patterns (DAMP) and in some cases the presentation of the antigen. They can influence innate immune cell function with the various proteases that allow these cells to increase host resistance to parasites and recruit other immune cells including macrophages and neutrophils (phylogenetically similar to them). In human, MCs are classified according to their protease content, those that mainly contain tryptases (mast cell T) and more rarely chymase, and those with both chymase and tryptase (mast cell TC) proteases (6). These protease content may vary after MCs treatment with various cytokines such as IL-1, IL-4, IL-6 and IL-33 (7).

The interleukin (IL)-1 family and its homologue TLR are closely linked to the innate immune response and mediates important inflammatory functions. IL-1 family cytokines are pro- and anti-inflammatory and intervene in the defense of the host (8). IL-1, together with an antigen, is an important T lymphocyte co-

stimulator and other immune cells, including MCs. However, seems that IL-1 family do not directly affect lymphocyte functions (8). IL-1 binds its receptor and lead to the activation of NF- κ B and MAPK pathways (mitogen activated protein kinase). IL-1 family contain activators and suppressors of inflammation and is composed of several members, including IL-1 α , IL-1 β , IL-18, IL-33, IL-1 receptor antagonist (IL-1RA), and IL-37 (8).

Over 20 years ago we and other authors reported that IL-1 is an inducer of gene expression and synthesis of COX-2 (cyclooxygenase type 2) leading to thromboxane and prostaglandin release and other proinflammatory compounds such as nitric oxide and PAF (platelet activating factor) (9). IL-1 increases the expression of intercellular adhesion molecule-1 (ICAM) and vascular cell adhesion molecule-1 (VCAM), and induces fever, vasodilatation, and hypotension. In addition IL-1 stimulates the generation of inflammatory cytokines/chemokines in several immunity cells, including MCs (10). Therefore, IL-1 can represent a growth factor for T cells and has the ability to activate MCs. The IL-1 family includes members who promote inflammation at both the receptor and nuclear levels, but also those that inhibit inflammatory diseases.

Blocking IL-1 in systemic diseases reduces the level of inflammatory IL-1 family members and it could be a therapeutic opportunity (11).

IL-18

IL-18 induces IL-4 and has a role in the Th2 response, and also contribute to Th1 activity. In addition, IL-18 is a pleiotropic proinflammatory cytokine generated by macrophages as well as epithelial cells (human keratinocytes). IL-18 leads to increased IgE production from B cells and enhances IL-13 in MCs and CD4⁺ T cells, and participates with IL-12 and IL-15 in the production of IFN- γ . IL-18 shares many proinflammatory properties with IL-1, IL-32 and IL-33 (12).

It has been reported that IL-1RA binds tightly to IL-1RI and is a specific inhibitor for IL-1 alpha and beta; while IL-37 binds IL-18R α and is a non-specific inhibitor of the innate immune response and inflammation. IL-1RA is particularly effective for

inhibiting the progressive joint space narrowing in rheumatoid arthritis which is an autoinflammatory disorder (13). IL-1RA is also used to treat diabetes, as it improves blood glucose levels and the function of beta cells producing insulin.

IL-33

IL-33, which is the ligand for the former orphan receptor ST2, acts and mediates many MCs-related immune responses, and it is also an activator of T cells promoting Th2 response. IL-33 is an intracellular cytokine involved in MC functions, TH2 immune responses and allergic inflammation (14). A member of this family is represented by IL-37 or IL-1F7 which neutralize IL-18 activity and inhibits IL-1, TNF, and TLR (innate immunity) (15).

The pathogenesis of many disorders involves inflammatory responses induced by several cytokines including IL-1 family members. TLR pathways play a key role in innate and adaptive immunity and may mediate the generation of IL-1 family and TNF which contribute to the inflammatory conditions. TLRs are pattern recognition receptors and can be activated by damaged-associated molecular patterns (DAMPs) and can be controlled by myeloid differentiation factor 88 (MyD88), which mediate the signal of TLRs. MyD88 mediates signaling TLR2, TLR3 and TLR4, while TRIF mediates TLR3 and TLR4. This MyD88-dependent pathway activates the transcription factors AP-1 and NF- κ B which are involved in the generation of inflammatory cytokines and therefore in inflammatory diseases.

Since inflammatory IL-1 family members are mediators of inflammatory disorders, blocking IL-1 activity is important in clinical medicine (16).

Mast cells

Mast cells (MCs) represent one of the first lines of defense which generate multiple inflammatory cytokines/chemokines in response to numerous antigens (17). MCs are involved in innate and adaptive immunity and reside in skin, mucosal tissue, lungs, gut and blood vessels (18). They are activated by cytokines, neurotransmitters and other molecules. Activated MCs secrete numerous vasoactive, chemoattractant and inflammatory compounds which participate in allergic and inflammatory diseases (Table I).

Activated antigen receptor Fc ϵ -RI provokes a number of important biochemical pathways starting with the induction of Src, Syk, and Tec family protein tyrosine kinase.

Phosphorylation causes the recruitment of adaptor molecules, such as SLP-76, and the activation of phospholipase C gamma (PLC γ) which regulates intracellular calcium and protein kinase C (PKC) activity. The recruitment of SLP-76 and other compounds activate GTPase which, provokes the stimulation of mitogen-activated protein (MAP) kinase family members, ERK, JNK and p38. This process activates many transcription factors including NF- κ B, leading MCs to generate inflammatory cytokines including TNF (19). MC activation leads to release phospholipase A₂ (PLA₂) to generate PGD₂, and leukotrienes LTC₄, D₄ and E₄.

Bronchoalveolar lavage fluid from symptomatic asthmatic patients is rich in MCs and several pro-inflammatory cytokines/chemokines including interleukin-1 beta IL-1 and tumour necrosis factor (TNF), showing an innate immune response.

IL-1 activates human MCs without degranulation and stimulates the production of inflammatory

Table I. Mast cell mediators.

Preformed: Biogenic amines (histamine, serotonin, and others); Proteoglycans (cathepsins, chymase, tryptase, MMP2, MMP-3, MMP-9); Cytokine (TNF).
De novo synthesis: Leukotrienes (LTC ₄ , LTD ₄ , LTE ₄); Prostaglandins (PGD ₂ , PGE ₂); Cytokines (IL-1, IL-33, and others, chemokines: CCL2, CCL3, CCL4, CCL5, CXCL2, CXCL8, etc.); Growth factors (SCF, NGF, VEGF, and others).

IL-6 which is required for the induction of several diseases (10).

IL-1 and IL-18 belong to the IL-1 cytokine family which stimulates the production of Th2 cytokines (IL-9 or IL-13) in mouse MCs and activates Th1 and Th17 cell differentiation in the presence of IgE-antigen-Fc ϵ RI, stem cell factor (SCF), or IL-3 (20).

IL-33, also called IL-1F11, binding its receptor T1/ST2 (Toll-IL-1 receptor), an orphan member of IL-1 receptor family, is localized in the nucleus and is a member of the IL-1 family which binds IL-1 receptor-related protein ST2 and activates T helper 2 (Th2) responses and releases IL-4, IL-5, and IL-13. IL-33 acts more as an immunoregulator than as a pro-inflammatory cytokine like other IL-1 family members, however, recently it has been found that IL-33 induces IL-13 production by mouse MCs independently of IgE-Fc ϵ RI signals and together with SP markedly enhances TNF synthesis and secretion from human MCs mediated by the interaction of their receptors (21-22). IL-33 is the most closely related to IL-18 and has been proposed to be secreted after maturation by caspase-1, which cleaves the precursor form of IL-33. This cytokine activates NF- κ B pathway and MAP kinases, driving the production of Th2 cytokines, and enhancing experimental allergic inflammation by

directly stimulating MCs to produce inflammatory cytokines. IL-33 in treated mice provokes eosinophilia in the intestines, lung, esophagus, and splenomegaly, and causes an augmentation of IgA and IgE in the blood (23).

Moreover, IL-33 stimulates in MCs arachidonic acid product release, such as PGD₂ and inflammatory cytokines including IL-6 and TNF. It has been reported that IL-33 is an important inflammatory cytokine which induces TNF, MCP-1, IL-8 and possibly other cytokines involved in allergy, suggesting that this protein plays a central role in the regulation of MC responses (24), even if the biological roles of IL-33 and the molecular mechanisms regulating its production, remain to be clarified.

IL-37

Inflammatory diseases may be improved by anti-inflammatory agents such as IL-37. This cytokine binds IL-18R- α chain, inhibits directly MyD88 (myeloid differentiation factor 88) and reduces the production of inflammatory IL-1 family members (25).

Interleukin (IL)-37, an IL-1 family member, is an important inflammatory cytokine inhibitor. We recently reported that IL-37 inhibits inflammation

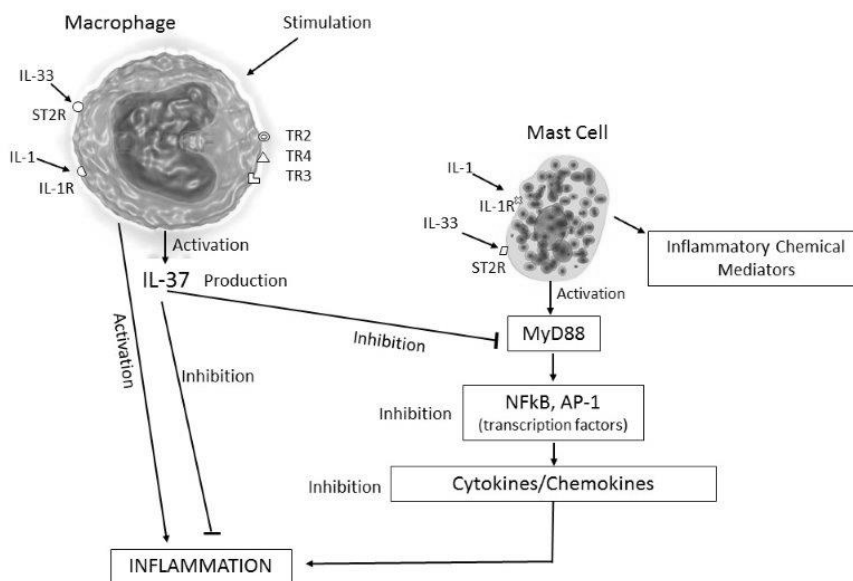


Fig. 1. The figure depicts mast cell activation pathway releasing inflammatory cytokines/chemokines. Inhibitory effect of IL-37 generated by activated macrophages.

in a number of diseases, including allergy, cancer, autoimmunity, arthritis, atherosclerosis and skin inflammation. The tissues that over-express IL-37 are less inflammatory than those which do not express it, and experimental animals, such as mice, expressing IL-37 genes are more protected from inflammatory diseases.

IL-37 down-regulates innate immunity by inhibiting macrophage response and its accumulation, and not only reduces the cytokines that mediate inflammation, but also the symptoms of inflammation (Fig. 1). This cytokine, along with other appropriated therapies, may inhibit inflammatory cytokines in response to various stimuli including inflammatory cytokines IL-1, IL-6 and IL-18 which participate in the Th1 response.

IL-37 prevents inflammatory cytokine activity and may provoke significant improvements in inflammatory preformed and de novo synthesized mediators released by MCs. Inhibiting inflammatory cytokines released by MC activity may reduce the incidence in acute and systemic inflammatory disorders.

Activated MCs produce cytokines and other compounds which may have an impact on macrophages (26-27) which, in turn, may generate IL-37, exerting an anti-inflammatory activity. It has been reported that IL-37 owns pleiotropic effects, and inhibits inflammatory cytokines IL-1, IL-18, IL-32 and TNF, inducing clinical improvement in the patient affected by inflammatory diseases and other pathological conditions. IL-37 provokes an increase of the anti-inflammatory IL-10 and a significant reduction of inflammatory severity and improvement in the quality of patients life (28).

This protein may be a good candidate in clinical medicine to treat a broad spectrum of inflammatory diseases including allergy, atherosclerosis, arthritis and psoriasis, mediated by MCs.

Therefore, the clinical treatment with this new cytokine inhibitor IL-37, in conjunction with conventional therapies, could reduce disease complication and mortality.

In this article, we report that inflammatory IL-1 family member blocking agents, such as IL-37, may improve the inflammatory markers and physiologic

parameters and the pathogenesis of inflammatory disorders mediated by MCs, describing a new concept of inhibition and cure of inflammation.

The therapeutic use of IL-37 in inflammatory disorders remains a promising approach, although great attention must be paid to cytokine therapies, since to date they have been highly toxic and can lead to death. In addition, we do not know the effects that may occur by inhibiting the immune system with IL-37. In this regard, more studies are needed to clarify both the side effects of IL-37, and the clinical dosage. However, its inhibitory effect on the immune system remains to be determined.

REFERENCES

1. Medzhitov R, Janeway C Jr. Innate immunity. *N Engl J Med* 2000; 343(5):338-44. Review.
2. Toniato E, Frydas I, Robuffo I, Ronconi G, Caraffa A, Kritas SK, Conti P. Activation and inhibition of adaptive immune response mediated by mast cells. *J Biol Regul Homeost Agents* 2017; 31(3):543-48.
3. Mackay IR, Rosen FS, Medzhitov R, Janeway C. Innate Immunity. *N Engl J Med* 2000; 343:338-344. doi: 10.1056/NEJM200008033430506.
4. Papazahariadou M, Athanasiadis GI, Papadopoulos E, et al. Involvement of NK cells against tumors and parasites. *Int J Biol Markers* 2007; 22(2):144-53. Review.
5. Netea MG, Lewis EC, Azam T, et al. Interleukin-32 induces the differentiation of monocytes into macrophage-like cells. *Proc Natl Acad Sci U S A* 2008; 105(9):3515-20. doi: 10.1073/pnas.0712381105.
6. Galli SJ, Borregaard N, Wynn TA. Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. *Nat Immunol* 2011; 12(11):1035-44. doi: 10.1038/ni.2109.
7. Theoharides TC, Stewart JM, Taracanova A, Conti P, Zouboulis CC. Neuroendocrinology of the skin. *Rev Endocr Metab Disord* 2016; 17(3):287-294. Review.
8. Dinarello CA. Immunological and inflammatory functions of the interleukin-1 family. *Annu Rev Immunol* 2009; 27:519-50. doi: 10.1146/annurev.immunol.021908.132612.
9. Conti P, Cifone MG, Alesse E, Reale M, Fieschi C, Dinarello CA. In vitro enhanced thromboxane

- B2 release by polymorphonuclear leukocytes and macrophages after treatment with human recombinant interleukin 1. *Prostaglandins* 1986; 32(1):111-15.
10. Kandere-Grzybowska K, Letourneau R, Kempuraj D, et al. IL-1 induces vesicular secretion of IL-6 without degranulation from human mast cells. *J Immunol* 2003; 171(9):4830-36.
 11. Dinarello CA, Bufler P. Interleukin-37. *Semin Immunol* 2013; 25(6):466-68. doi: 10.1016/j.smim.2013.10.004.
 12. Novick D, Kim S, Kaplanski G, Dinarello CA. Interleukin-18, more than a Th1 cytokine. *Semin Immunol* 2013; 25(6):439-48. doi: 10.1016/j.smim.2013.10.014.
 13. Conti P, Youinou P, Theoharides TC. Modulation of autoimmunity by the latest interleukins (with special emphasis on IL-32). *Autoimmun Rev* 2007; 6(3):131-37. Review.
 14. Dinarello CA. An IL-1 family member requires caspase-1 processing and signals through the ST2 receptor. *Immunity* 2005; 23(5):461-62.
 15. Giacoppo S, Thangavelu SR, Diomede F, Bramanti P, Conti P, Trubiani O, Mazzon E. Anti-inflammatory effects of hypoxic-preconditioned human periodontal ligament cells secretome in an experimental model of multiple sclerosis: a key role of IL-37. *FASEB J* 2017; doi: 10.1096/fj.201700524R.
 16. Conti P, Caraffa A, Ronconi G, Kritas SK, Mastrangelo F, Tettamanti L, Theoharides TC. Impact of mast cells in mucosal immunity of intestinal inflammation: inhibitory effect of IL-37. *Eur J Pharmacol* 2017; doi: 10.1016/j.ejphar.2017.09.044.
 17. Conti P, Caraffa A, Kritas SK, Ronconi G, Lessiani G, Toniato E, Theoharides TC. Mast cell, pro-inflammatory and anti-inflammatory: Jekyll and Hyde, the story continues. *J Biol Regul Homeost Agents* 2017; 31(2):263-67. Review.
 18. Theoharides TC, Zhang B, Kempuraj D, et al. IL-33 augments substance P-induced VEGF secretion from human mast cells and is increased in psoriatic skin. *Proc Natl Acad Sci U S A* 2010; 107(9):4448-53. doi: 10.1073/pnas.1000803107.
 19. Galli SJ, Kalesnikoff J, Grimaldeston MA, Piliponsky AM, Williams CM, Tsai M. Mast cells as “tunable” effector and immunoregulatory cells: recent advances. *Annu Rev Immunol* 2005; 23:749-86. Review.
 20. Iwamura C, Nakayama T. Toll-like receptors in the respiratory system: their roles in inflammation. *Curr Allergy Asthma Rep* 2008; 8(1):7-13. Review.
 21. Taracanova A, Alevizos M, Karagkouni A, et al. SP and IL-33 together markedly enhance TNF synthesis and secretion from human mast cells mediated by the interaction of their receptors. *Proc Natl Acad Sci U S A* 2017; 114(20):E4002-E4009. doi: 10.1073/pnas.1524845114.
 22. Presta I, Guadagno E, Di Vito A, et al. Innate immunity may play a role in growth and relapse of chordoid meningioma. *Int J Immunopathol Pharmacol* 2017; 1:394632017730241. doi: 10.1177/0394632017730241.
 23. Cherry WB, Yoon J, Bartemes KR, Iijima K, Kita H. A novel IL-1 family cytokine, IL-33, potently activates human eosinophils. *J Allergy Clin Immunol* 2008; 121(6):1484-90.
 24. Theoharides TC, Stewart JM, Tsilioni I. Tolerability and benefit of a tetramethoxyluteolin-containing skin lotion. *Int J Immunopathol Pharmacol* 2017; 30(2):146-51. doi: 10.1177/0394632017707610.
 25. Zhan Q, Zeng Q, Song R, et al. IL-37 suppresses MyD88-mediated inflammatory responses in human aortic valve interstitial cells. *Mol Med* 2017; 23. doi: 10.2119/molmed.2017.00022.
 26. W-H, Liu Y-H, Liang J-L, et al. β -Patchoulene, isolated from patchouli oil, suppresses inflammatory mediators in LPS-stimulated RAW264.7 macrophages. *Eur J Inflamm* 2017; 2:136-41.
 27. Yang L, Yue Ting Y, Shen Y, et al. 17 β -estradiol modulates the viability, phenotype, endocytosis, and inflammatory cytokine expression of RAW264.7 macrophages. *Eur J Inflamm* 2016; 1:10-17.
 28. Conti P, Carinci F, Lessiani G, Spinass E, Kritas SK, Ronconi G, Caraffa A, Theoharides TC. Potential therapeutic use of IL-37: a key suppressor of innate immunity and allergic immune responses mediated by mast cells. *Immunol Res* 2017; doi: 10.1007/s12026-017-8938-7.

THYMOQUINONE PROTECTS AGAINST COBALT CHLORIDE-INDUCED NEUROTOXICITY VIA Nrf2/GCL-REGULATED GLUTATHIONE HOMEOSTASIS

L. JIANG¹, H. LI² and N. ZHAO¹

¹Department of Anesthesiology, No. 215 Hospital of Shaanxi Nuclear Industry, Xianyang, Shaanxi, China; ²ICU, No. 215 Hospital of Shaanxi Nuclear Industry, Xianyang, Shaanxi, China

Received May 6, 2017 – Accepted August 28, 2017

The prevalence of neurodegenerative diseases worldwide has increased dramatically in the last decades. Hypoxia and oxidative stress play a central role in the pathogenesis of neurodegenerative diseases. Thymoquinone (TQ) is a monoterpenoid hydrocarbon compound that possesses potent antioxidant activity. In the current study, we investigated the neuroprotective effects of TQ against CoCl₂, a widely used hypoxia-inducing agent. We found that TQ inhibited CoCl₂-induced cytotoxicity *in vitro*, as reflected by an increase of cell viability and decrease of apoptosis in CoCl₂-treated PC12 cells. TQ exhibited a potent protective effect against CoCl₂-induced neurotoxicity *in vivo*, as evidenced by decreased time spent to find the platform site in the Probe trials, reduced escape latencies, decreased traveling distance and reduction of apoptotic cell death in brains in CoCl₂-treated rats. CoCl₂-resulted decrease of glutathione (GSH) and increase of malondialdehyde (MDA) levels were significantly inhibited by TQ. Inhibition of GSH synthesis by buthionine sulfoximine (BSO) significantly attenuated TQ-induced neuroprotective effects against CoCl₂ in rats and in PC12 cells. TQ could upregulate nuclear factor (erythroid-derived 2)-like 2 (Nrf2)/glutamate-cysteine ligase catalytic subunit (GCLc) and Nrf2/glutamate-cysteine ligase modifier subunit (GCLm) pathway which contributed to antioxidant and neuroprotective effects of TQ. In summary, we found that TQ exhibited protective effects against neurotoxicity via upregulation of Nrf2/GCL signaling. Upregulation of Nrf2/GCL signaling promoted the synthesis of GSH and contributed to attenuation of oxidative stress, neuronal cell apoptosis and neurotoxicity. These data have appointed a new path toward the understanding of the neuroprotective activities of TQ.

There has been a dramatic worldwide increase in the prevalence of neurodegenerative diseases such as Alzheimer's and Parkinson's (1, 2). Neurodegenerative diseases and central neuronal system injuries are characterized by neuronal cell death, which is the major cause of consequent functional deficits including cognitive and motor disabilities. Strategies aiming to protect neurons from cell death have gained much attention. Hypoxic/ischemic injury plays a central role in neuronal cell

death under neurodegenerative condition (3, 4). It has long been accepted that cobalt chloride (CoCl₂) is a suitable *in vitro* hypoxia-mimetic agent which could activate the production of reactive oxygen species (ROS) in various cultured cells (5-7). PC12 cell line, a cell line derived from rat pheochromocytoma, has been widely used as an *in vitro* model for the investigation of neuronal cell death (8).

Thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone, TQ), a monoterpenoid hydrocarbon

Key words: thymoquinone, neurotoxicity, oxidative stress, nuclear factor (erythroid-derived 2)-like 2, glutamate-cysteine ligase

Mailing address:

Dr Ning Zhao,
Department of Anesthesiology,
No. 215 Hospital of Shaanxi Nuclear Industry,
No. 35 Weiyangxi Road, Xianyang, 712000, Shaanxi, China
Tel.: +8615319068098
e-mail: zhaoning197410@163.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

compound, is the major biological component of *nigella* (N.) *sativa* and the related extracts. Numerous studies have suggested that N. *sativa* has a series of biological properties, including anti-inflammatory and analgesic (9), anti-epileptic (10), anti-tumor (11), anti-histaminic (12), and diuretic and hypotensive effects (13, 14). In particular, TQ plays an antioxidant role through regulation of glutathione (GSH) metabolism, and NADPH and NADH balance (15). Previous studies have shown that TQ protects against brain injury induced by trauma and ischemia-reperfusion (16, 17). Moreover, TQ could protect against brain injury through activation of the antioxidant pathway in status epilepticus rats (18) and decrease seizure activity and lower hippocampal neuronal loss in an epilepsy model and in children with refractory seizures (19, 20). Those findings suggest that TQ possesses a neuroprotective activity. However, the effect of TQ on neuronal cell death and the underlying mechanism are not clear.

In the study, we investigated TQ-induced neuroprotective effects in CoCl_2 -treated rats and PC12 cells. We showed that TQ exhibited neuroprotective activities and inhibited CoCl_2 -induced neurotoxicity via nuclear factor (erythroid-derived 2)-like 2 (Nrf2)/glutamate cysteine ligase (GCL)-regulated GSH homeostasis.

MATERIALS AND METHODS

Chemicals

β -actin, GCLc, GCLm and Nrf2 protein antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 2', 7'-Dichlorodi-hydrofluorescein diacetate, MTT and CoCl_2 were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animal treatment

All procedures were carried out in accordance with the conventional guidelines for experimentation with animals (National Institute of Health, Publication no. 85-23, revised 1996). All animal procedures were approved by the Review Board of No. 215 Hospital of Shaanxi Nuclear Industry. All efforts were made to reduce suffering and use the minimal number of animals. Forty male Wistar rats (220–250 g) were obtained from the Animal Centre of

School of Medicine, Xi'an Jiaotong University. Rats were housed in a controlled environment with 12 h/12 h light/dark cycle, controlled humidity and temperature, and free access to food and water.

Rats were randomly divided into four groups: Control, CoCl_2 , CoCl_2 +TQ and CoCl_2 +TQ+buthionine sulfoximine (BSO, an inhibitor of glutathione synthesis). The animal model was established as previously reported (21). In brief, rats were placed in a stereotaxic apparatus and drilled a small hole on the skull in the frontoparietal cortex at Bregma -1.3 mm to induce a unilateral lesion. After that, we injected sterile solution of CoCl_2 (50 mM) or saline solution to the right hemisphere of the rat, 1 mm below the pia level in the cerebral cortex (layers 3–4) using a Hamilton syringe. The CoCl_2 solution was adjusted to reach a physiological osmolarity of 310 mOsm/kg. Then, the lesion site was repaired using the temporal muscle and the attached fascia. The rats were given sevoflurane 8% (v/v) to induce anesthesia and maintain the surgical operation and each animal was later put into a separate cage for recovery. The body temperature of the rats was maintained using a heating pad during surgery. The rats were allowed to recover for two days and then received i.p. injection of TQ (50 mg/kg) with or without BSO (10 mg/kg) daily for 10 days. After the animal treatment, sections of frozen brain were collected for the measurement of apoptosis.

Morris water maze (MWM) test

Spatial learning and memory abilities of the rats were detected by MWM test as previously reported (22). In brief, the test was performed in a maze, a black circular pool (120 cm in diameter and 45 cm high) filled with clear tap water at a temperature of $27 \pm 0.5^\circ\text{C}$ with a depth of 30 cm. Two cm below the surface of the water, there was a black platform, 10 cm in diameter, in the middle of one of the four quadrants. The Videomex Water Maze Monitoring Software (Taimeng tech, Chengdu, China) was used to record and analyze the rat's path in the pool. During the experiments, the surrounding environment was controlled to be unaltered among tests of different rats.

The rats were allowed to take a 60 s swimming trial each day for 5 days. In the trials, the time spent to reach the platform, the distance of swimming, and the swimming speed were recorded. The computer would stop tracking and record the time as 60 s if the rat did not

find the platform within the set time. The rat would be allowed to remain on the platform for 10 s if it found the platform within 60 s. Otherwise, the rat would be guided to the platform to remain for 10 s. On the 6 day, the probe trial test was performed. The platform was removed and the rats were allowed to conduct a 60 s exploration and the crossing index to the previous platform site was recorded.

Cell culture and transfection

PC12 rat pheochromocytoma cells were purchased from American Type Culture Collection (ATCC CRL-1721TM) and grown in RPMI 1640-medium (Gibco), supplemented with 10% heat inactivated horse serum (Gibco), 5% heat inactivated fetal bovine serum (Gibco), 100 U/mL penicillin/100 g/mL streptomycin (Gibco) and 2 mM L-glutamine (Gibco) in a humidified incubator with 5% CO₂ at 37°C. The cells were grown as adherent cultures to a confluence of approximately 80%. For assay performance, the cells were harvested from the flasks using a 0.05% trypsin/EDTA (Gibco), were counted using a CASY cell counter (Roche) and were seeded after resuspension in cell culture medium at a defined concentration. In the experimental treatment, cells were exposed to CoCl₂ in the presence or absence of TQ with or without BSO in serum-free RPMI 1640-medium. Lentivirus (LV) expressing shRNA of Nrf2 was obtained from Santa Cruz Biotechnology (Santa Cruz, USA). The cells were transfected with LV-shNrf2 or vectors to according to the manufacturer's protocols.

Measurement of cell viability

Cells were seeded in a 96-well plate at a density of 1×10^4 cells per well. After the experiment, cell viability was examined by MTT assay. Absorbance was measured at 550 nm and results were shown as fold-changes of control. After the treatment, 0.5 mg/ml MTT was added and the plates were incubated for 4 h at 37°C in an incubator. After the removal of supernatant, the blue-colored formazan that formed was dissolved in 150 μ l DMSO per well. The absorbance was detected at 490 nm using an ELISA plate reader.

Measurement of apoptosis

After the experiments, cells were stained with TUNEL dye according to the manufacturer's instructions (Roche). TUNEL-positive cells were counted and results

were expressed as fold increase versus control.

Measurement of ROS level

ROS level was detected by the oxidation of DCF, the nonfluorescent cell-permeant compound that is intracellularly cleaved by endogenous esterases and is retained by viable cells. Oxidation of DCF by ROS produced the fluorescent compound 2', 7'-dichlorofluorescein. After the treatment, PC12 cells were harvested and preincubated with 10 μ mol/L DCF for 30 min in serum-free medium at 37°C. After washing twice, cells were resuspended with PBS. Fluorescence intensity was detected by a flow cytometry. Results were shown as percentage of control.

Quantitative real-time PCR

Total RNA was extracted from brains and cells using Trizol reagent (Invitrogen, CA, USA). 0.5 μ g of RNA was reversely transcribed to cDNA using an miScript II RT Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to precisely quantify the mRNA expression level of Nrf2, GCLc and GCLm using gene-specific primer pairs on an Applied Biosystems 7500 cycler (Applied Biosystems, CA, USA) using an miScript SYBR Green PCR Kit (Thermo Fisher Scientific Inc.). The reaction conditions were: an initial step at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 30 s, and then extension at 72°C for 10 s. The $2^{-\Delta\Delta CT}$ method was used to measure gene expression compared with the endogenous control β -actin.

Western blot

Total proteins were extracted using ice-cold RIPA lysis buffer (Thermo Fisher Scientific, Rockford, IL, USA). The concentration of protein was determined using BCA assay. Extracts were separated on a sodium dodecyl sulfate–polyacrylamide gel and then transferred to polyvinylidene difluoride membrane (Millipore Corporation, MA, USA) and non-specific binding of antibodies was blocked with 5% BSA in tris-buffered saline (TBS) for 1 h at room temperature. The membranes were incubated with appropriate primary antibodies overnight at 4°C, and washed four times with TBS Tween (TBST) each for 10 min. Membranes were incubated with

peroxidase-conjugated IgG secondary antibody for 30 min at 37°C, and then washed another four times. Finally, membranes were detected by ECL system (Millipore Corporation, MA, USA). Bands were finally captured and quantified with Quantity One software (Bio-Rad). Target gene expression levels were normalized to β -actin expression.

Evaluation of oxidative stress

GSH and MDA contents were measured using assay kits (Nanjing Jiancheng Company, Jiangsu, China) as per the manufacturer's protocols. When the pH is 8, o-phthalaldehyde (OPT) may react with GSH, generating fluorescent materials. In brief, 10% trichloroacetic acid was added to the sample. After centrifugation (5000 $\times$ g, 10 min), OPT was added to the supernatant and incubated. Fluorescence at 425 nm was determined with the excitation at 350 nm. Levels of MDA were determined with the colorimetric absorption of the thiobarbituric acid-MDA chromophore used to determine the index of lipid peroxidation.

Statistical analysis

Statistical analysis was performed by GraphPad Software. Data were expressed as mean $\pm$ SEM of three independent experiments. The statistical significance of differences between groups was analyzed via one-way analysis of variance followed by a Student–Newman–Keuls method for multiple comparisons. A P value <0.05 was considered to be significant.

RESULTS

Neuroprotective effect of TQ against CoCl₂ in PC12 cells

In the study, we firstly investigated the neuroprotective effect of TQ against CoCl₂ in PC12 cells. As shown in Fig. 1A and B, TQ concentration-dependently inhibited CoCl₂-induced decrease of cell viability and increase of apoptotic cell death. This inhibition was significantly suppressed by BSO, an inhibitor of GSH synthesis through suppression of GCL, the enzyme which is required in the first step of GSH synthesis (Fig. 1C and D). The results demonstrated that TQ could protect against CoCl₂-induced neuronal cytotoxicity *in vitro* and the regulation of GSH metabolism may be involved.

Protective effect of TQ against CoCl₂-induced GSH depletion and oxidative stress in PC12 cells

To further detect the effect of TQ on CoCl₂-induced GSH metabolism, we measured the content of GSH, which is considered to be a predominant antioxidant protein in a cell. As shown in Fig. 2A, CoCl₂ resulted in a marked decrease of GSH content in PC12 cells. The treatment of TQ notably inhibited CoCl₂-induced decrease of GSH (Fig. 2A). BSO treatment reversed the effect of TQ on GSH levels *in vitro* (Fig. 2A). Furthermore, we showed that the levels of malondialdehyde (MDA), an important product of lipid peroxidation, in PC12 cells, were increased by CoCl₂ (Fig. 2B). CoCl₂-induced increase of MDA levels was inhibited by TQ (Fig. 2B). TQ-induced inhibition of the increase of MDA levels in cells was blocked by BSO (Fig. 2B). Moreover, we detected ROS level in PC12 cells, which was measured by oxidation of DCF, the nonfluorescent cell-permeant compound that was intracellularly cleaved by endogenous esterases and was retained by viable cells. We showed that CoCl₂ resulted in a marked increase in ROS level which was inhibited by TQ (Fig. 2C). In the presence of BSO, TQ-induced decrease of ROS level in cells was significantly suppressed (Fig. 2C). These results indicate that TQ could protect against CoCl₂-induced oxidative stress which was mainly attributed to the upregulation of GSH levels.

TQ regulates GSH homeostasis through Nrf2/GCL pathway in CoCl₂-treated PC12 cells

To investigate the mechanism of TQ-induced regulation of GSH metabolism, we detected the changes of Nrf2/GCL signaling, which was the major regulator of GSH synthesis. We showed that the mRNA and protein expression of Nrf2 was decreased by CoCl₂ in PC12 cells (Fig. 3A and D). TQ significantly inhibited CoCl₂-induced decrease of Nrf2 expression (Fig. 3A and D). BSO treatment had no significant effect on Nrf2 expression in the presence of CoCl₂ and TQ (Fig. 3A and D). In consistence, the mRNA and protein expression of GCLc and GCLm were decreased by CoCl₂ in PC12 cells (Fig. 3B, C and D). TQ significantly inhibited CoCl₂-induced decrease of GCLc and GCLm

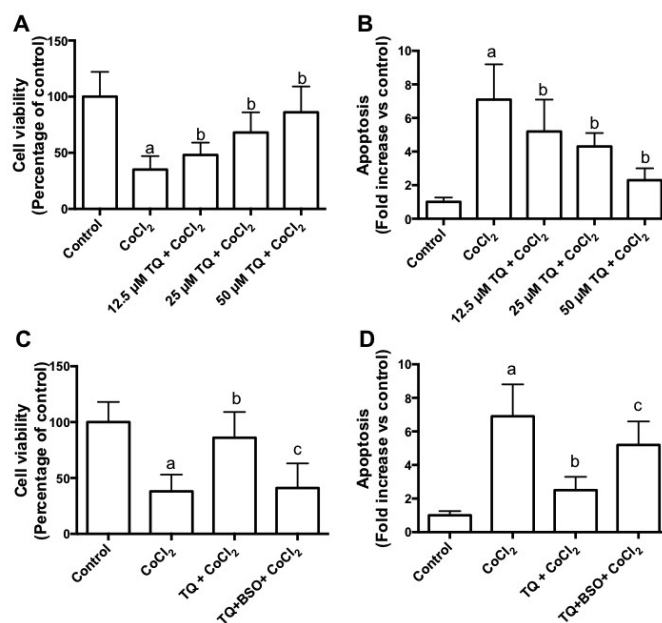


Fig. 1. The protective effects of TQ against CoCl₂-induced neurotoxicity in PC12 cells.

A and B) PC12 cells were incubated with 500 μM CoCl₂ with or without 0-50 μM TQ for 24 h. A) Cell viability was measured by MTT assay and results were expressed as percentage of control. B) Apoptosis was measured by TUNEL staining and results were shown as fold increase versus control. (C and D) PC12 cells were incubated with 500 μM CoCl₂ with or without 50 μM TQ and 10 μM BSO for 24 h. C) Cell viability was measured by MTT assay and results were expressed as percentage of control. D) Apoptosis was measured by TUNEL staining and results were shown as fold increase versus control. Data were expressed as mean ± SEM of three independent experiments. a $p < 0.05$, the indicated group vs control. b $p < 0.05$, the indicated group vs CoCl₂ group. c $p < 0.05$, the indicated group vs TQ administration.

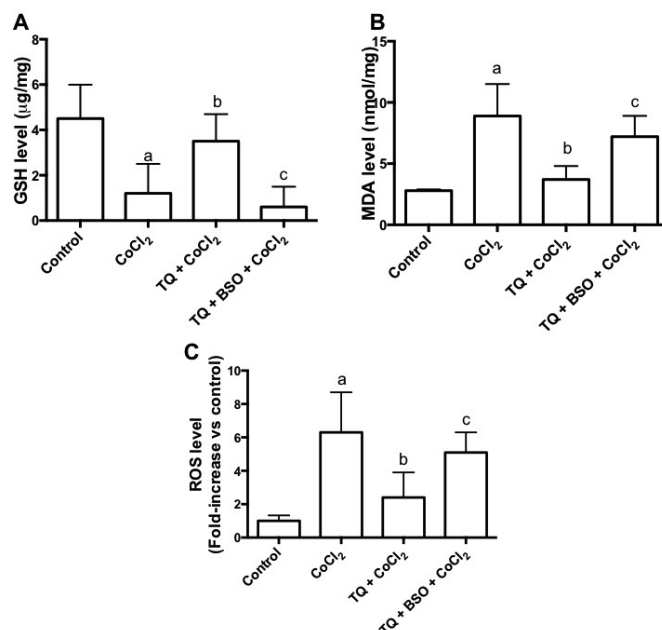


Fig. 2. The protective effects of TQ against CoCl₂-induced GSH depletion and oxidative stress in PC12 cells. PC12 cells were incubated with 500 μM CoCl₂ with or without 50 μM TQ and 10 μM BSO for 24 h. GSH (A) and MDA (B) contents in cells were determined using assay kits. C) ROS level in cells were determined by DCFH-DA staining and relative level of ROS was shown. Data were expressed as mean ± SEM of three independent experiments. a $p < 0.05$, the indicated group vs control. b $p < 0.05$, the indicated group vs CoCl₂ group. c $p < 0.05$, the indicated group vs TQ administration.

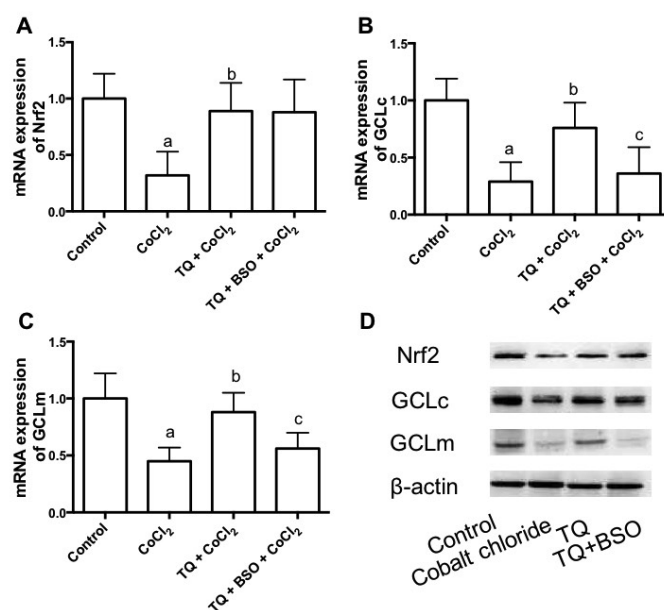


Fig. 3. The effects of TQ on CoCl₂-induced changes of GSH synthesis regulators. PC12 cells were incubated with 500 μ M CoCl₂ with or without 50 μ M TQ and 10 μ M BSO for 24 h. mRNA expression of Nrf2 (A), GCLc (B), and GCLm (C) was examined by real-time PCR. D) Protein expression of Nrf2, GCLc, and GCLm was examined by western blot. Data were expressed as mean $\pm$ SEM of three independent experiments. a $p < 0.05$, the indicated group vs control. b $p < 0.05$, the indicated group vs CoCl₂ group. c $p < 0.05$, the indicated group vs TQ administration.

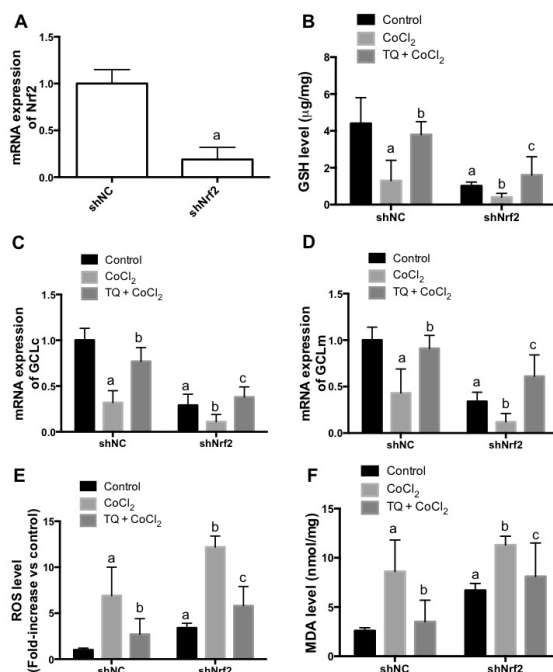


Fig. 4. Nrf2 was involved in TQ-induced protective effects against GSH depletion and oxidative stress. PC12 cells were transfected with shNrf2 and then exposed to 500 μ M CoCl₂ with or without 50 μ M TQ for 24 h. A) The efficiency of Nrf2 knockdown was estimated by qRT-PCR. B) GSH content was determined by commercial kits. C, D) mRNA expression of GCLc and GCLm was determined by real-time PCR. E) ROS level in cells were determined by DCFH-DA staining and relative level of ROS was shown. F) MDA content was determined by commercial kits. Data were expressed as mean $\pm$ SEM of three independent experiments. a $p < 0.05$, the indicated group vs control. b $p < 0.05$, the indicated group vs CoCl₂ group. c $p < 0.05$, the indicated group vs TQ administration.

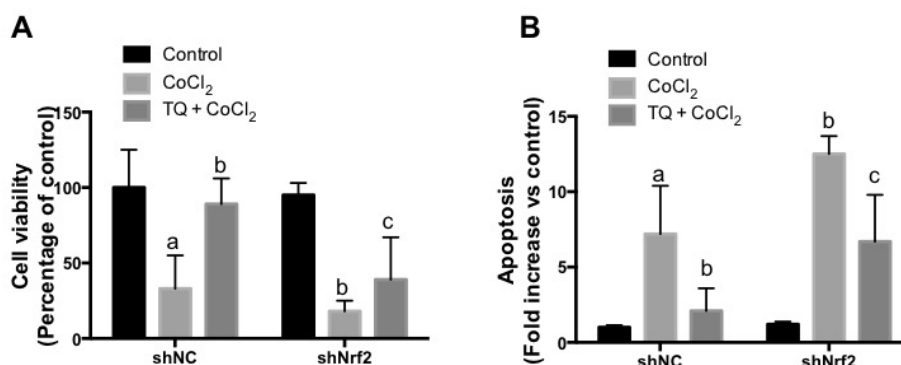


Fig. 5. *Nrf2* was involved in TQ-induced protective effects against CoCl₂-induced neurotoxicity. PC12 cells were transfected with shNrf2 and then exposed to 500 μ M CoCl₂ with or without 50 μ M TQ for 24 h. **A)** Cell viability was measured by MTT assay and results were expressed as percentage of control. **B)** Apoptosis was measured by TUNEL staining and results were shown as fold increase versus control. Data were expressed as mean $\pm$ SEM of three independent experiments. *a* $p < 0.05$, the indicated group vs control. *b* $p < 0.05$, the indicated group vs CoCl₂ group. *c* $p < 0.05$, the indicated group vs TQ administration.

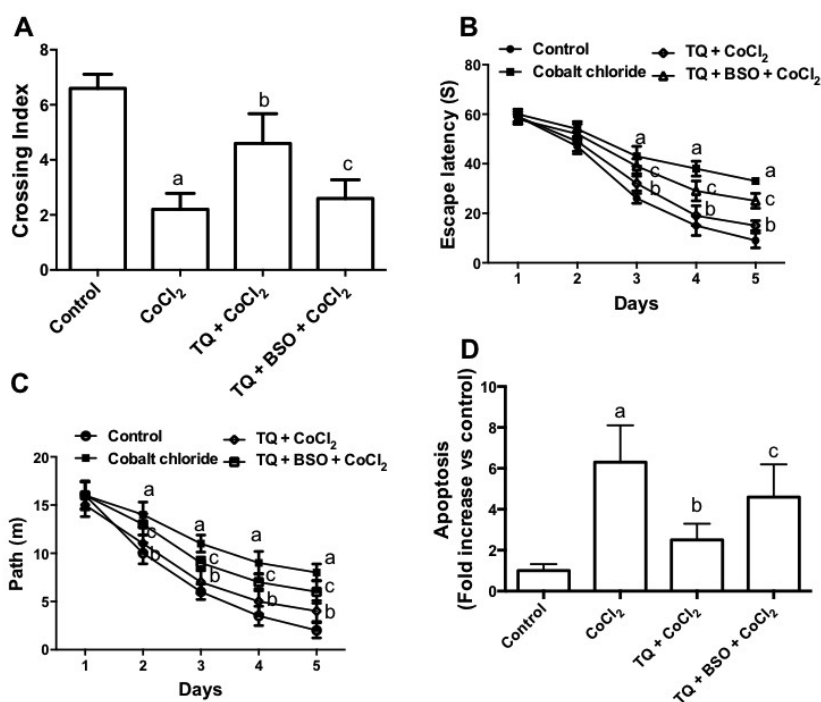


Fig. 6. The protective effects of TQ against CoCl₂-induced neurotoxicity in rats. Rats were injected with CoCl₂ to induce neurotoxicity and then received injection of TQ (50 mg/kg) or saline solution with or without BSO (10 mg/kg). **A)** Spatial learning and memory abilities were evaluated by MWM test. The crossing index indicates the average frequency of swims over the platform site in the target quadrant. **B and C)** Spatial memory for platform location was evaluated by the probe trials after 5 days of training in the MWM test. **B)** The average escape latencies. **C)** The distance traveled by the rats to find the platform. **D)** Apoptosis in brain was determined using a TUNEL staining kit and results were shown as fold increase versus control. $N = 10$. *a* $p < 0.05$, the indicated group vs control. *b* $p < 0.05$, the indicated group vs CoCl₂ group. *c* $p < 0.05$, the indicated group vs TQ administration.

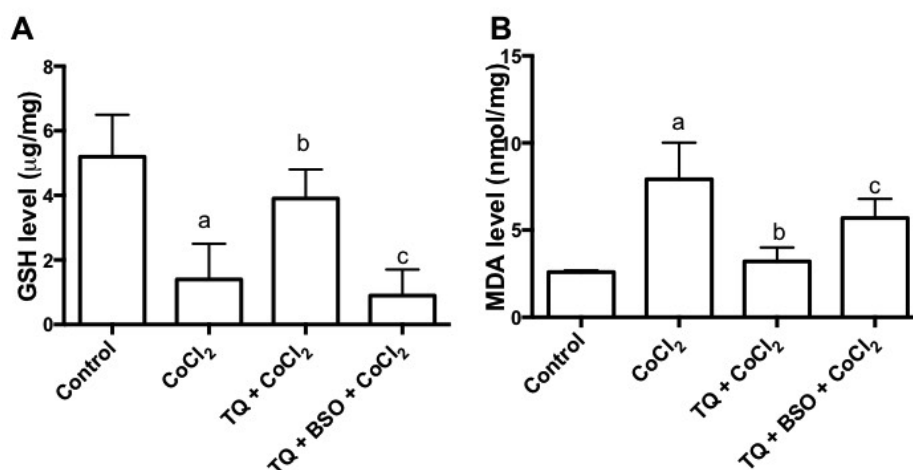


Fig. 7. The protective effects of TQ against CoCl₂-induced GSH depletion and oxidative stress in rats. Rats were injected by CoCl₂ to induce neurotoxicity and then received injection of TQ (50 mg/kg) or saline solution with or without BSO (10 mg/kg). GSH (A) and MDA (B) contents in brains were determined using assay kits. a $p < 0.05$, the indicated group vs control. b $p < 0.05$, the indicated group vs CoCl₂ group. c $p < 0.05$, the indicated group vs TQ administration.

expression (Fig. 3B, C and D). BSO treatment notably suppressed TQ-induced increase of GCLc and GCLm expression in CoCl₂-treated cells (Fig. 3B, C and D).

To further verify the role of Nrf2 in TQ-induced regulation of GSH synthesis, we knocked down the expression of Nrf2 in PC12 cells using lentivirus-mediated shRNA. We showed that after the transfection of Nrf2 shRNA (Fig. 4A), TQ-induced increase of GSH content (Fig. 4B), GCLc (Fig. 4C) and GCLm expression (Fig. 4D), and decrease of ROS level (Fig. 4E) and MDA content (Fig. 4F) were inhibited by knockdown of Nrf2. The results demonstrated that Nrf2 was responsible for TQ-induced regulation of GSH.

TQ inhibits CoCl₂-induced cytotoxicity through upregulation of Nrf2 in PC12 cells

To further verify the role of Nrf2 in TQ-exhibited protective effects against CoCl₂-cytotoxicity in PC12 cells, we examined the effect of Nrf2 knockdown on cell viability and apoptosis. We showed that knockdown of Nrf2 significantly inhibited TQ-induced increase of cell viability and decrease of apoptosis in CoCl₂-treated PC12 cells (Fig. 5A and B). The results demonstrated that Nrf2 may be an important target that mediated the antioxidant

and neuroprotective effects of TQ against CoCl₂ cytotoxicity *in vitro*.

Neuroprotective effect of TQ against CoCl₂ in rats

Rats were injected with CoCl₂ to establish an animal model of hypoxia-associated neurotoxicity. Then, the rats were administered TQ with or without BSO injection. After the animal treatment, spatial learning and memory abilities of rats were assessed by the MWM test. As shown in Fig. 1A, the rats treated with CoCl₂ spent more time to find the platform site in the Probe trials. TQ decreased the time spent by CoCl₂-treated rats (Fig. 6A). TQ-induced reduction of spent time was inhibited by BSO (Fig. 6A). On test days 3, 4, and 5, the escape latencies in rats in the CoCl₂ group were significantly increased compared with that of the control group (Fig. 6B). The administration of TQ notably reduced the escape latencies in rats treated by CoCl₂ (Fig. 6B). TQ-induced decrease of escape latencies was suppressed by BSO (Fig. 6B). Moreover, the distances traveled by the rats in each group to find the platform were also gradually reduced by training (Fig. 6C). On test days 2, 3, 4, and 5, the traveling distances of rats in the CoCl₂ group were significantly increased compared with that of control group (Fig. 6C). The administration of TQ notably reduced the traveling

distances in rats treated by CoCl_2 (Fig. 6C). This reduction was suppressed by BSO (Fig. 6C). CoCl_2 resulted in a significant increase of TUNEL-positive cells in brains of rats and this increase was inhibited by TQ, indicating the amelioration of apoptosis (Fig. 6D). BSO notably blocked the anti-apoptotic effect of TQ in CoCl_2 -treated rats (Fig. 6D).

The treatment of TQ notably inhibited CoCl_2 -induced decrease of GSH in rats (Fig. 7A). BSO treatment reversed the effect of TQ on GSH levels *in vivo* (Fig. 7A). Furthermore, we showed that MDA levels in brains of rats were increased by CoCl_2 , which was inhibited by TQ (Fig. 7B). TQ-induced inhibition of MDA increase in CoCl_2 -treated rats was blocked by BSO (Fig. 7B). The results demonstrated that TQ could protect against CoCl_2 -induced neurotoxicity in rats, and that the regulation of GSH metabolism may be one of the major mechanisms.

DISCUSSION

Although the molecular mechanism of neurodegenerative diseases is not clear, it is believed that oxidative stress plays a central role in the process of neuronal cell death (23). The redox state in a body is controlled by the oxidant and antioxidant systems (24). In response to pathological stimuli, ROS generation could be increased or the antioxidant system could be damaged, resulting in imbalance of the redox system and oxidative injury of key biomacromolecules, including DNA, protein and lipids. The level of antioxidant enzyme and proteins is lower in the central nervous system (CNS) compared with other tissues, thus the CNS is more susceptible to oxidant influence or attack. Substantial literature has reported that oxidative stress is closely associated with the development of neurodegenerative disease. The levels of MDA, protein carbonylation, and oxidized albumin in blood of patients with neurodegenerative disease is higher than that in healthy individuals (25). Oxidative modification of proteins occurs under the condition from mild cognitive impairment and throughout the course of Alzheimer's disease (26). Moreover, increased ROS production is considered to be a major mechanism that mediates CoCl_2 -induced

neurotoxicity (21, 27). Thus, exploration of herbal extracts and natural compounds with antioxidant and neuroprotective activities has been regarded as a strategy to finding new therapeutic interventions for neurodegenerative diseases (28-30).

The antioxidant activities of TQ was found previously (18). In the current study, we examined the possible protective effects of TQ against CoCl_2 -induced neurotoxicity. We found that TQ exhibited a potent protective effect against CoCl_2 -induced neurotoxicity *in vitro* and *in vivo*, evidenced by inhibition of behavioral injury and apoptotic cell death. Previous studies have also shown that TQ possessed neuroprotective effects under different pathological conditions. TQ protects cultured hippocampal and human induced pluripotent stem cell-derived neurons against alpha-synuclein-induced synapse damage (31). Neuropathic pain could be attenuated by TQ (32). TQ also ameliorates lead-induced brain damage in rats (33). TQ exhibits a neuroprotective effect against 1-methyl-4-phenylpyridinium-induced dopaminergic cell death in primary mesencephalic cell culture (34).

To explore the mechanism of TQ-exhibited neuroprotective effects against CoCl_2 , we tested the effect of TQ on oxidative stress under CoCl_2 -induced pathological condition. We found that CoCl_2 -resulted oxidative stress was significantly inhibited by TQ. Nrf2/GCL-mediated upregulation of GSH was involved in the antioxidant and neuroprotective effects of TQ. GSH is an important antioxidant and detoxifying protein in the body (35, 36). Glutamate-cysteine ligase (GCL) is the rate-limiting enzyme for GSH synthesis, which consists of glutamate-cysteine ligase catalytic subunit (GCLc) and glutamate-cysteine ligase modifier subunit (GCLm) (35, 36). Nrf2 is a central transcriptional regulator of redox balance through regulation of a battery of antioxidant enzymes, including GCLc and GCLm (35, 36). Several studies have revealed that Nrf2 could be regulated by TQ under different conditions. Based on these studies and our findings, we propose that Nrf2 is an important target of TQ and activation of Nrf2, thus the downstream signaling, including GCLc/GCLm, mediates the antioxidant and neuroprotective effects of TQ.

In summary, we found that TQ exhibited protective effects against neurotoxicity via upregulation of Nrf2/GCL signaling. Upregulation of Nrf2/GCL signaling promoted the synthesis of GSH and contributed to attenuation of oxidative stress, neuronal cell apoptosis and neurotoxicity. These data have appointed a new path toward the understanding of the neuroprotective activities of TQ. TQ may be potentially developed into a candidate for the treatment of hypoxia/ischemia-related neurodegenerative diseases such as Alzheimer's and Parkinson's.

REFERENCES

1. Gratwicke J, Jahanshahi M, Foltynie T. Parkinson's disease dementia: a neural networks perspective. *Brain* 2015; 138:1454-76.
2. Paulsen JS, Nance M, Kim JJ, et al. A review of quality of life after predictive testing for and earlier identification of neurodegenerative diseases. *Prog Neurobiol* 2013; 110:2-28.
3. Won SJ, Kim DY, Gwag BJ. Cellular and molecular pathways of ischemic neuronal death. *J Biochem Mol Biol* 2002; 35:67-86.
4. Li RC, Morris MW, Lee SK, Pouranfar F, Wang Y, Gozal D. Neuroglobin protects PC12 cells against oxidative stress. *Brain Res* 2008; 1190:159-66.
5. Sandner P, Wolf K, Bergmaier U, Gess B, Kurtz A. Hypoxia and cobalt stimulate vascular endothelial growth factor receptor gene expression in rats. *Pflugers Arch* 1997; 433:803-8.
6. Zou W, Yan M, Xu W, Huo H, Sun L, Zheng Z, Liu X. Cobalt chloride induces PC12 cells apoptosis through reactive oxygen species and accompanied by AP-1 activation. *J Neurosci Res* 2001; 64:646-53.
7. Jung JY, Kim WJ. Involvement of mitochondrial- and Fas-mediated dual mechanism in CoCl₂-induced apoptosis of rat PC12 cells. *Neurosci Lett* 2004; 371:85-90.
8. Wu Y, Shang Y, Sun SG, Liu RG, Yang WQ. Protective effect of erythropoietin against 1-methyl-4-phenylpyridinium-induced neurodegeneration in PC12 cells. *Neurosci Bull* 2007; 23:156-64.
9. Abdel-Fattah AM, Matsumoto K, Watanabe H. Antinociceptive effects of *Nigella sativa* oil and its major component, thymoquinone, in mice. *Eur J Pharmacol* 2000; 400:89-97.
10. Zaoui A, Cherrah Y, Lacaille-Dubois MA, Settaf A, Amarouch H, Hassar M. [Diuretic and hypotensive effects of *Nigella sativa* in the spontaneously hypertensive rat]. *Therapie* 2000; 55:379-82.
11. Swamy SM, Tan BK. Cytotoxic and immunopotentiating effects of ethanolic extract of *Nigella sativa* L. seeds. *J Ethnopharmacol* 2000; 70:1-7.
12. Chakravarty N. Inhibition of histamine release from mast cells by nigellone. *Ann Allergy* 1993; 70:237-42.
13. Ilhan A, Gurel A, Armutcu F, Kamisli S, Iraz M. Antiepileptogenic and antioxidant effects of *Nigella sativa* oil against pentylenetetrazol-induced kindling in mice. *Neuropharmacology* 2005; 49:456-64.
14. Akhondian J, Parsa A, Rakhshande H. The effect of *Nigella sativa* L. (black cumin seed) on intractable pediatric seizures. *Med Sci Monit* 2007; 13:Cr555-9.
15. Khalife KH, Lupidi G. Nonenzymatic reduction of thymoquinone in physiological conditions. *Free Radic Res* 2007; 41:153-61.
16. Gulsen I, Ak H, Colcimen N, Alp HH, et al. Neuroprotective effects of thymoquinone on the hippocampus in a rat model of traumatic brain injury. *World Neurosurg* 2016; 86:243-49.
17. Xiao XY, Zhu YX, Bu JY, Li GW, Zhou JH, Zhou SP. Evaluation of neuroprotective effect of thymoquinone nanoformulation in the rodent cerebral ischemia-reperfusion model. *Biomed Res Int* 2016; 2016:2571060.
18. Shao YY, Li B, Huang YM, Luo Q, Xie YM, Chen YH. Thymoquinone attenuates brain injury via an anti-oxidative pathway in a status epilepticus rat model. *Transl Neurosci* 2017; 8:9-14.
19. Dariani S, Baluchnejadmojarad T, Roghani M. Thymoquinone attenuates astrogliosis, neurodegeneration, mossy fiber sprouting, and oxidative stress in a model of temporal lobe epilepsy. *J Mol Neurosci* 2013; 51:679-86.
20. Ullah I, Badshah H, Naseer MI, Lee HY, Kim MO. Thymoquinone and vitamin C attenuates pentylenetetrazole-induced seizures via activation of GABAB1 receptor in adult rats cortex and hippocampus. *Neuromolecular Med* 2015; 17:35-46.
21. Guan D, Su Y, Li Y, Wu C, Meng Y, Peng X, Cui Y. Tetramethylpyrazine inhibits CoCl₂-induced neurotoxicity through enhancement of Nrf2/GCLC/

- GSH and suppression of HIF1 α /NOX2/ROS pathways. *J Neurochem* 2015; 134:551-65.
22. Dai Y, Li W, Zhong M, Chen J, Liu Y, Cheng Q, Li T. Preconditioning and post-treatment with cobalt chloride in rat model of perinatal hypoxic-ischemic encephalopathy. *Brain Dev* 2014; 36: 228-40.
 23. Bhat AH, Dar KB, Anees S, Zargar MA, Masood A, Sofi MA, Ganie SA. Oxidative stress, mitochondrial dysfunction and neurodegenerative diseases; a mechanistic insight. *Biomed Pharmacother* 2015; 74:101-10.
 24. Wang X, Hai C. Redox modulation of adipocyte differentiation: hypothesis of “Redox Chain” and novel insights into intervention of adipogenesis and obesity. *Free Radic Biol Med* 2015; 89:99-125.
 25. Greilberger J, Koidl C, Greilberger M, et al. Malondialdehyde, carbonyl proteins and albumin-disulphide as useful oxidative markers in mild cognitive impairment and Alzheimer’s disease. *Free Radic Res* 2008; 42:633-38.
 26. Sultana R, Perluigi M, Allan Butterfield D. Lipid peroxidation triggers neurodegeneration: a redox proteomics view into the Alzheimer disease brain. *Free Radic Biol Med* 2013; 62:157-69.
 27. Lin HC, Su SL, Lu CY, et al. Andrographolide inhibits hypoxia-induced HIF-1 α -driven endothelin 1 secretion by activating Nrf2/HO-1 and promoting the expression of prolyl hydroxylases 2/3 in human endothelial cells. *Environ Toxicol* 2017; 32:918-930.
 28. Ferrante C, Recinella L, Locatelli M, et al. Protective effects induced by microwave-assisted aqueous harpagophytum extract on rat cortex synaptosomes challenged with amyloid beta-peptide. *Phytother Res* 2017; 31:1257-64.
 29. Brunetti L, Menghini L, Orlando G, et al. Antioxidant effects of garlic in young and aged rat brain in vitro. *J Med Food* 2009; 12:1166-69.
 30. Chiavaroli A, Brunetti L, Orlando G, et al. Resveratrol inhibits isoprostane production in young and aged rat brain. *J Biol Regul Homeost Agents* 2010; 24:441-46.
 31. Alhebshi AH, Odawara A, Gotoh M, Suzuki I. Thymoquinone protects cultured hippocampal and human induced pluripotent stem cells-derived neurons against alpha-synuclein-induced synapse damage. *Neurosci Lett* 2014; 570:126-31.
 32. Celik F, Gocmez C, Karaman H, et al. Therapeutic effects of thymoquinone in a model of neuropathic pain. *Curr Ther Res Clin Exp* 2014; 76:11-6.
 33. Radad K, Hassanein K, Al-Shraim M, Moldzio R, Rausch WD. Thymoquinone ameliorates lead-induced brain damage in Sprague Dawley rats. *Exp Toxicol Pathol* 2014; 66:13-17.
 34. Radad KS, Al-Shraim MM, Moustafa MF, Rausch WD. Neuroprotective role of thymoquinone against 1-methyl-4-phenylpyridinium-induced dopaminergic cell death in primary mesencephalic cell culture. *Neurosciences (Riyadh)* 2015; 20:10-16.
 35. Cao W, Hong Y, Chen H, Wu F, Wei X, Ying W. SIRT2 mediates NADH-induced increases in Nrf2, GCL, and glutathione by modulating Akt phosphorylation in PC12 cells. *FEBS Lett* 2016; 590:2241-55.
 36. Zhang J, Zhou X, Wu W, Wang J, Xie H, Wu Z. Regeneration of glutathione by alpha-lipoic acid via Nrf2/ARE signaling pathway alleviates cadmium-induced HepG2 cell toxicity. *Environ Toxicol Pharmacol* 2017; 51:30-37.

EXPRESSION OF GENES RESPONSIBLE FOR CELL MORPHOGENESIS INVOLVED IN DIFFERENTIATION IN PORCINE BUCCAL POUCH MUCOSAL CELLS DURING LONG-TERM PRIMARY CULTURE AND REAL-TIME PROLIFERATION *IN VITRO*

M. DYSZKIEWICZ-KONWIŃSKA¹, A. BRYJA², K. JOPEK³, J. BUDNA³, R. KHOZMI²,
M. JESETA⁴, D. BUKOWSKA⁵, P. ANTOSIK⁵, M. BRUSKA¹, M. NOWICKI³,
M. ZABEL^{3,6} and B. KEMPISTY^{2,3,4}

¹Department of Biomaterials and Experimental Dentistry, Poznań University of Medical Sciences, Poznań, Poland; ²Department of Anatomy, Poznań University of Medical Science, Poznań, Poland; ³Department of Histology and Embryology, Poznań University of Medical Science, Poznań, Poland; ⁴Department of Obstetrics and Gynecology, University Hospital and Masaryk University, Brno, Czech Republic; ⁵Institute of Veterinary, Poznań University of Life Sciences, Poznań, Poland; ⁶Department of Histology and Embryology, Wrocław Medical University, Wrocław, Poland

Received May 22, 2017 – Accepted September 1, 2017

Recently, using experimental animal model, we demonstrated that porcine buccal pouch mucosal cells reflect increased proliferation capability during primary cultivation *in vitro*. Although the histological structure and morphogenesis in oral cavity is well recognized, the molecular mechanisms which regulate this process still need further investigation. This study was aimed to analyze the molecular marker expression profile involved in morphogenesis and differentiation capacity of porcine buccal pouch mucosal cells during their long-term primary cultivation *in vitro*. The experiment was performed on buccal pouch mucosal cells isolated from 80 pubertal crossbred Landrace gilts. After collection, the cells were treated enzymatically and transferred into a primary *in vitro* culture (IVC) system and cultured for 30 days. The cells were collected for RNA isolation after 7, 15 and 30 days of IVC and were checked for their real-time proliferative status using the RTCA system. We found an increased expression of FN1 and SOX9 genes when calculated against ACTB after 7, and 30 days of IVC, ($P < 0.01$, $P < 0.001$, respectively). The CXCL12 mRNA was down-regulated after 7, 15 and 30 days of IVC, but not statistically significant. Similar expression profile was observed when calculated against HPRT, however, DAB2 was found to be higher expressed at day 15 of IVC, ($P < 0.05$). The cell index measured during real-time cell proliferation was substantially increased between 96 h and 147h of IVC and reached the log phase. Since FN1 and SOX9 revealed significant increase of expression after long-term culture *in vitro*, it is suggested that expression of these differentiation and stemness genes is accompanied by cell proliferation. Moreover, FN1 and SOX9 might be recognized as new markers of buccal pouch mucosal cell proliferation and differentiation in pigs in *in vitro* primary culture model.

The cells collected from adult organs and constituting primary *in vitro* culture are characterized by distinct morphology and biochemical properties

(1). Moreover, the primary cultivation system is often composed of different cell types, including cells with epithelial and/or connective tissue origin, that are

Key words: mucosal cells, pig, primary culture

Mailing address:

Bartosz Kempisty PhD,
Department of Histology and Embryology, Department of Anatomy,
Poznań University of Medical Sciences, 6 Święcickiego St.,
60-781 Poznań, Poland
Tel./Fax: +48 61 8546418/+48 61 8546440
e-mail: bkempisty@ump.edu.pl

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

distinct regarding cells physiology, metabolomics and cell cycle (2, 3). This specificity of highly heterogenous populations of primary cultured cells may be successfully reduced by mechanical separation during microsurgical tissue recovery and/or enzymatic distinguishing of cells directed into culture *in vitro*. The buccal pouch mucosa is composed of fibroblasts and keratinocytes that may be separated from culture using collagenase and trypsin (4, 5). After these procedures, the selected cells may be successfully cultured *in vitro* (IVC) for a long-term.

Our recent studies and experiments indicated that porcine buccal pouch mucosal cells substantially increased their proliferation during short-term cultivation and reached the logarithmic phase between 48-72h of IVC (6). Another feature of primary cultured cells is the high ability to differentiate even to distinct cell types. It is still undiscovered whether buccal pouch mucosal cells including fibroblasts and/or undifferentiated keratinocytes may differentiate into other cells during long-term IVC. When the high proliferation ability of mucosal cells *in vitro* is accompanied by increased capability to differentiation, there is a huge capability to build a bioactive graft in allotransplantation. The application of mucosal grafts and/or grafts originating from oral tissue may be a new direction of microsurgical dentistry, and regenerative, reconstructive medicine (7-9).

Recently, we found that porcine buccal pouch mucosa cultured primary *in vitro* is composed of cells with both epithelial and stromal origin. In this heterogenous cell population, we identified both fibroblasts and keratinocytes. Although, the increased proliferation ability of these cells *in vitro* was well determined in our studies, the differentiation capability of these cells during long-term IVC in pig model has never been investigated previously.

In this study, we therefore investigated the selected markers of genes involved in cellular morphogenesis and their differentiation ability. These genes included MAP1B, DAB2, FN1, SOX9, CXCL12, and were selected based on our recent porcine oocyte study (10). Using RT-qPCR, we analyzed the expression of these genes in cells after each stage of IVC in order to investigate the “differentiation status” of porcine buccal pouch mucosal cells.

MATERIALS AND METHODS

Animals

For this study, a total of 40 pubertal crossbred Landrace gilts bred on a commercial local farm were used. They had a mean age of 155 days (range 140 – 170 days), and the mean weight was 100 kg (95-120 kg). All of the animals were housed under identical conditions and fed the same forage (depending on age and reproductive status). The experiments were approved by the Local Ethic Committee.

Cell isolation and culture

After slaughter, samples of buccal pouch mucosa were obtained within 40 min and transported to the laboratory. The excised tissue was washed twice in Dulbecco's phosphate buffered saline (D-PBS) (137 mM NaCl, 27 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4). The surface of the buccal pouch was surgically removed using sterile surgical blades. The tissue fragments were incubated with 0.05% collagenase I (Sigma Aldrich, Madison, USA) for 25 min at 38°C in a shaking water bath and then were treated with 0.05% Trypsin/EDTA (Cascade Biologics, Portland, USA) for 10 min. The cell suspension obtained from this digestion was filtered through mesh to remove non-dissociated tissue fragments. Isolated cells were washed three times by centrifugation (10 min at 200 g) with Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich, Madison, USA) supplemented with gentamicin (20 µg/mL) and 0.1% BSA. The final cell pellet was resuspended in DMEM supplemented with 10% fetal calf serum (FCS; Sigma Aldrich, Madison, USA) and 100 U/mL penicillin G, 10 mg/mL streptomycin, and 25 µg/mL amphotericin B (antibiotic antimycotic solution; Sigma Aldrich, Madison, USA). Cell viability was 90-95% as determined by trypan blue staining (Sigma Aldrich, Madison, USA). The cells were cultured at 38.5°C in a humidified atmosphere of 5% CO₂. Once the keratinocyte cultures attained 70–80% confluency, they were passaged by washing with PBS, digested with 0.025% Trypsin/EDTA (Cascade Biologics, Portland, USA), neutralized by a 0.0125% trypsin inhibitor (Cascade Biologics, Portland, USA), centrifuged, and resuspended at a seeding density of 2x10⁴ cells/cm². The culture medium was changed every three days.

In vitro porcine buccal pouch mucosal cell cultivation using a real-time cell analyzer (RTCA)

The recovered buccal pouch mucosal cells were transferred into an E-Plate 16 of a real-time cell analyzer (RTCA; Roche-Applied Science, GmbH, Penzberg, Germany), which consisted of an RTCA analyzer, an RTCA SP station, and RTCA software. The cells were then cultured in 200 µl of standard porcine culture medium that consisted of Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, USA), as mentioned above. The cells were cultured for 0-168 h at 38.5°C under 5% CO₂ in air. The cultivation medium was replaced every three days. After each period of cultivation, the cells were treated with trypsin (0.25% trypsin in a balanced salt solution; Sigma-Aldrich, St. Louis, MO, USA), and the collected pool of proliferating cells was used for RT-qPCR analysis. After each cultivation period, the cell index (CI) was used to evaluate the relative and quantitative changes in electrical cell impedance. The cell status was determined using RTCA software.

Real-time quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was isolated from porcine buccal pouch

mucosal cells using an RNeasy mini column from Qiagen GmbH (Hilden, Germany). The RNA samples were resuspended in 20 µl of RNase-free water and stored in liquid nitrogen. RNA samples were treated with DNase I and reverse-transcribed (RT) into cDNA. RT-qPCR was conducted in a LightCycler real-time PCR detection system (Roche Diagnostics GmbH, Mannheim, Germany) using SYBR® Green I as a detection dye, and target cDNA was quantified using the relative quantification method. The relative abundance of MAP1B, DAB2, FN1, SOX9 and CXCL12 transcripts in each sample was standardized to the internal standards ACTB and HPRT. For amplification, 2 µl of cDNA solution was added to 18 µl of QuantiTect® SYBR® Green PCR (Master Mix Qiagen GmbH, Hilden, Germany) and primers (Table I). One RNA sample of each preparation was processed without the RT-reaction to provide a negative control for subsequent PCR.

To quantify the specific genes expressed in buccal pouch mucosal cells, the expression levels of specific buccal pouch mucosal cell mRNAs in each sample were calculated relative to HPRT and ACTB levels. To ensure the integrity of these results, an additional housekeeping

Table I. Oligonucleotide sequences of primers used for RT-qPCR analysis.

Gene	Gene accession number	Primer sequence (5'-3')	Product size (bp)
MAP1B	XM_003134032.4	TGGACCTGTGCTATATTCCCA CCTCATCACCTCCGAGTCA	218
DAB2	XM_003133906.5	ATCAGACATCTTTGCTCCTCC CTAAAGACGCTGGGTTCAT	191
FN1	XM_013984465.1	GAATATCTCGGTGCCATTTGCT ACTCTCGGGAATCTTCTATGTCA	229
SOX9	NM_213843.1	AGCAGACGCACATCTCTCCCA CGCCCCTCTCGCTTCAGGTCA	190
CXCL12	NM_001009580	GCCAGAGCCAACATCAAGCA AAACATCCCGCCGTCCTCA	250
ACTB	XM_003124280.3	CCCTTGCCGCTCCGCCTTC GCAGCAATATCGGTCATCCAT	69
HPRT	NM_001032376.2	CCATCACATCGTAGCCCTC ACTTTTATATCGCCCGTTGAC	171

gene was used as an internal standard to demonstrate that HPRT and ACTB were not differentially regulated in buccal pouch mucosal cell groups. Expression of HPRT and ACTB was measured in cDNA samples from isolated buccal pouch mucosal cells.

Statistical analysis

A one-way ANOVA, followed by Tukey's post-hoc test, was used to compare the results of the RT-qPCR. The experiments were carried out in a minimum three replicates. The results for 7, 15 and 30 days of IVC were compared to the control (24 h of IVC). The differences were considered to be significant at $*P<0.05$, $**P<0.01$, and $***P<0.001$ for the quantitative analysis of mRNA expression. The software program GraphPad Prism

version 4.0 (GraphPad Software, San Diego, CA, USA) was used for the statistical calculations.

RESULTS

Using RT-qPCR, the mRNA expression encoding proteins such as MAP1B, DAB2, FN1, SOX9 and CXCL12, responsible for cell morphogenesis involved in differentiation, was determined in porcine buccal pouch mucosal cells for the first time. The gene expression profile was calculated against two housekeeping genes as ACTB and HPRT (Figs. 1 and 2). When measured against ACTB (Fig. 1), we observed significantly increased expression of FN1 mRNA after 30 days of IVC as compared to

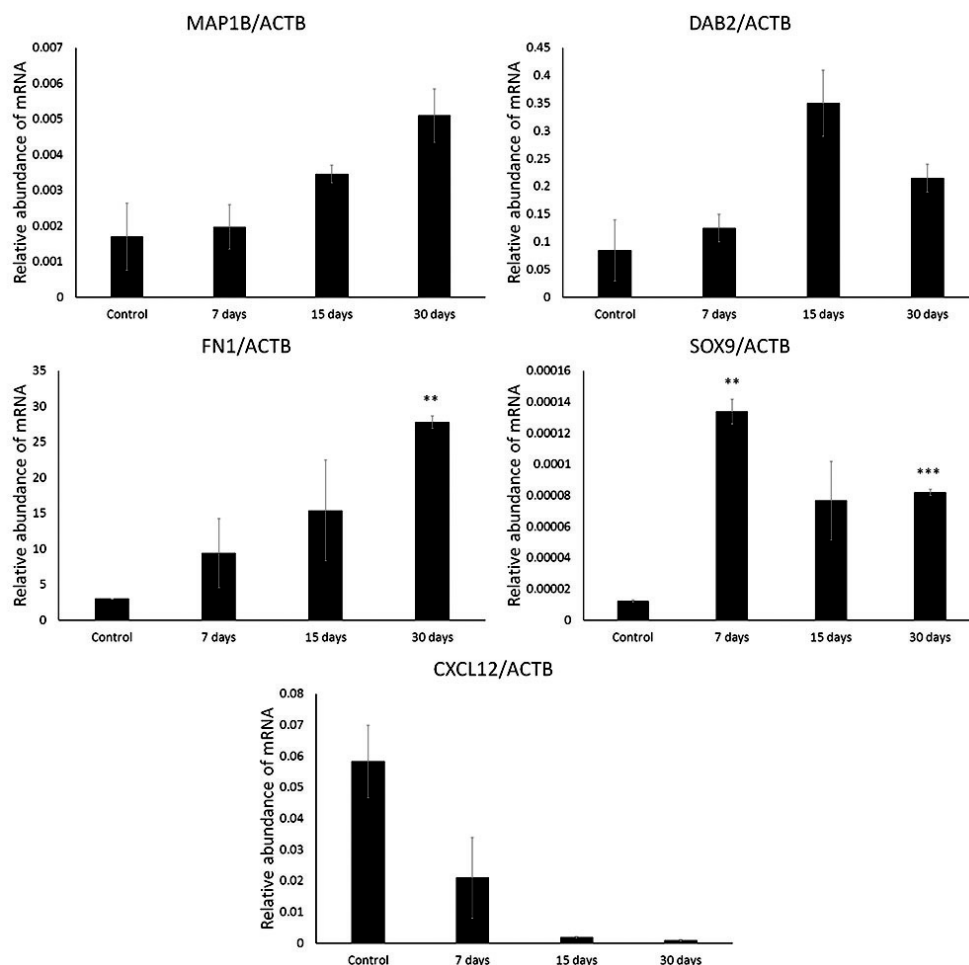


Fig. 1. Relative abundance of MAP1B, DAB2, FN1, SOX9, CXCL12 transcripts related to ACTB in porcine buccal pouch mucosal cells analyzed during 30 days of IVC. Porcine buccal pouch mucosal cells were isolated from pubertal gilts and immediately used to isolate RNA, which was reverse-transcribed into cDNA. The relative abundance of mRNAs was evaluated by RT-qPCR analysis before and after 7, 15 and 30 days of IVC. The results are presented as the mean $\pm$ SEM with the level of significance shown as $*P<0.05$, $**P<0.01$, $***P<0.001$.

controls, ($P < 0.01$). The FN1 transcript profile after 7 days and 15 days was also higher, but not statistically significant compared to controls. Moreover, we found up-regulation of the SOX9 mRNA expression after 7 days and 30 days of IVC as compared to controls ($P < 0.01$, and $P < 0.001$, respectively). The MAP1B and DAB2 transcripts revealed increased expression after long-term (15 days and 30 days) of IVC, however, the results were not significant. Conversely, the CXCL12 expression profile was down-regulated after 7 days, 15 days and 30 days of IVC as compared to controls. Similarly, when gene expression was analyzed against HPRT (Fig. 2), we found significantly increased expression of SOX9 after 7 days and 30 days of IVC ($P < 0.01$, and

$P < 0.05$, respectively). The FN1 mRNA profile was also higher after 30 days of IVC ($P < 0.05$). However, we found higher expression of DAB2 after 15 days of IVC as compared to controls ($P < 0.05$).

The buccal pouch mucosal cell proliferation was analyzed in real-time using the RTCA system. The cell proliferation index was measured between 0h and 147h of IVC. We found lag phase of proliferation between 0h and 96h of IVC (Fig. 3 A-E), when the log phase was reached between 96h and 147h of IVC (Fig. 3F-G). The value of cell index at the level between 0-1.3 between 96h and 123h of IVC, and 0.7-1.6 between 120h and 147h of IVC was observed to be substantially different from cell index reached during lag phase (Fig. 3 C-D).

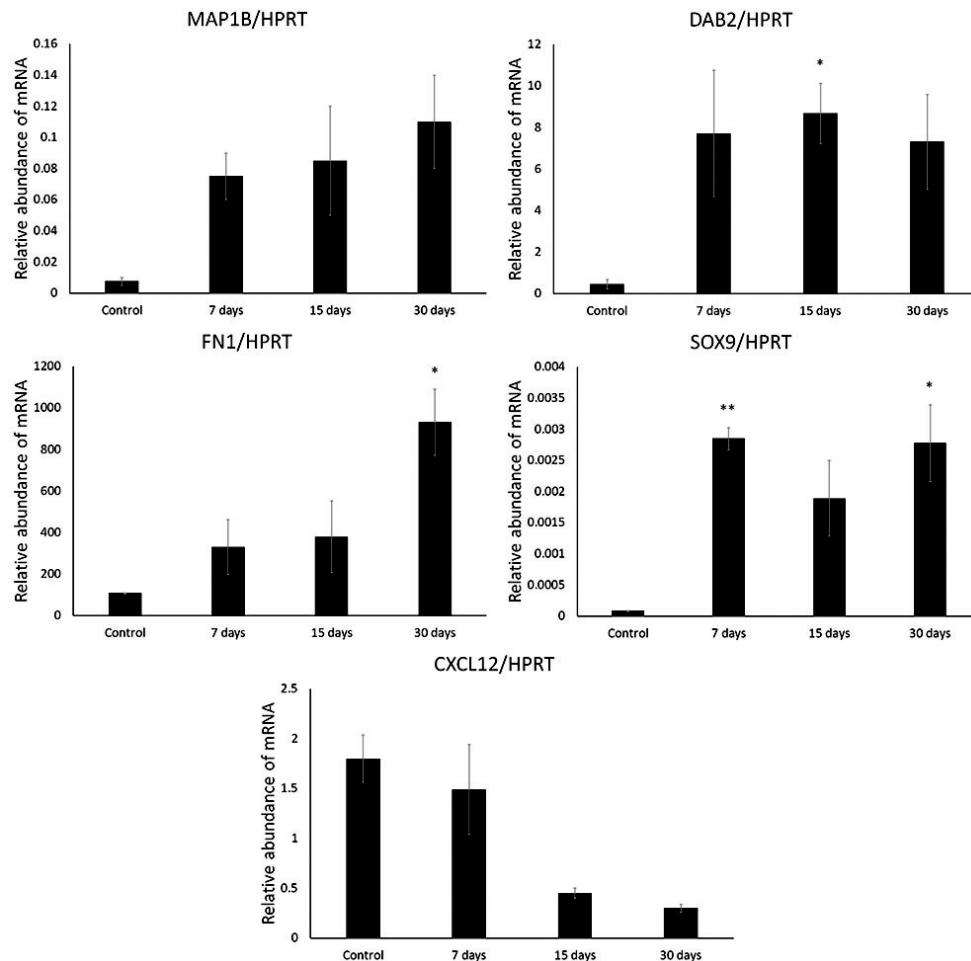


Fig. 2. Relative abundance of MAP1B, DAB2, FN1, SOX9, CXCL12 transcripts related to HPRT in porcine buccal pouch mucosal cells analyzed during 30 days of IVC. Porcine buccal pouch mucosal cells were isolated from pubertal gilts and immediately used to isolate RNA, which was reverse-transcribed into cDNA. The relative abundance of mRNAs was evaluated by RT-qPCR analysis before and after 7, 15, and 30 days of IVC. The results are presented as the mean $\pm$ SEM with the level of significance shown as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

DISCUSSION

Buccal pouch mucosal cells undergo substantial morphological and biochemical modification during their physiological lifespan. The biochemical changes include the differences in gene expression profile as response to extracellular environmental changes such as drugs delivery and administration. The permanent

oral mucosal cell modifications are much more often recognized as the cellular ability to differentiation and/or transdifferentiation *in vivo* and/or *in vitro*. Therefore, it is clearly suggested that these cells have an increased stemness potency, which is the basic feature that must be met in application of tissue grafts to advanced regenerative dentistry. The differentiation capability of oral mucosa cells is well recognized,

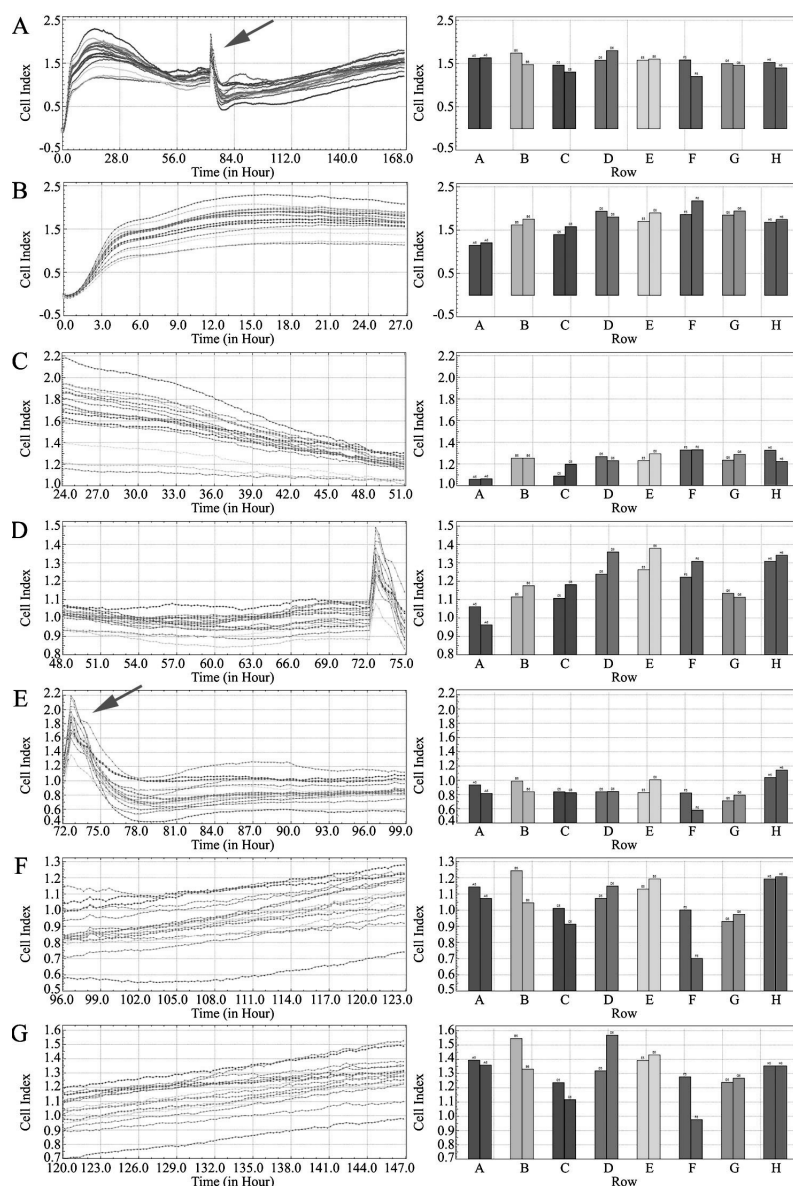


Fig. 3. Cell proliferation index (CI) of porcine buccal pouch mucosal cells cultivated for 150 h. Porcine buccal pouch mucosal cells were recovered from pubertal gilts and treated with collagenase for 10 min at 38.5°C. The cells were immediately transferred into an E-Plate 48 of a real-time cell analyzer (RTCA; Roche-Applied Science, GmbH, Penzberg, Germany). The experiment consisted of eight replicates involving the cultivation of the same population of collected cells. The arrow shows the peak, that is unique to the mucosal cells.

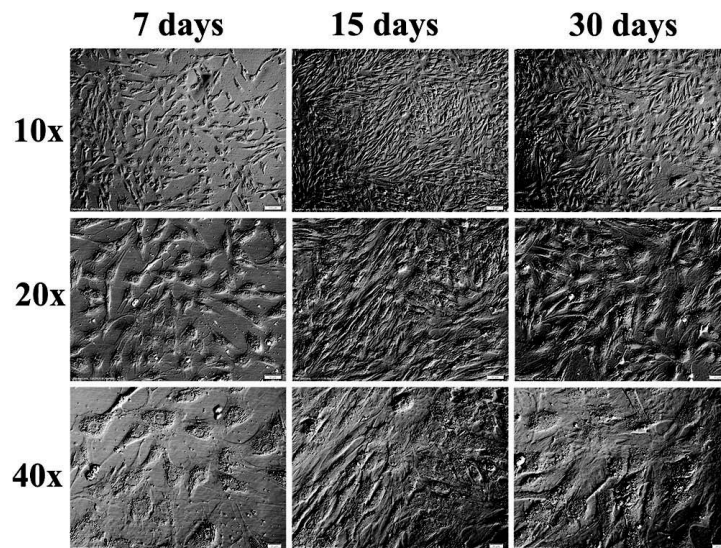


Fig. 4. Long-term cellular proliferation, in vitro culture (IVC) monitoring, and Nomarski phase/contrast images. The porcine buccal pouch oral mucosal cells were cultured for 30 days using Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, USA). On 7th, 15th and 30th days of IVC, cells were monitored for proliferation index and morphological changes following achievement of confluency. Magnifications shown are 10x, 20x, and 40x.

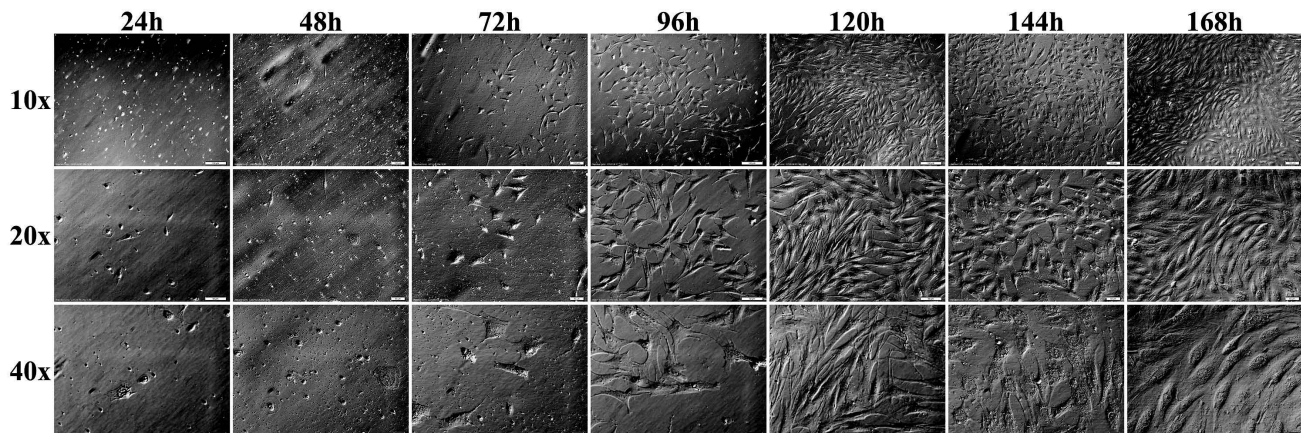


Fig. 5. Short-term cellular proliferation, in vitro culture (IVC) monitoring, and Nomarski phase/contrast images. The porcine buccal pouch oral mucosal cells were cultured for 168 h using Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, USA). Every 24 h of IVC, cells were monitored for proliferation index and morphological changes following achievement of confluency. Magnifications shown are 10x, 20x, and 40x.

however, the differential expression profile of genes involved in this process such as MAP1B, DAB2, FN1, SOX9 and CXCL12, has never been investigated previously in a porcine model.

First of all, in the context of tissue engineered cultivated grafts, the ability to identify stem cell population is of great value. There has been an ongoing search for stemness markers in the last

decade. Nakamura et al. (11) reported in their study that p75, a low-affinity neurotrophin receptor, is a potential marker for oral mucosal epithelial stem/progenitor cells. Ishii et al. confirmed this finding in their study, with the results demonstrating that p75NGFR (+) basal keratinocytes in the oral mucosa possess the ability to migrate and regenerate the epithelium following an injury (12). To increase

the probability of long-term successful tissue regeneration, it is important that stem/progenitor cells are retained in the cultivated oral mucosal cell lines, as these cells have greater proliferative potential. That is why the interesting feature of SOX factors is that they can function as regulators of stem/progenitor cell potency and competence (13). The imbalance of SOX factor levels can result in severe congenital defects. Several conditions in humans and mice are associated with subtle changes in regulation or haploinsufficiency of some Sox genes. For example, distinct hypomorphic levels of Sox2 result in esophageal atresia and tracheoesophageal fistula (14) and a range of eye phenotypes from anophthalmia to microphthalmia (15). In both cases of SOX factor deficiency, aberrant maintenance and differentiation of the stem/progenitor cell pool leads to the clinical manifestation of disorder. Taken together, these findings indicate that discrete levels of SOX factors play an important role in normal differentiation of stem/progenitor cell populations. Formeister et al. confirmed in their observations that there are two distinct levels of Sox9EGFP expression, termed Sox9EGFP^{HI} and Sox9EGFP^{LO}, in the base of the crypt of intestine mucosa (13). Because distinct levels of SOX factors appear to be critical for proper proliferation and differentiation in stem cell biology, we hypothesized that differential SOX9 expression may be playing a similar role in stem/progenitor cell populations in the porcine buccal mucosa. When analyzing gene expression against HPRT, we found significantly increased expression of SOX9 after 7 days and 30 days of IVC ($P < 0.01$, and $P < 0.05$, respectively). We followed with the analysis of Disabled-2 (DAB2) that was first identified as DOC-2, for differentially expressed in ovarian carcinoma (16), and subsequently as a down regulated protein in oesophagus and colon cancer (17).

DAB2 is involved in regulation of cytoskeleton-based functions by regulating cell positioning, inducing macrophage adhesion, or regulating protein trafficking by interacting with different proteins involved in endocytosis (18). It plays an important role in regulating the homeostasis of epithelial differentiation, a role confirmed by several genetic studies demonstrating a function for DAB2

in endodermal cell formation, differentiation, and organization (19). DAB2 is also engaged in diverse signal transduction pathways. It has been reported that DAB2 interferes with mitogenic growth factor signal transduction (20) as well as with Wnt signaling (21).

DAB2 can also be a positive mediator of TGF β signaling by bridging the heteromeric TGF β receptor complex to the Smad proteins (22). However, the biological role of DAB2 in TGF β signaling has not yet been fully recognized.

Data show that the decrease in integrin signaling which is initiated by down-regulation of DAB2, leads to a blockade of TGF β -induced EMT and, in consequence, to an increase in apoptosis. In our study, DAB2 transcripts revealed increased expression after long-term (15 days and 30 days) of IVC.

The FN1 mRNA profile was also higher after 30 days of IVC ($P < 0.05$). However, we found higher expression of DAB2 after 15 days of IVC as compared to controls.

Fibronectin is a protein secreted by fibroblasts during development, wound healing, and other processes involving extracellular matrix remodeling. The process consist of few stages: Fn binding to cells, mechanical coupling of Fn to the cytoskeleton by integrins, elongation of fibrils by Fn polymerization. The initial cell attachment, exposure of the integrin-binding arginine-glycine-aspartic acid loop, and availability of self-assembly sites are all most likely regulated by Fn arrangement (23).

We also analyzed the expression of MAP1B transcripts which revealed increased expression after long-term (15 days and 30 days) of *in vitro* cultivation. MAP1B protein is localized to axons and their precursors (so-called 'minor neurites') in developing cultured neurons. MAP1B is specifically enhanced in growing axons (24). Additionally, Vecino et al. observed increased expression of MAP1B in regenerating optic nerves. The regenerating CNS neurons of the fish have a pattern of MAP1B-phos expression similar to that found in regenerating neurons in the PNS of the mammal (25). MAP1B in our study presented increased expression after long term IVC.

It can be suggested that these findings stand in

line with well-known potential for regeneration of mucosal tissue. In further hypothesizing, we could speculate that these results present the potential for regeneration that is orchestrated by genes involved in neuron regeneration, healing processes and angiogenesis.

The angiogenic properties of CXCL12 are well known. It is involved in the outgrowth and metastasis of CXCR4-expressing tumors. CXCL12/CXCR4 interactions play an important role in the migration of hematopoietic stem cells and their progenitors from the bone marrow (26). In our study, the CXCL12 expression profile was down-regulated after 7 days, 15 days and 30 days of IVC compared to controls. Most studies analyzing the role of CXCL12 in mucosal angiogenesis are focused on intestinal tissue and emphasize its potential role in tumorigenesis.

By using studies of gene expression in embryonic stem cells or cells which have begun to differentiate, a panel of genes has been identified as biomarkers of the difference between the two states. These biomarkers could be used as a simple test of the differentiation state of a cell culture (27).

In conclusion, our results reveal the presence of selected genes involved in cellular morphogenesis and differentiation ability in the porcine buccal mucosa IVC. The high proliferative activity of oral mucosa and its regenerative potential make it a tissue of great interest. Literature is available for a number of studies on pathophysiology of mucosal tissue but the mechanisms that regulate its function in physiological conditions are still unknown. Such mechanisms are necessary to be reproduced in order to receive satisfactory effects in regenerative and reconstructive medicine.

REFERENCES

1. Sa G, Xiong X, Wu T, Yang J, He S, Zhao Y. Histological features of oral epithelium in seven animal species: As a reference for selecting animal models. *Eur J Pharm Sci* 2016; 81:10-17.
2. Kaur P, Li A. Adhesive properties of human basal epidermal cells: an analysis of keratinocyte stem cells, transit amplifying cells, and postmitotic differentiating cells. *J Invest Dermatol* 2000; 114:413-20.
3. Borys S, Khozmi R, Kranc W, Bryja A, Dyszkiewicz-Konwińska M, Jeseta M, Kempisty B. Recent findings of the types of programmed cell death. *Adv Cell Biol* 2017; 5:45-52.
4. Klingbeil MFG, Herson MR, Cristo EB, dos Santos Pinto D JR, Yoshito D, Mathor MB. Comparison of two cellular harvesting methods for primary human oral culture of keratinocytes. *Cell Tissue Bank* 2009; 10:197-204.
5. Bayar GR, Aydintug YS, Gulses A, Elci P, Sarper M. A pilot study of the primary culture of the oral mucosa keratinocytes by the direct explant technique. *Oral Health Dent Manag* 2011; 2011.
6. Bryja A, Dyszkiewicz-Konwińska M, Chachula A, et al. Differential expression and distribution of cytokeratins and vimentin in buccal pouch mucosal cells during real-time cell proliferation: research based on a porcine model. *J Biol Regul Homeost Agents* 2016; 30:951-60.
7. Bardag-Gorce F, Oliva J, Wood A, et al. Carrier-free cultured autologous oral mucosa epithelial cell sheet (CAOMECS) for corneal epithelium reconstruction: a histological study. *Ocul Surf* 2015; 13:150-63.
8. Güneş M, Altok M, Güneş A, et al. A novel approach to penile augmentation urethroplasty using buccal mucosa and amniotic membrane: a pilot study in a rabbit model. *Urology* 2016; 87:210-15.
9. Heller M, Frerick-Ochs EV, Bauer H-K, et al. Tissue engineered pre-vascularized buccal mucosa equivalents utilizing a primary triculture of epithelial cells, endothelial cells and fibroblasts. *Biomaterials* 2016; 77:207-15.
10. Budna J, Bryja A, Celichowski P, et al. Genes of cellular components of morphogenesis in porcine oocytes before and after IVM. *Reproduction* 2017 [epub].
11. Nakamura T, Endo K-i, Kinoshita S. Identification of human oral keratinocyte stem/progenitor cells by neurotrophin receptor p75 and the role of neurotrophin/p75 signaling. *Stem Cells* 2007; 25:628-38.
12. Ishii A, Muramatsu T, Lee J-M, Higa K, Shinozaki N, Jung H-S, Shibahara T. Expression of p75(NGFR), a proliferative and basal cell marker, in the buccal mucosa epithelium during re-epithelialization. *Acta*

- Histochem Cytochem 2014; 47:145-53.
13. Formeister EJ, Sionas AL, Lorange DK, Barkley CL, Lee GH, Magness ST. Distinct SOX9 levels differentially mark stem/progenitor populations and enteroendocrine cells of the small intestine epithelium. *Am J Physiol Gastrointest Liver Physiol* 2009; 296:G1108-18.
 14. Que J, Okubo T, Goldenring JR, et al. Multiple dose-dependent roles for Sox2 in the patterning and differentiation of anterior foregut endoderm. *Development* 2007; 134:2521-31.
 15. Fantes J, Ragge NK, Lynch S-A, et al. Mutations in SOX2 cause anophthalmia. *Nat Genet* 2003; 33:461-63.
 16. Mok SC, Chan WY, Wong KK, et al. DOC-2, a candidate tumor suppressor gene in human epithelial ovarian cancer. *Oncogene* 1998; 16:2381-87.
 17. Anupam K, Tusharkant C, Gupta SD, Ranju R. Loss of disabled-2 expression is an early event in esophageal squamous tumorigenesis. *World J Gastroenterol* 2006; 12:6041-45.
 18. Prunier C, Howe PH. Disabled-2 (Dab2) is required for transforming growth factor beta-induced epithelial to mesenchymal transition (EMT). *J Biol Chem* 2005; 280:17540-48.
 19. Morris SM, Tallquist MD, Rock CO, Cooper JA. Dual roles for the Dab2 adaptor protein in embryonic development and kidney transport. *EMBO J* 2002; 21:1555-64.
 20. Zhou J, Hsieh JT. The inhibitory role of DOC-2/DAB2 in growth factor receptor-mediated signal cascade. DOC-2/DAB2-mediated inhibition of ERK phosphorylation via binding to Grb2. *J Biol Chem* 2001; 276:27793-98.
 21. Hocevar BA, Mou F, Rennolds JL, Morris SM, Cooper JA, Howe PH. Regulation of the Wnt signaling pathway by disabled-2 (Dab2). *EMBO J* 2003; 22:3084-94.
 22. Hocevar BA, Smine A, Xu XX, Howe PH. The adaptor molecule Disabled-2 links the transforming growth factor beta receptors to the Smad pathway. *EMBO J* 2001; 20:2789-801.
 23. Baneyx G, Baugh L, Vogel V. Coexisting conformations of fibronectin in cell culture imaged using fluorescence resonance energy transfer. *Proc Natl Acad Sci USA* 2001; 98:14464-68.
 24. Gonzalez-Billault C, Avila J, Caceres A. Evidence for the role of MAP1B in axon formation. *Mol Biol Cell* 2001; 12:2087-98.
 25. Vecino E, Avila J. Distribution of the phosphorylated form of microtubule associated protein 1B in the fish visual system during optic nerve regeneration. *Brain Res Bull* 2001; 56:131-37.
 26. Liekens S, Schols D, Hatse S. CXCL12-CXCR4 axis in angiogenesis, metastasis and stem cell mobilization. *Curr Pharm Des* 2010; 16:3903-20.
 27. YAP DY, Smith DK, Zhang XV, Hill J. Using biomarker signature patterns for an mRNA molecular diagnostic of mouse embryonic stem cell differentiation state. *BMC Genomics* 2007; 8:210.

TEA CATECHINS INDUCE CROSSTALK BETWEEN SIGNALING PATHWAYS AND STABILIZE MAST CELLS IN ULCERATIVE COLITIS

A. GERGES GEAGEA^{1,2}, M. RIZZO², A. EID¹, I. HAJJ HUSSEIN³, Z. ZGHEIB¹,
M.N. ZEENNY¹, R. JURJUS⁴, M.L. UZZO⁵, G.F SPATOLA⁵, G. BONAVENTURA⁵,
A. LEONE⁵, L. MASSAAD-MASSADE^{6,7,8} and A. JURJUS¹

¹Department of Anatomy, Cell Biology and Physiological Sciences, Faculty of Medicine, American University of Beirut, Beirut, Lebanon; ²Department of Internal Medicine, University of Palermo, Italy; ³Oakland University William Beaumont School of Medicine, Michigan USA; ⁴Department of Anatomy and Regenerative Biology, George Washington University, Washington D.C., USA; ⁵"BIONECA" Biomedicine e Clinical Neurosciences Department – Section of Histology, Embryology and Anatomy, School of Medicine and Surgery, Palermo, Italy; ⁶Université Paris-Sud 11, Laboratoire de Vectorologie et Thérapeutiques Anticancéreuses, UMR 8203, Villejuif, France; ⁷CNRS UMR 8203, Villejuif, Laboratoire de Vectorologie et Thérapeutiques Anticancéreuses, UMR 8203, France; ⁸Gustave Roussy, Laboratoire de Vectorologie et Thérapeutiques Anticancéreuses, UMR 8203, Villejuif, France

Received October 1, 2017 – Accepted October 24, 2017

It is well documented that nutraceuticals, in general, and Green tea catechins, in particular, possess a potential therapeutic value in inflammatory bowel diseases (IBD) due to their anti-oxidative and anti-inflammatory effects. This study aimed to investigate the possible mechanism of action of catechins in a rat model of colitis induced by 2,4,6 trinitrobenzene sulfonic acid (TNBS). Thirty-five young adult Sprague-Dawley rats were divided into four groups: normal control (n=5), catechins (n=9), TNBS (n=9) and TNBS plus catechins (n=12) treated. Catechin in the form of Epigallocatechin-3-gallate (EGCG) was administered daily by intraperitoneal injection, 1 week before the induction date of UC. Biopsies of the descending colon were collected on days 3, 10 and 17, and partly frozen for molecular studies or fixed for light microscopy. The status of intestinal tissue alterations and mast cells number were also assessed, as well as the mRNA expressions of IL-6, TNF- α and NF- κ B, and determination of ROS expression. Histological data depicted a significant amelioration in the TNBS- and EGCG-treated rats compared to the non-treated animals. Catechin expressed strong anti-inflammatory and anti-oxidant effects, ameliorated ulcerative colitis and stabilized mast cells. The mechanism of action occurred basically through the NF- κ B pathway and possibly through a crosstalk with other pathways.

Many areas of current investigations focus on the role and effects of natural products, especially dietary habits that have survived the test of time, on human

health and disease (1). Nutraceuticals, including tea catechins, are being increasingly used in preventing and treating various health disorders including

Key words: IBD, EGCG, TNBS, colitis, mast cells, ROS, TNF- α , IL-6, NF- κ B, nutraceuticals

Mailing address:

Dr. Abdo Jurjus,
Department of Anatomy,
Cell Biology and Physiological Sciences, Faculty of Medicine,
American University of Beirut, P.O. Box 110236/41
Riad El Solh, Beirut 1107-2020 - Beirut, Lebanon
Tel.: +961 3 308716 - Fax: +961 1 480 87
e-mail: aj00@aub.edu.lb

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

ulcerative colitis. They are also being described as providing extra health benefits in addition to their original nutritional value (2).

For years, inflammatory bowel diseases (IBDs) have been included in the spectrum of such health disorders responsive to catechins. Their mechanisms of action in IBDs are somehow complex, be it in ulcerative colitis (UC) or in Crohn's disease (CD). Many experimental models that mimic IBDs have been reported to test their therapeutic value. The colitis model in rats induced by trinitrobenzene sulfonic acid (TNBS) has been commonly used to assess drug effects because of its great similarity to human IBD (3). This experimental model is associated with the increase of clinical signs and symptoms and of histological alterations. It is also characterized by the presence of oxidative stress in addition to infiltration of the mucosa by polynuclear leukocytes, activation of mast cells and other cells to secrete pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 Beta (IL-1 β), and interleukin-6 (IL-6) among others. This secretory cascade leads to the activation of the NF- κ B pathway as well as other possible pathways, thus inducing an inflammatory response in the gut and consequently tissue damage (4).

On the other hand, the current therapeutic strategies for IBD present limitations due to the occurrence of multiple and serious side effects, often alongside unreachable remission. Therefore, the continuous search for effective molecules to improve its management is progressing. Thus, EGCG, a natural antioxidant in green tea, possesses an array of biological and medicinal properties and could provide a potential alternative approach for the management of IBD (5).

Epigallocatechin-3-gallate (EGCG), as a nutraceutical, has been implicated in many preventative and treatment regimens due to its multiple properties, including antioxidant, anti-carcinogen, anti-obesity, anti-bacterial, anti-viral and anti-enzymatic effects (6, 7). Many studies have shown its positive effects on diabetes, Parkinson's disease, Alzheimer's disease and stroke (5) as well as inhibiting tumor promotion and progression by inhibiting the release of TNF- α (8).

Moreover, EGCG has been documented to

inhibit multiple signaling pathways linked to inflammation like NF- κ B and activator protein-1 (AP-1) among others.

The objective of this study was to evaluate the mechanisms of the anti-inflammatory effect of EGCG in a model of TNBS-induced colitis in rats and to assess the mechanisms of action of this nutraceutical in the management of intestinal inflammation through its effect on the activation and nuclear translocation of NF- κ B. It is expected that the crosstalk of NF- κ B signaling pathway with other pathways is also affected, thus leading to an improvement in macroscopic and histological alterations, decrease in mast cell numbers, and enhancement of the antioxidant activity of EGCG on the production of reactive oxygen species (ROS) as well as on the modulation of TNF- α and IL-6 in the intestinal tissues.

MATERIALS AND METHODS

Animals

The experiment used a total of 35 adult male Sprague-Dawley rats, weighing 200-225 g. The animals were housed in the Animal Care Facility of the American University of Beirut, in rack-mounted wire cages, with a maximum of 5 rats per cage and kept on a 12 hours light/dark cycle in a controlled temperature and humidity room. Standard laboratory pelleted formula and tap water were provided ad libitum. The experiment was carried out in accordance to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the American University of Beirut.

Experimental design

The rats were randomized into 4 groups: a control group (n = 5) receiving only saline intraperitoneally, an EGCG group (n = 9) receiving only EGCG, a TNBS group (n = 9) with colonic inflammation and a fourth TNBS + EGCG group (n = 12) with colonic inflammation but treated with EGCG.

Colitis was induced by intrarectal administration of 20mg/kg of TNBS (Sigma-Aldrich, 92822-1ML), dissolved in 100 μ L of 50% (v/v) ethanol, once a week for a period of three weeks. After an overnight fasting with free access to water, the induction of colitis in

rats was conducted according to the method originally described by Morris et al. (9).

For the groups receiving EGCG, 1mg/kg EGCG (Sigma-Aldrich, 4268-100MG) was dissolved in 100 μ L of saline and injected intraperitoneally, 7 days before induction, to assess the preventive effect of EGCG daily throughout the rest of the experiment (10). The rats were anesthetized and sacrificed at the specified time points, days 3, 10 and 17. The descending colon was excised. Biopsies, of approximately 1 cm each, from the colon were removed; one was immersed in 4% paraformaldehyde for histological analysis and another was stored at -80°C for oxidative stress analysis. The remaining piece was cleaned with a syringe filled with phosphate buffered saline (PBS) pH 7.4 to get rid of stools and opened longitudinally. The mucosa and submucosa were scraped and transferred into labeled aliquots and then stored at -80°C for extraction of RNA and molecular analysis of TNF- α , NF- κ B and IL-6.

Macroscopic and histologic assessments of colonic damage

Macroscopic damage was assessed by two observers blinded according to previously established scoring criteria that take into account the size of the affected area, morphology of the descending colon, presence of adhesions, vasodilation, mushroom-like structures (polyps), necrosis, and wall thickness (11).

For assessment of microscopic damage, colonic biopsies were fixed with 4% paraformaldehyde for 24 h and then embedded in paraffin. Five- μ m thick sections were cut and stained with hematoxylin and eosin (H&E) for routine analysis or with toluidine blue (TB) for mast cell counting, or with periodic acid Schiff (PAS) for goblet cell inspection. The extent of inflammation of the colon was graded semi-quantitatively on a scale from 0 to 3 according to previously published criteria (11). Six parameters were evaluated: status of the epithelium, loss of goblet cells, tissue edema, cryptic abscess and infiltration of inflammatory cells (11).

RNA extraction and quantitative real-time PCR (qRT-PCR)

For the analysis of gene expression of TNF- α , NF- κ B and IL-6, total RNA was first extracted from colonic tissue using TRIzol reagent (TRI Reagent®, Sigma-Aldrich, T9424- 100ML) according to the manufacturer's protocol. 1 μ L RNA was diluted in 20 μ L of sterile water containing diethyl pyrocarbonate (DEPC), the quantity and quality of the extracted RNA were then determined

using a spectrophotometer (NanoDrop ND- 1000) by reading absorbance at 260 nm.

Reverse transcription (RT) of RNA to cDNA was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, K1622).

The real-time PCR was performed with 0.05 μ g cDNA in a final volume of 25 μ L using the QuantiFast SYBR Green Master Mix PCR Kit (QIAGEN) and the following primers: Rel-a sense 5'-CCGTGAGGCTGTTTGGTTTG-3', antisense 5'-GGTCTGCCCTCCTGACTCTA-3'; IL-6 sense 5'-GCCCTTCAGGAACAGCTATGA-3', antisense 5'-TGTCACAACATCAGTCCCAAGA-3'; TNF- α sense 5'-AACACACGAGACGCTGAAGT-3', antisense 5'-TCCAGTGAGTTCCGAAAGCC-3'. The expression of these genes was normalized to the expression of GAPDH; sense 5'-GGGGCTCTCTGCTCCTCCCTG-3', antisense 5'-CGGCCAAATCCGTTCACACCG-3'. Fluorescence was monitored and analyzed by a detection system iCycler iQ™ (Bio-Rad).

Assessment of antioxidant activity

To measure intracellular ROS level, 10 μ m frozen sections were cut from frozen biopsies in the cryostat and placed on slides. The slides were then incubated in DHE (10 μ mol/L) diluted in PBS for 30 min in the dark at 37°C. Visualizing the fluorescence was performed subsequently at 0 and at 10 min by two independent observers using confocal microscopy, and the relative mean intensity of the red fluorescence was reported.

Statistical analysis

The results were carefully analyzed and expressed as standard error of the mean. The comparison between groups was made using Student's *t*-tests. Differences were considered statistically significant if *p* < 0.05 in all statistical analyses.

RESULTS

Improvement of clinical symptoms

Macroscopic examination of the abdominal cavity of TNBS-treated rats revealed multiple pathological signs, justifying the previous findings of Satoh et al., 1997. Vasodilatation was found surrounding the inoculation site of some rats and throughout the DC in others. TNBS produced an ulcerative inflammation of

the descending colon, exhibiting a reddish edematous mucosa. Due to the effect of TNBS-induced colitis, the colon wall was relatively thick and the GI tract moderately distended with loose stools on days 10 and 17 and soft stools on day 3. The number of mushroom-like structures, pseudo-polyps, in 10 cm of the proximal jejunum and colon increased from day 3 to day 10 and 17. Upon scraping the colon and the jejunum, watery stools were found with some traces of mucosal necrotic alterations in some of the TNBS-treated rats. Most of the rats presented megacolon symptoms and swollen abdomen (Fig. 1a, b, and c).

Abdominal examination of rats in the group receiving EGCG injection resulted in a significant decrease in the extent as well as the severity of the injury of the descending colon. Although soft stools were seen inside the descending colon, the thickness of the walls and vasodilatation were decreased compared to non-EGCG treated rats, and exhibited an overall abdominal architecture

close to normal and healthy conditions of organs. The colon appeared normally thin and elastic, thus confirming the clinical inspection of the rats before they were sacrificed, whereby no abnormal side effects of EGCG treatment were seen (Fig. 1d). They presented relatively the lowest scores, close to healthy conditions (Fig. 2).

There was no visible damage to the colonic tissue in the normal control group. The internal viscera appeared normal with regularly packed intestinal loops, no redness or abnormal adhesions. The descending colon showed normal features (no obstruction, no vasodilatations and no altered mucosa). In addition, rats treated with EGCG alone had similar findings, meaning that EGCG had no side effects on the colonic and jejunal tissue.

Improvement of histological alterations

The histological examination of descending colon tissues using light microscopy depicted inflammatory

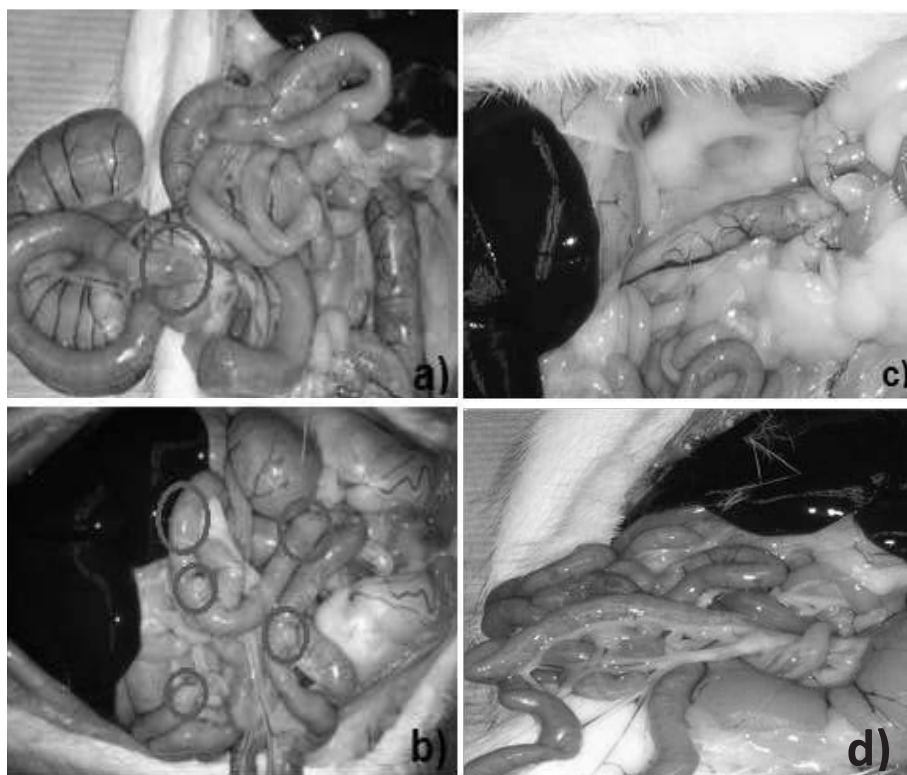


Fig. 1. A prototype of abdominal structures at the sacrifice days in the TNBS group: **(a)** vasodilation (circle), **(b)** mushroom-like structures (circle) in the serosa of the jejunum and descending colon (circles), **(c)** adhesions between organs, **(d)** after treatment with TNBS and EGCG: lack of pseudopolyps, vasodilation, and no or minimal adhesion between organs.

alterations in colonic mucosa induced by TNBS. The TNBS-induced intestinal inflammation was associated with infiltration of the mucosa and submucosa by inflammatory cells, mainly neutrophils, eosinophils and macrophages, edema of the submucosa, dilation of blood vessels, loss or disorganization of crypts and epithelial ulceration.

The progression of inflammation depended on the TNBS exposure time. After several administrations of TNBS, the colonic mucosa appeared generally disorganized. Many glands were destroyed by inflammation and the others appeared irregular, circular, atrophic and invaded by inflammatory cells. On day 17, the inflammation peaked; a more voluminous lymphoid aggregate was observed in the colonic wall.

The EGCG treatment significantly reduced the size and severity of the histological alterations. The morphology and architecture of the crypts were less altered and the pathologies were more localized. By

day 17, there was almost complete remission. Note that the TNBS group scores were always higher (with an average score of 13.7/18 on day 17) than the group receiving EGCG (with an average score of 4/18 on day 17) (Fig. 3). These findings suggest that EGCG reduces significantly the colonic injury induced by TNBS. The rats treated with EGCG alone did not show histological changes, they were similar to the normal controls injected with saline at all-time points.

PAS staining was carried out to study the goblet cells; the results showed that EGCG treatment restored the normal architecture of the cells and thus the crypts were straight and parallel at day 17, with minimal inflammatory cell infiltrate.

Fig. 3 shows the significant improvement in all parameters after the EGCG treatment at all time points. The architecture of the epithelium, the status of the crypts, goblet cells, cellular infiltrate and edema were greatly improved.

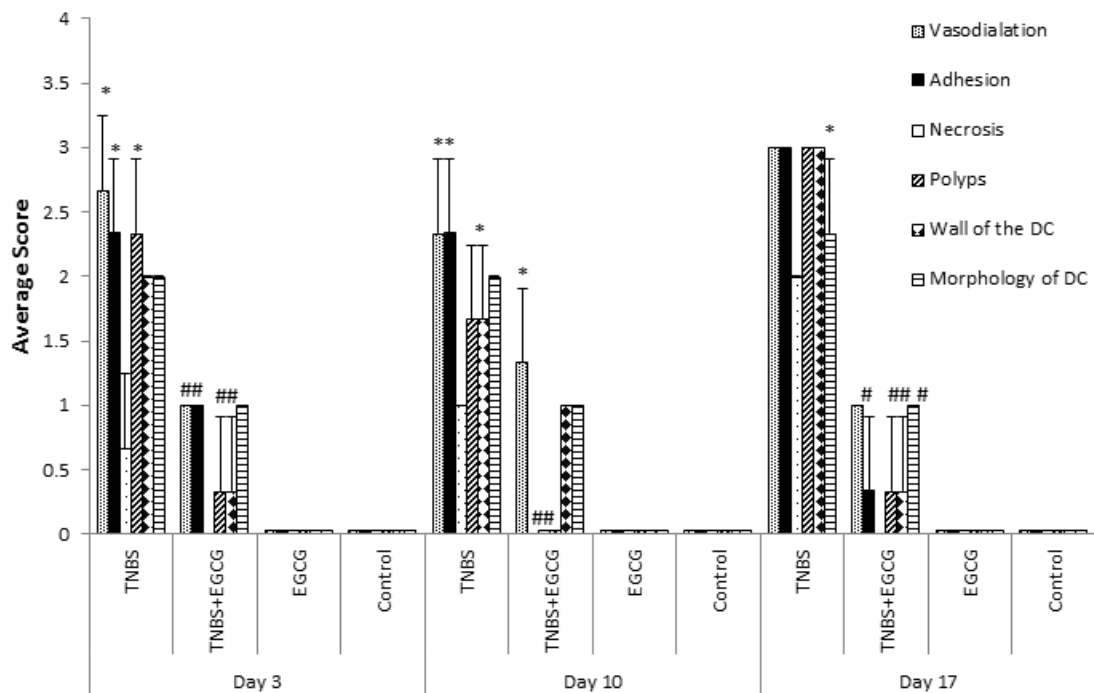


Fig. 2. Macroscopic damage scores per group and per time point. Data are expressed as mean $\pm$ Standard error of the mean (SEM). Histological findings were significantly different when comparing EGCG-treated groups to the Control(*) and to the TNBS(#) groups. The scores of the various parameters (vasodilation, adhesion) of the TNBS group were always significantly higher compared to the TNBS+EGCG-treated group at days 3, 10, and 17 ($p < 0.05$).

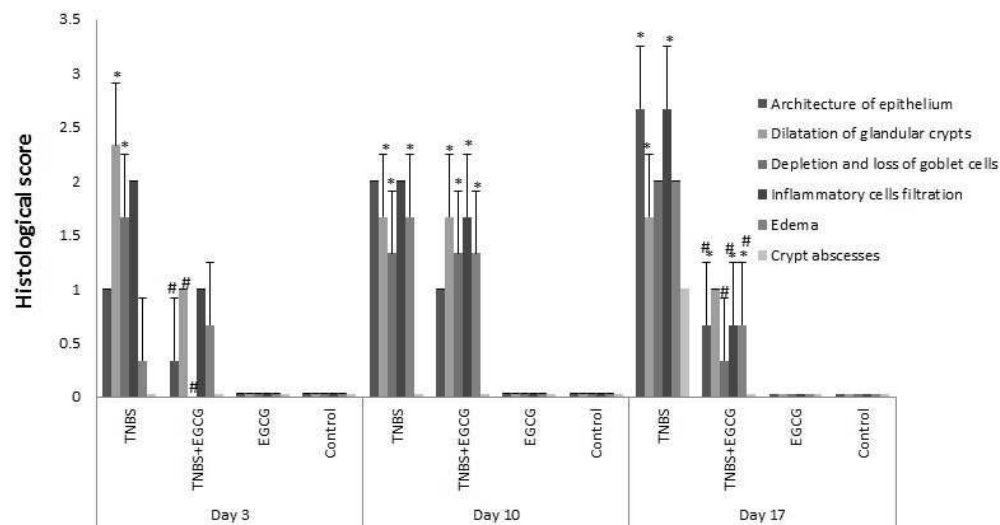


Fig. 3. Morphological alterations scores per group and per time point. Data are expressed as mean $\pm$ Standard error of the mean (SEM). A significant improvement was shown in the EGCG-treated animals when compared to control (*) and TNBS (#) groups.

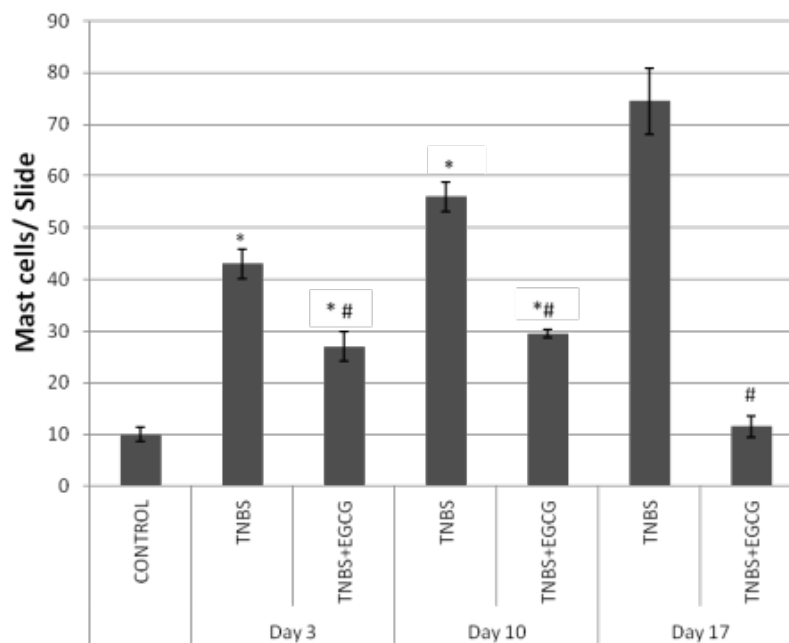


Fig. 4. Number of mast cells in the colonic mucosa and submucosa of rats treated with TNBS, TNBS + EGCG and control rats treated either with saline or EGCG, examined on days 3, 10 and 17 after induction of colitis. Data are expressed as Standard error of the mean (SEM). Note the significant decrease ($p < 0.05$) in the number of mast cells after the EGCG treatment at all time points compared to controls (*) or to only TNBS-treated animals (#) ($p < 0.05$).

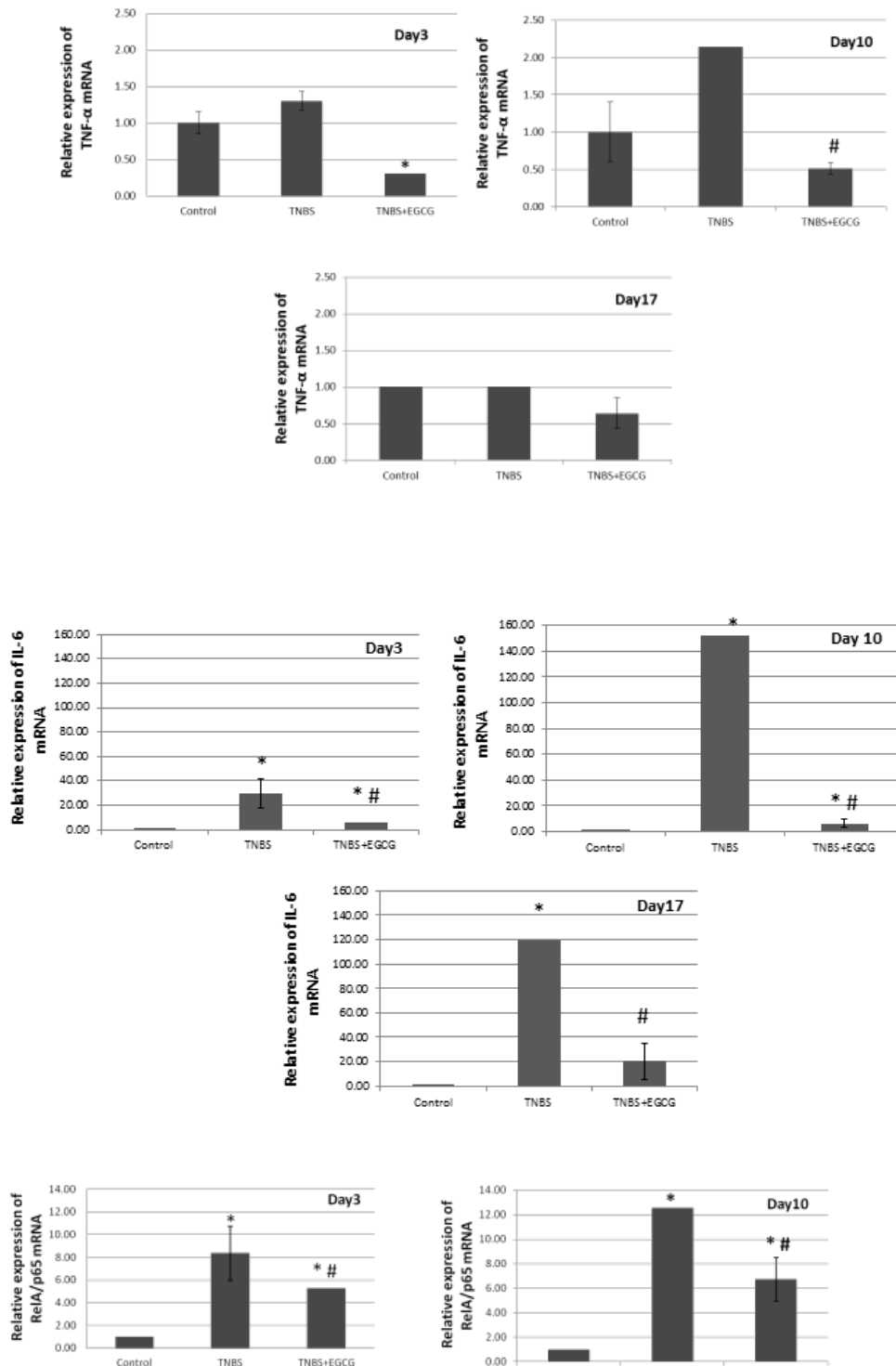


Fig. 5. Effect of EGCG on the mRNA expression of TNF- α (A), IL-6 (B) and RelA/p65 (C) in the colonic mucosa of rats treated with TNBS, TNBS + EGCG and rat control (Saline or EGCG) on days 3, 10 and 17. Data are expressed as Standard error of the mean (SEM). Note the significant decrease ($p < 0.05$) in the expression of TNF- α , IL-6 and NF- κ B after the EGCG treatment at all-time points compared to control (*) and TNBS only (#).

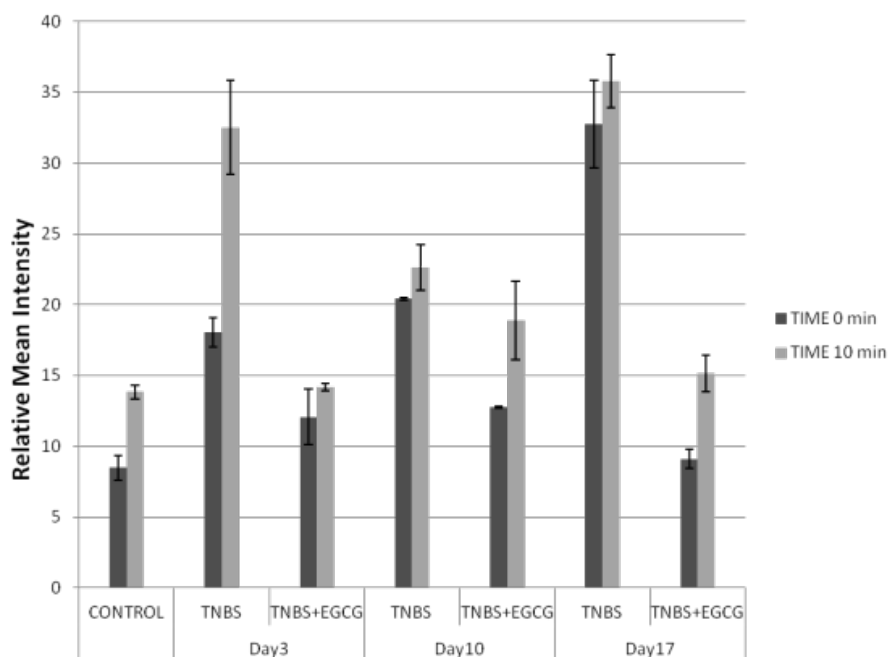


Fig. 6. Relative mean intensity of the red fluorescence of ROS in the rat colons of the TNBS-, TNBS+EGCG-treated and controls on days 3, 10, and 17 ($n=2$ for each group/time point). It was more intense on day 17 followed by days 3 and 10. The EGCG treatment reduced this intensity significantly.

Stabilization of mast cells

In the colon of control rats, a limited number of mast cells were observed mainly in the submucosa (an average of 10 mast cells/slide) (Fig. 4). Three days after induction of colitis, there was a significant increase in their number in the submucosa, reaching an average of 43 cells/slide. This increase was time-dependent, the longer the inflammation persisted, the more the number of mast cells increased (an average of 75 mast cells/slide on day 17). Once the inflammation became chronic at day 17, mast cells, usually predominant in the submucosa and around blood vessels, invaded the different layers of the colon. The rats treated with TNBS showed mast cell aggregates distributed in the inflamed tissue. Approximately 80% of degranulated mast cells were found in the TNBS group, signifying that they were activated. However, the presence of EGCG significantly reduced the number of mast cells at all-time points (e.g. an average of 12 mast cells/slide on day 17).

Reduction in the expression of $TNF-\alpha$, IL-6 and transcription factor NF- κB

As shown in Fig. 5, a quantitative RT-PCR analysis revealed that EGCG caused a significant decrease in the mRNA expression of pro-inflammatory cytokines $TNF-\alpha$, IL-6 and the transcription factor NF- κB compared to the respective TNBS group ($p<0.05$). The $TNF-\alpha$ expression was significantly inhibited by EGCG starting at day 3 compared to TNBS alone or even normal control ($p<0.05$). However, it reached its peak of expression by day 10 in the TNBS group, and that expression was also significantly inhibited by EGCG ($p<0.05$). On day 17, the inhibition of $TNF-\alpha$ by EGCG continued (Fig. 5A). A trend similar to $TNF-\alpha$ was observed with IL-6 mRNA expression. There was a significant suppression by EGCG of the elevated expression of IL-6 induced by TNBS at all-time points ($p<0.05$) (Fig. 5B).

Regarding NF- κB mRNA expression, the p65 subunit expression, also known as RelA NF- κB p65,

was selected to follow. It was overexpressed in the TNBS group, but the EGCG treatment had a significant inhibitory effect ($p < 0.05$) (Fig. 5C).

In brief, EGCG produced significant improvements in all of the studied parameters in the TNBS+EGCG-treated rats compared to only TNBS treatment ($p < 0.05$).

Reduction in expression of the oxidative stress marker

Following dihydroethidium staining, the fluorescence was mainly detected in the TNBS group on day 17 (Relative mean intensity = 36). The intensity decreased significantly upon treatment with EGCG (more than 50%) and was close to normal compared to the control group. The expression of ROS, in the TNBS colitis model, increased from day 3 to day 10 and peaked on day 17. The production of ROS increased from 0 to 10 minutes in both treated and untreated groups, but the fluorescence intensity remained lower in the group that received EGCG (Fig. 6).

DISCUSSION

The results of this study are in agreement with data reported by other animal-model studies associating green tea consumption with health benefits especially due to its antioxidant and anti-inflammatory effects (10). These effects were basically attributed to the prominent EGCG catechin found in green tea. In this study of TNBS-induced colitis, it is demonstrated that EGCG administration exerts, in fact, a protective anti-inflammatory effect on rat colonic mucosa. This study showed that EGCG could significantly decrease the TNBS-induced increased levels of inflammation and tissue injury through decreasing the number of inflammatory cells and activated mast cells in the colonic tissue, limiting ROS release, as well as reducing the mRNA expression of NF- κ B, TNF- α and inhibiting IL-6 mRNA production in the colon of EGCG-treated rats.

On the other hand, EGCG injected in normal rats did not lead to any significant changes in the tested parameters; EGCG behaved very similar to saline-injected animals. It is very likely that the inflammatory environment created by the TNBS could potentiate

the anti-inflammatory effect of EGCG.

Colitis induced in rats by the hapten TNBS has been widely used by other investigators in testing drug efficacy to screen new therapeutic treatments relevant to human IBD. It is well established that this model provides a reproducible long-lasting colonic damage along with inflammatory cell infiltration and ulcerations. It is efficiently able to mimic the pattern of inflammation with human IBD. Data in the literature supported this model because it induces, among other parameters, the increase in oxidative stress, high levels of inflammatory cytokines (TNF- α , IL-1 and IL-6), and activation of the NF- κ B pathway (4).

Moreover, mast cells play a key role in the onset and progression of inflammation. Indeed, during mast cell degranulation, several chemical mediators are released, such as the chemokine responsible for neutrophil recruitment to the site of inflammation, as well as other pro-inflammatory cytokines (11). A remarkable increase in mast cell number was noted in the mucosa of IBD patients (12) and, in our study, the number of activated mast cells increased in response to TNBS alone and decreased significantly in the presence of EGCG treatment. These results suggest that EGCG presents a protective effect in stabilizing mast cells in colonic tissues, thus limiting their secretion or de-granulation and consequently the severity of the inflammation. This is consistent with the histological findings that showed attenuation in the morphological damage followed by reepithelization of the intestinal surface upon EGCG treatment.

Inflammatory cytokines coming from mast cells or other sources elicit NF- κ B activation. The range of NF- κ B inducing stimuli further extends to oxidative stresses which are all elevated in the colonic tissues as a result of TNBS induction and reduced after EGCG treatment.

Furthermore, the NF- κ B activation is closely linked to the onset of colitis. NF- κ B is a transcription factor that regulates the expression of several genes important in cellular responses such as inflammation and innate immunity. ROS and TNF- α released during inflammation could activate NF- κ B (Fig. 7), and thus activate the inflammatory cascade in TNBS induced

colitis. On the other hand, macrophages and mast cells which appear to be anergic in normal mucosa change their phenotype in IBD and this is associated with activation of NF- κ B (13). It was also reported that the NF- κ B activity increases in inflamed colonic mucosa of patients with IBD in correlation with the severity of the disease (14). Furthermore, it was also demonstrated that administration of an antisense oligonucleotide directed against NF- κ Bp65 may be effective in ulcerative colitis (15). Some studies have found that EGCG inhibits lipopolysaccharide (LPS)-mediated NF- κ B activation by reducing I κ B degradation (16). In this study, mRNA expression of NF- κ B in EGCG-treated colonic tissues was much lower than in those not receiving EGCG. These data can explain, at least in part, the anti-inflammatory effects of EGCG. In addition, this significant decrease of NF- κ B in the EGCG-treated animals is associated with stabilization of mast cells and reduction of colonic damages. Further studies are needed to establish the exact role of NF- κ B, but all data suggest that blocking NF- κ B may represent an attractive strategy for treating intestinal inflammation.

Many studies have documented the importance of oxidative stress in IBD. The increase in ROS production and the impairment of antioxidant defense mechanisms have been considered as etiologic factors in inflammatory diseases (17). Overproduction of ROS in the intestinal mucosa induced by inflammatory and immune responses may cause damage of intestinal epithelial cells, therefore, influencing the integrity of the mucosa or initiating an inflammatory signaling cascade in NF- κ B or possibly other pathways, and leading to a severe colitis (18). Our data confirm that EGCG could modulate the NF- κ B signaling pathway induced by oxidative stress. Actually, in this study, the amount of ROS significantly increased and peaked on day 17 in the TNBS model. However, EGCG presented a protective effect and reduced significantly ROS expression in the colonic tissue of EGCG-treated rats at all time-points.

Several studies have reported that intestinal macrophages isolated from patients with IBD secrete high amounts of free radicals. Moreover, a

large number of peripheral neutrophils, capable of producing ROS, migrate across the intestinal wall of these patients (29). The ROS have several pro-inflammatory roles including the recruitment of neutrophils to sites of inflammation, and the release of cytokines such as TNF- α and IL-1 β which are both stimulated by NF- κ B (20). Therefore, this increased production of ROS in rats treated with TNBS reflects the infiltration of the submucosa and crypts by inflammatory cells, mainly neutrophils, leading to ulcerations of the colonic mucosa, whereas EGCG treatment decreases their production and limits infiltration at the site of inflammation. Thus, the antioxidant properties of EGCG can explain its anti-inflammatory actions.

TNF- α , one of the most important cytokines in the inflammatory cascade of IBD, stimulates the release of several pro-inflammatory mediators including ROS, IL-1 β , IL-6, and nitric oxide (NO). It also induces self-amplification, and contributes to the recruitment of inflammatory cells, and thus amplifies and prolongs the inflammatory response through the activation of the other immune cells (4). Vascular endothelial cells respond to TNF- α by undergoing several pro-inflammatory changes, which increase leukocyte adhesion, transendothelial migration and vascular leak. Therefore, TNF- α may be a potential target in the management of intestinal inflammation. However, TNF- α signaling has been shown to be associated with an increase in oxidative stress involved in inflammatory processes inducing TNF- α expression. Gossart et al. (21) demonstrated that free radical scavenger pretreatment ameliorated histological alterations and decreased TNF- α expression at the level of mRNA in lung inflammation in rats. Our data depicted a reducing effect of EGCG on TNF- α levels at all time-points. This effect of EGCG may be the result of its antioxidant action, thus reducing the production of ROS. In the TNBS group, the TNF- α level peaked on day 10, a stimulus necessary to maintain a chronic inflammation (Fig. 5).

To better elucidate the mechanism of action of EGCG in IBD, the level of IL-6 mRNA level was checked. In the downstream pathway of NF- κ B, IL-6, a multifunctional NF- κ B-regulated cytokine,

is a major modulator of inflammation by acting on epithelial and immune cells. IL-6 regulates neutrophil trafficking during acute inflammation and participates in controlling the transition from acute to chronic inflammation by changing the nature of leucocyte infiltrate from neutrophils to monocytes (22). Therefore, blocking IL-6 signaling should offer substantial clinical benefit to patients with IBD. Gross et al. (23) confirmed that IL-6 is expressed at very high levels in both serum and intestinal tissue from patients with Crohn's disease. Our data showed that the IL-6 level, in the TNBS group, increased

considerably and then decreased after treatment with EGCG, compared to the control group and that in response to NF- κ B and TNF- α . Indeed, the promoter region of IL-6 has a putative NF- κ B binding site (24), so it is believed that the reduction of IL-6 by EGCG could be linked to the modulation of NF- κ B.

The transcription factor NF- κ B will be activated following the binding of numerous factors such as lipopolysaccharides (LPS) or TNF- α to its receptors, which will initiate a signaling cascade leading to I κ B degradation. Free radicals (ROS) may also lead to the degradation of I κ B and cause the activation of NF-

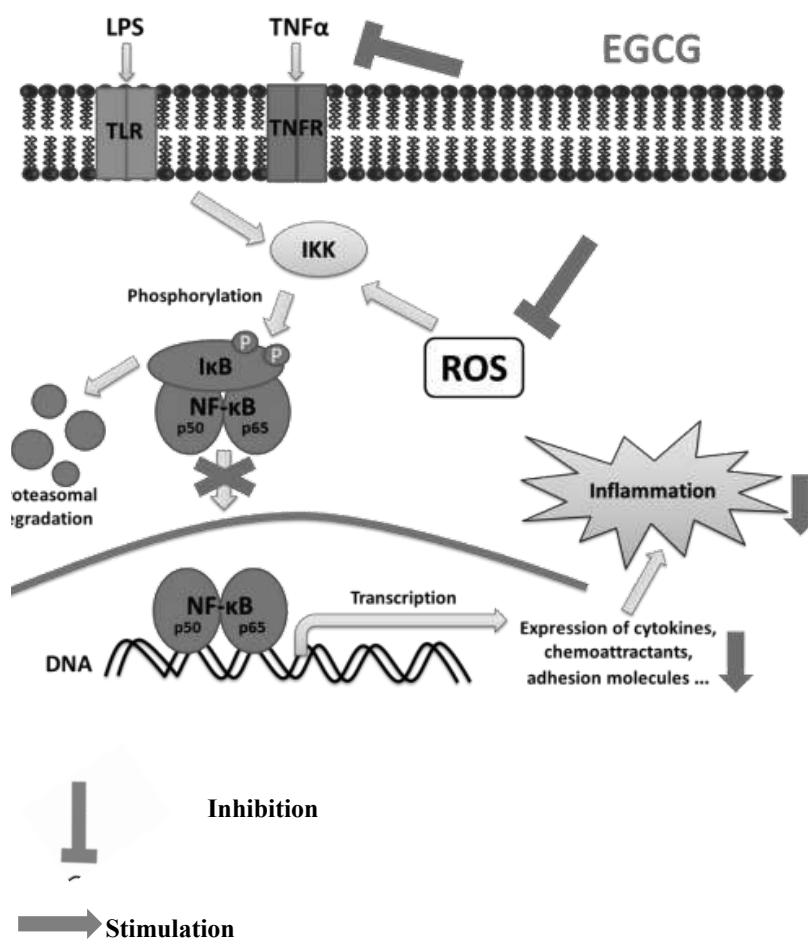


Fig. 7. Potential mechanisms of action of EGCG inhibiting inflammatory markers. The transcription factor NF- κ B will be activated following the binding of numerous factors such as lipopolysaccharides (LPS) or TNF- α to its receptors, which will initiate a signaling cascade leading to I κ B degradation. Free radicals (ROS) may also lead to the degradation of I κ B and cause the activation of NF- κ B. Once free, NF- κ B is translocated to the nucleus where it binds to DNA and induces the expression of several genes involved in the inflammatory response such as the genes for cytokines like TNF- α , IL-6 and IL-1 β among others.

κ B. Once free, NF- κ B is translocated to the nucleus where it binds to DNA and induces the expression of several genes involved in the inflammatory response such as the genes for cytokines like TNF- α , IL-6 and IL-1 β among others (Fig. 7).

On the other hand, as shown in Fig. 7, IKKs could be also involved. They were long thought to be highly specific for I κ B proteins; however, evidence has accumulated indicating that IKKs target not only upstream mediators in NF- κ B cascades but also other mediators independently of NF- κ B such as MAPK pathway. In addition, IKKs play a role in immune responses and, specifically, autoimmune and allergic reactions (25). Further studies are needed in this direction to confirm the involvement EGCG in reducing inflammatory reactions and the specific pathways involved.

In brief, EGCG inhibited the NF- κ B cascade, thus preventing the transcription of inflammatory genes and leading to the reduction of inflammatory reactions. Furthermore, EGCG has also shown an inhibitory effect on the production of ROS and TNF- α , thus interrupting the crosstalk of NF- κ B cascade with other pathways (26).

Moreover, in a review of 2013 several biological actions of EGCG were highlighted, whereby it can directly interact with plasma membrane proteins and phospholipids thus stimulating intracellular signaling pathways; another possible mechanism of action is through its transport to the intracellular compartments (cytosol, mitochondria, and nucleus). The type of cells, stress conditions, and concentrations of EGCG are the main factors affecting these effects (27).

With the advancement in immunology and molecular biology, multiple treatment modalities for IBD have been reported including: anti- TNF- α antibodies, anti-IL-6R and antisense oligonucleotides against NF- κ B. They are effective tools for the treatment of colitis. However, their expensive prices coupled with the lack of sufficient research on their safety, limit their use (28, 29). Thus, EGCG, a nutraceutical and a natural antioxidant, is safer and cheaper and provides an interesting potential alternative approach for the treatment of IBD.

In summary, data of the present study demonstrated that EGCG exerts a protective effect

on the TNBS-induced colitis in rats. It inhibited the production of inflammatory mediators involved in intestinal inflammatory and immune responses by decreasing the production of TNF- α and IL-6 as well as ROS through a mechanism that, at least in part, involves inhibition of NF- κ B activation. However, further molecular, cellular, and animal studies need to be conducted in this direction to prove that EGCG is really a promising agent for the treatment of IBD, and to investigate the presence of specific receptor proteins of EGCG on the membranes of cancerous and non-cancerous cells, thereby revealing its detailed mechanism of action and downstream signaling. Furthermore, chemical reform of an EGCG pharmacophore may modify relative therapeutic activities so that combinatorial supplementation may boost positive health effects.

ACKNOWLEDGEMENTS

This work was partially supported by CEDRE PHC 2017, No. 37355 NF.

REFERENCES

1. Tomasello G, Mazzola M, Leone A, et al. Nutrition, oxidative stress and intestinal dysbiosis: Influence of diet on gut microbiota in inflammatory bowel diseases. *Biomed Pap* 2016; 160(4):461-66.
2. Mazzola M, Carini F, Leone A, et al. Inflammatory bowel disease and colorectal cancer, nutraceutical aspects. *Euromed Biomed J* 2016; 11(10):123-29.
3. Jurjus AR, Khoury NN, Reimund J-M. Animal models of inflammatory bowel disease. *J Pharmacol Toxicol Methods* 2004; 50(2):81-92.
4. Neurath MF, Fuss I, Pasparakis M, et al. Predominant pathogenic role of tumor necrosis factor in experimental colitis in mice. *Eur J Immunol* 1997; 27(7):1743-50.
5. Singh BN, Shankar S, Srivastava RK. Green tea catechin, epigallocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical applications. *Biochem Pharmacol* 2011; 82(12):1807-21.
6. Tadakatsu S, Wei-Hua Z, Zhi-Qing H. mechanism of action and potential for use of tea catechin as an anti-infective agent. *Anti-Infect Agents Med Chem* 2007; 6(1):57-62.

7. Hara Y. Elucidation of physiological functions of tea catechins and their practical applications. *J Food and Drug Anal* 2012; 20:296-300.
8. Bertolini F, Fusetti L, Rabascio C, Cinieri S, Martinelli G, Pruneri G. Inhibition of angiogenesis and induction of endothelial and tumor cell apoptosis by green tea in animal models of human high-grade non-Hodgkin's lymphoma. *Leuk* 2000; 14(8):1477.
9. Morris GP, Beck PL, Herridge MS, Depew WT, Szewczuk MR, Wallace JL. Hapten-induced model of chronic inflammation and ulceration in the rat colon. *Gastroenterology* 1989; 96(3):795-803.
10. Mochizuki M, Hasegawa N. Protective effect of (-)-epigallocatechin gallate on acute experimental colitis. *J Health Science* 2005; 51(3):3626-4.
11. Hussein IAH, Tohme R, Barada K, et al. Inflammatory bowel disease in rats: bacterial and chemical interaction. *W J Gastroenterol: WJG* 2008; 14(25):4028.
12. He S-H. Key role of mast cells and their major secretory products in inflammatory bowel disease. *W J Gastroenterol* 2004; 10(3):309.
13. Rogler G, Brand K, Vogl D, et al. Nuclear factor κ B is activated in macrophages and epithelial cells of inflamed intestinal mucosa. *Gastroenterology* 1998; 115(2):357-69.
14. Schreiber S, Nikolaus S, Hampe J. Activation of nuclear factor κ B in inflammatory bowel disease. *Gut* 1998; 42(4):477-84.
15. Neurath MF, Pettersson S, Zum Büschenfelde K-HM, Strober W. Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF- κ B abrogates established experimental colitis in mice. *Nature Medicine* 1996; 2(9):998-1004.
16. Ichikawa D, Matsui A, Imai M, Sonoda Y, Kasahara T. Effect of various catechins on the IL-12p40 production by murine peritoneal macrophages and a macrophage cell line, J774. 1. *Biol Pharma Bull* 2004; 27(9):1353-58.
17. Han SN, Meydani SN. Antioxidants, cytokines, and influenza infection in aged mice and elderly humans. *J Infect Dis* 2000; 18(Suppl 1):S74-80.
18. Oz HS, Chen TS, McClain CJ, De Villiers W. Antioxidants as novel therapy in a murine model of colitis. *J Nut Biochem* 2005; 16(5):297.
19. Crama-Bohbouth GE, Arndt JW, Pena AS, et al. Value of indium-111 granulocyte scintigraphy in the assessment of Crohn's disease of the small intestine: prospective investigation. *Digestion* 1988; 40(4):227-36.
20. Cuzzocrea S, Riley DP, Caputi AP, Salvemini D. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. *Pharmacol rev* 2001; 53(1):135-59.
21. Gossart S, Cambon C, Orfila C, et al. Reactive oxygen intermediates as regulators of TNF-alpha production in rat lung inflammation induced by silica. *J Immunol* 1996; 156(4):1540-48.
22. Fielding CA, McLoughlin RM, McLeod L, et al. IL-6 regulates neutrophil trafficking during acute inflammation via STAT3. *J Immunol* 2008; 181(3):2189-95.
23. Gross V, Andus T, Caesar I, Roth M, Scholmerich J. Evidence for continuous stimulation of interleukin-6 production in Crohn's disease. *Gastroenterology* 1992; 102(2):514-19.
24. Libermann TA, Baltimore D. Activation of interleukin-6 gene expression through the NF-kappa B transcription factor. *Mol Cell Biol* 1990; 10(5):2327-34.
25. Oeckinghaus A, Hayden MS, Ghosh S. Crosstalk in NF-[kappa]B signaling pathways. *Nat immunol* 2011; 12(8):695-708.
26. Atreya I, Atreya R, Neurath M. NF κ B in inflammatory bowel disease. *J Interl Med* 2008; 263(6):591-96.
27. Kim H-S, Quon MJ, Kim J-a. New insights into the mechanisms of polyphenols beyond antioxidant properties; lessons from the green tea polyphenol, epigallocatechin 3-gallate. *Redox Biology* 2014; 2(Supplement C):187-95.
28. Schlaak J, Barreiros A, Pettersson S, Schirmacher P, Meyer Zum Büschenfelde KH, Neurath M. Antisense phosphorothioate oligonucleotides to the p65 subunit of NF κ B abrogate fulminant septic shock induced by *S. typhimurium* in mice. *Scand J Immunol* 2001; 54(4):396-403.
29. Yamamoto M, Yoshizaki K, Kishimoto T, Ito H. IL-6 is required for the development of Th1 cell-mediated murine colitis. *J Immunol* 2000; 164(9):4878-82.

EXPRESSION AND CLINICAL SIGNIFICANCE OF ARTN AND MMP-9 IN ENDOMETRIAL CARCINOMA

XH. WANG¹, YN. LIU¹, K. TIAN², CX. YAO¹, LM. LI¹, YQ. ZHENG³ and ZM. SHI¹

¹Pathological Staff Room, School of Medicine and Affiliated Hospital of HeBei University of Engineering, Hebei, China; ²Oncology Department, School of Medicine and Affiliated Hospital of HeBei University of Engineering, Hebei, China; ³Department of Clinical Laboratory, Handan Maternal and Child Health-Care Hospital, Hebei, China

Received October 4, 2017 – Accepted November 7, 2017

The aim of this study was to detect the differential expression of Artemin (ARTN) and matrix metalloproteinase protein 9 (MMP-9) in endometrial carcinoma (EC) and to assess their clinical significance in order to provide insight into the pathological mechanism of tumor infiltration and metastasis in EC. A total of 48 patients who had undergone surgery for EC at the School of Medicine and Affiliated Hospital of HeBei University of Engineering between July 2015 and July 2016 were included in the study. The 48 patients were classified into 3 groups according to tumor stage: 27 patients with EC stage I, 12 patients with EC stage II and 9 patients with EC stage III. The samples collected from each patient included fresh normal endometrial tissue, endometrial simple hyperplastic tissue and endometrial atypical hyperplastic tissue. The transcription levels of ARTN and MMP-9 mRNA in each group were investigated using RT-PCR. The expression levels of ARTN and MMP-9 protein in each group were examined using Western blotting. Spearman's correlation analysis was used to analyze the correlation between the expression levels of ARTN and MMP-9 proteins and EC tissue type. RT-PCR and Western blotting assays revealed that the expression levels of ARTN and MMP-9 were increased in normal endometrial tissue, simple hyperplastic tissue, atypical hyperplastic tissue and EC of stages I, II and III. The differences noted were statistically significant ($P < 0.05$). Furthermore, Western blot analysis indicated that the expression levels of ARTN and MMP-9 proteins in lymphatic metastatic tissues were higher than those in non-lymphatic metastatic tissues ($P < 0.05$). The expression levels in the infiltration tissues of the deep muscular layer were higher than those noted in the light muscular layer ($P < 0.05$). The expression levels of ARTN and MMP-9 proteins were positively correlated ($P < 0.05$). The data suggest that ARTN and MMP-9 are involved in the occurrence, development, invasion and metastasis of EC, and play a synergistic role in the development of EC and lymphatic metastasis.

Endometrial carcinoma (EC) is one of the main causes of death among malignant gynecological tumors in developed countries (1). EC is more frequent in younger subjects in China and exhibits an increased rate of morbidity and mortality (2).

The occurrence, development and metastasis are extremely complicated multistage abnormal processes. A variety of molecular changes occur during the progression of endometrial simple hyperplasia to atypical hyperplasia and during

Key words: EC, ARTN, MMP-9, invasion, metastasis

Mailing address:

Dr Zhimin Shi,
Pathological Staff Room,
School of Medicine and Affiliated Hospital
Hebei University of Engineering, Handan 056000, Hebei, China
Tel./Fax: 0310 8577600
e.mail:shizhiminszm1980@163.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
**DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF
INTEREST RELEVANT TO THIS ARTICLE.**

the progression of early EC to late carcinoma (3). Histochemical and immunohistochemical analyses are extremely important for the accurate and early diagnosis of these malignancies (4, 5). Infiltration and metastasis occur due to malignancy, and are the main processes that impair treatment of tumor patients and/or determine their overall survival.

Cytostatic treatment is aggressive and exerts several side effects such as immunosuppression and the increased risk of bacterial and fungal infections (6, 7). Notably, fungal infections from *Aspergillum* and/or *Fusarium genus* (8) have been reported in these patients. Additional side effects include vitamin K-dependent coagulopathy (9), particularly in the presence of pre-existing liver diseases such as non-alcoholic fatty liver (10). Alternative treatments have been explored, namely the use of plant extracts with cytotoxic effects in order to circumvent the severity of symptoms (11).

Therefore, the identification of highly sensitive and specific molecular markers closely related with the appearance, development, invasion and metastasis of EC can lead to a correct diagnosis and treatment for patients with EC. The improvement in the EC patient survival rate has become a main research focus during recent years.

Artemin (ARTN) is a subtype of Glial cell line-Derived Neurotrophic Factor (GDNF) family ligands (GFL) that belongs to the transforming growth factor- β (TGF- β) superfamily (12). ARTN is a secretory phosphorylation glycoprotein. It can combine with its receptors RET and GFR α 3 in order to activate downstream kinases and finally transmit signals to the cell. The physiological function of ARTN involves the support of the survival of peripheral neuron and dopaminergic CNS neuron populations. Yoshida (13) and colleagues examined the expression of ARTN in various tumor tissues, such as tumors of the digestive and reproductive systems and internal secretory adenomas. The contributions of ARNT in the growth, infiltration and metastasis of the tumors were explored. Matrix metalloproteinase-9 (MMP-9) belongs to the matrix metalloproteinase family of enzymes that can degrade various collagen types of proteins in the tumor microenvironment in order to promote tumor metastasis (14). For example, Takada

and Wang demonstrated that MMP-9 exhibited an important prognostic value for bladder (15) and cervical cancers (16). Although the morbidity of EC is increasing every year, the research in this field is currently limited. The expression levels of ARTN and MMP-9 in EC tissues have been extensively studied, although reports that have investigated the combined detection of these two proteins in EC tissue are limited. The previous studies mainly adopted immunohistochemical techniques (17). In the present study, RT-PCR and Western blotting were used for the combined detection of the differential expression levels of ARTN and MMP-9 in EC. The study aimed to discuss the changes in their expression levels and their correlation with infiltration and metastasis of the EC tissue, thus providing experimental evidence for the pathological mechanism of infiltration and metastasis in EC.

MATERIALS AND METHODS

Study subjects

The present study included 48 patients (age range: 32–63 years) who had undergone surgical treatment for EC at the School of Medicine and Affiliated Hospital of HeBei University of Engineering between July 2015 and July 2016. Following surgery, the pathologic results indicated that the patients exhibited endometrial adenocarcinoma. According to the 2009 FIGO pathological stage of EC, the 48 patients were classified into 3 groups based on disease severity: The sample size included 27 cases of EC stage I, 12 of stage II and 9 of stage III. Nine patients exhibited lymphatic metastases; 30 patients exhibited high cellular differentiation, 9 patients moderate, and 9 patients low. None of the patients had undergone chemoradiotherapy or hormonal therapy. The control group included 144 patients who underwent treatment in our hospital during the same period. Samples of fresh normal endometrial tissue, endometrial simple hyperplastic tissue and endometrial atypical hyperplastic tissue were collected from the 48 study patients. The study protocol was approved by the Ethics Committee of the Affiliated Hospital of HeBei University of Engineering and all the patients signed informed consent for their participation. The samples collected were placed in non-enzyme centrifugal tubes and stored at -80°C until further molecular analysis.

Experimental method

The expression of ARTN and MMP-9 mRNA in EC tissue was detected using RT-PCR (Shenzhen Standard Biological Technology Co. Ltd.). Total RNA extraction was performed following the instructions of the Trizol extraction RNA kit. The tissue was homogenized by addition of 1 ml of Trizol reagent and centrifuged for 15 min at 13,000 rpm. The top layer of the supernatant was mixed with 500 µl of ice-cold isopropanol and further centrifuged at 13,000 rpm for 10 min. The resulting RNA pellet was washed with 75% ice-cold ethanol and resuspended in RNase free water. The detection of RNA purity and concentration was estimated by spectrophotometry. The first chain of cDNA was synthesized by reverse transcription. 1 µg of RNA was mixed with water and oligo dT primers, heated for 10 min at 30°C and finally incubated at 42°C for 30 min in the presence of DNTPs, reverse transcriptase and reaction buffer containing MgCl₂ (5 mM). The reaction was terminated by heating at 99°C for 5 min and the samples were stored at 5°C until further use. For PCR reaction a total of 0.5 µl of cDNA was mixed with water, primers and polymerase in a total volume of 20 µl per reaction. The specific primer sequences and the conditions of amplification are shown in Table I.

Measurement and analysis for PCR amplified product

In all groups the expression levels of ARTN and MMP-9 proteins were detected by Western blotting.

The total protein was extracted by addition of lysis buffer containing protease inhibitors. The samples were centrifuged at 13,000 rpm for 15 min at 4°C in order to obtain the protein fraction. Bicinchoninic (BCA) acid was used to measure the protein concentration [BCA protein concentration measurement kit, MultiSciences (Lianke) Biotech Co., Ltd.]. 5×SDS sample buffer was added at a 4:1 ratio of protein: sample buffer and subsequently β-mercaptoethanol (Nanjing SenBeiJia Biological Technology Co., Ltd.) was added at a final concentration of 5% in order to denature the proteins.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

According to the molecular weight of the proteins, a 12% separating gel and a 5% spacer gel (all reagents were purchased from Amresco Company, Germany) were prepared. The 12% separating gel (10 ml) included the following components: DdH₂O, 3.3 ml, 30% acrylamide, 4.0 ml, 1.5 M Tris-HCL (PH 8.8), 2.5 ml, 10% SDS, 0.1 ml and TEMED, 0.004 ml. The 5% spacer gel (10 ml) comprised DdH₂O, 3.4 ml, 30% acrylamide, 0.83 ml, 1.0 M Tris-HCL (PH 6.8), 0.63 ml, 10% SDS, 0.05 ml, 10% ammonium persulfate, 0.05 ml; and TEMED, 0.005 ml. Membrane transfer was conducted using a PVDF membrane and six pieces of filter paper that were cut to the same size. The addition of methanol was used to soak the PVDF membrane. Subsequently, the buffer solution was added

Table I. Primer sequence and amplification conditions.

Gene	Primer sequence	Amplified length	Annealing temperature
ARTNA	Upstream: 5'-AAGCCATCACC ACTCAGAATTG-3'	241bp	53°C
	Downstream: 5'-CACTGATCTACCGTATTTGCTGT-3'		
MMP-9	Upstream: 5'-CACTGATCTACCGTATTTGCTGT-3'	141bp	60°C
	5'-TGGCTACCTGAGTGAAGATGG-3'		
GAPDH	5'-AGTTATTGTGTGCTGGGGACA-3'	309bp	62°C
	5'-CAGGCTCCAGSSGAAGTTGG-3'		

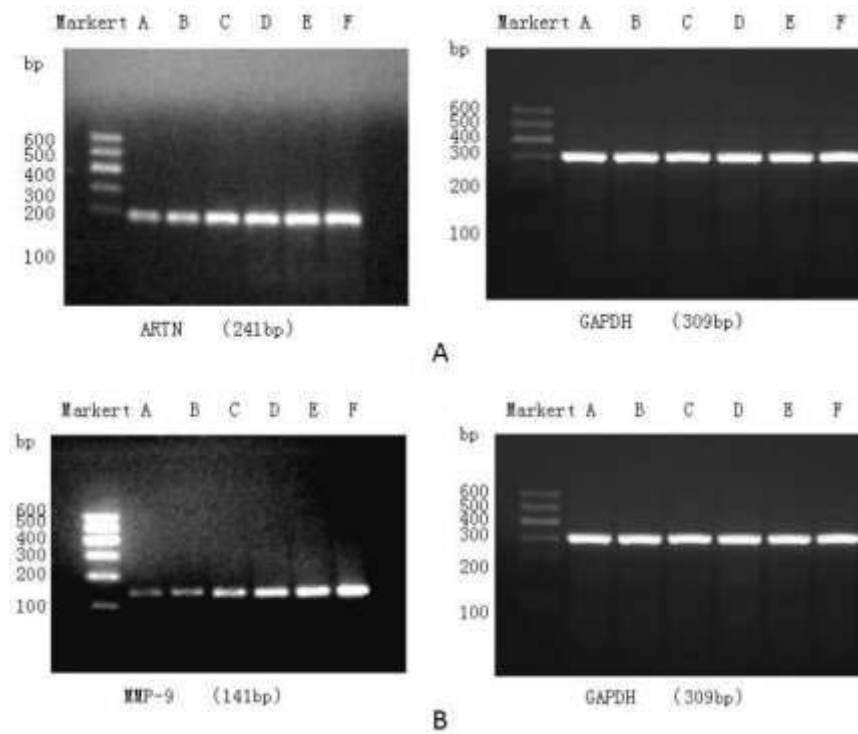


Fig. 1. The expression levels of ARTN mRNA (A) and MMP-9 mRNA (B) in each group as determined by RT-PCR. A: Normal endometrium group; B: Simple hyperplasia group; C: Endometrial atypical hyperplasia group; D: EC stage I group; E: EC stage II group; F: EC stage III group.

Table II. Expression levels of ARTN and MMP-9 as determined by PCR expression in the six groups ($\bar{x} \pm s$).

Group	Number of cases (n)	ARTN/GAPDH	MMP-9/GAPDH
Normal endometrium group	48	0.328 ± 0.031	0.157 ± 0.020
Simple hyperplasia group	48	0.485 ± 0.029^b	0.161 ± 0.035^b
Endometrial atypical hyperplasia group	48	0.495 ± 0.030^c	0.319 ± 0.026^c
EC stage I group	27	0.628 ± 0.032^d	0.451 ± 0.023^d
EC stage II group	12	0.671 ± 0.030^e	0.504 ± 0.024^e
EC stage III group	9	0.792 ± 0.028^f	0.699 ± 0.060^f

$P^b > 0.05$, $P^c < 0.05$, $P^d < 0.05$, $P^e < 0.05$, $P^f < 0.05$

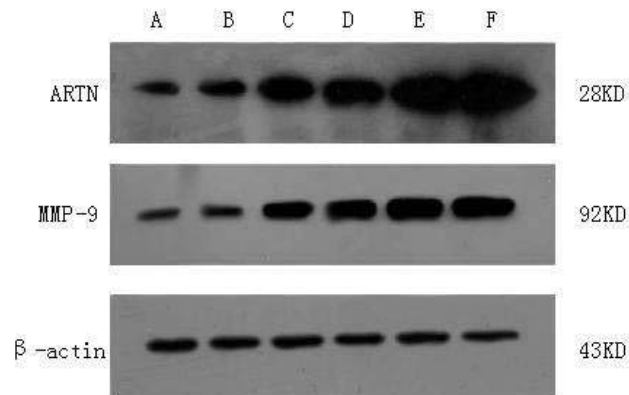


Fig. 2. Expression of ARTN and MMP-9 by Western blotting in each group (A: NE group; B: ESH group; C: EAH group; D: STAGD- I group; E: STAGD- II group; F:STAGD- III group)

Table III. The expression levels of of ARTN and MMP-9 as determined by Western blotting expression in the six Groups ($x \pm s$)

Group	Number of cases (n)	ARTN/ β -actin	MMP-9/ β -actin
Normal endometrium group	48	0.329 ± 0.036	0.507 ± 0.024
Simple hyperplasia group	48	0.400 ± 0.021^b	0.569 ± 0.030^b
Endometrial atypical hyperplasia group	48	0.851 ± 0.036^c	0.932 ± 0.052^c
EC stage I group	27	1.205 ± 0.034^d	1.219 ± 0.055^d
EC stage II group	12	1.634 ± 0.031^e	1.374 ± 0.057^e
EC stage III group	9	1.891 ± 0.032^f	1.560 ± 0.048^f

$P^b > 0.05$, $P^c < 0.05$, $P^d < 0.05$, $P^e < 0.05$, $P^f < 0.05$

and the membrane was placed in a basin with ice cubes. The proteins were transferred to the membrane following electrophoresis at 300 mA for 1 h. The membrane was blocked with 10% milk in TBS-T and incubated with primary antibodies against ARTN and/or MMP9 in 1% milk in TBS-T at 4°C overnight. The membrane was washed 3 times using PBS and incubated with secondary antibody diluted in 1% TBS-T for 1 h. The samples were subsequently washed 3 times with PBS and ECL reagents were added for 5 min for protein detection. The membranes were exposed on films and developed in dark. Quantity One software was used to analyze the lanes and calculate the

gray level ratio between the ARTN, MMP-9 lanes and the β -actin internal reference stripe that served as a loading control of the proteins ARTN and MMP-9.

Statistical analysis

The results are expressed as mean $\pm$ SEM. Statistical Processing for Social Sciences (SPSS) version 18.0 statistical software was used for statistical analysis. One-way analysis of variance (ANOVA) was performed in order to compare the mean values among the groups. A p value of lower than 0.05 ($p < 0.05$) was considered statistically significant.

RESULTS

PCR

ARTN expression levels in the 6 groups was evident by the presence of a fragment at 241 bp (Fig. 1A), whereas the MMP-9 expression was evident by the presence of a fragment at 141 bp (Fig. 1B). GAPDH was noted at 309 bp (in the relevant target gene lanes). GAPDH is widely accepted as a housekeeping gene and is used for normalization and loading control of PCR. The expression levels of ARTN and MMP-9 mRNA in the EC and atypical hyperplastic groups was significantly increased compared with the normal endometrium group ($P < 0.05$). In addition, the expression levels of ARTN and MMP-9 mRNA in EC tissue of stage III were higher than those of stages I and II (Table II).

Western blot

Fig. 2 shows that ARTN was present at 28 kD, whereas MMP-9 was present at 92 kD and β -actin at 43 kD, as noted by the relevant target protein lanes. The expression levels of ARTN and MMP-9 proteins in the EC group and atypical hyperplastic group were increased significantly compared with the normal endometrium group ($p < 0.05$). The expression levels of ARTN and MMP-9 proteins in EC of tissue stage III were higher than those of EC of stage I and II (Table III). Previous studies have shown that the expression levels of ARTN and MMP-9 proteins were higher in the high operative pathological stage with lymphatic metastatic tissue of an infiltration depth of muscular layer greater than and/or equal to $\frac{1}{2}$ ($\geq 1/2$) compared with the low operative pathological stage and the non-lymphatic metastatic tissue that exhibited an infiltration depth of muscular layer lower than $\frac{1}{2}$ ($< 1/2$). The expression levels of ARTN and MMP-9 proteins were not associated with the parameters of cancer differentiation and age. The results are shown in Table IV.

Correlation analysis

Spearman correlation analysis demonstrated that the expression levels of ARTN and MMP-9 proteins were positively correlated in the EC tissue (Fig.3).

DISCUSSION

ARTN is a secretory glycoprotein that belongs to the GDNF family ligands. Its main role is to combine with the the cysteine residue of the higher specificity receptor GDNF α 3. ARTN is combined with GDNF α 3 in order to induce the phosphorylation of tyrosine kinase and in turn activate kinase enzymes such as MAPK and PI-3. The latter proteins act downstream in order to conduct various signaling pathways. Recent studies have shown that ARTN is involved in the development of a majority of tumor types (18). Banerjee et al. (19) studied the significance of ARTN in breast cancer tissues, and demonstrated that ARTN might be involved in the occurrence, development and invasion of breast cancer. In the present study, the experimental results indicated that the expression levels of ARTN in atypical hyperplastic tissue and EC tissue were significantly higher than those in normal tissue. Furthermore, the expression levels of ARTN in EC tissue stage III were higher than those of stages I and II. The data confirmed that the expression of ARTN and MMP-9 were positively correlated. Therefore, ARTN has an important significance in the occurrence and development of EC. A recent study (20) highlighted that the expression of ARTN was positively correlated with the volume of liver tumors and was further related with relapses. Thus, it is hypothesized that ARTN may increase the amount of liver cancer stem cells as a hypoxia stimulated factor. Transcription of ARTN protein is directly activated by hypoxia-inducible factor-1. ARTN is induced by hypoxia through the AKT signaling pathway in order to promote the epithelial tissue to mesenchymal transition and increase the amount of tumor stem cells, thereby facilitating tumor invasion and metastasis. However, additional studies are required in order to understand the specific mechanism of action.

MMPs are proteolytic enzymes that are reliant on zinc and calcium in order to degrade all the components of the ECM (21). The regulatory function of MMPs involves the release of structural extracellular matrix molecules that can influence certain aspects of cellular activities that possess receptors for ECM components, such as ECM

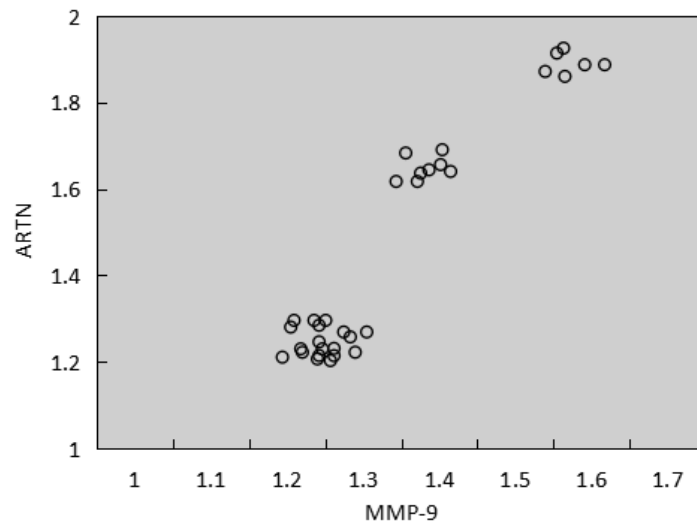


Fig. 1. The expression levels of ARTN m RNA (A) and MMP-9 m RNA (B) in each group as determined by RT-PCR. A: Normal endometrium group; B: Simple hyperplasia group; C: Endometrial atypical hyperplasia group; D: EC stage I group; E: EC stage II group; F: EC stage III group.

Table III. The expression levels of ARTN and MMP-9 as determined by Western blotting expression in the six Groups ($\bar{x} \pm s$)

Group		n	$\bar{x} \pm s$		P
			MMP-9 protein	ARTN protein	
Age	≤ 50	33	1.279 $\pm$ 0.026	1.2318 $\pm$ 0.0301	P>0.05
	>50	15	1.272 $\pm$ 0.024	1.2355 $\pm$ 0.0322	
Depth of invasion	<1/2	27	0.926 $\pm$ 0.042	0.8523 $\pm$ 0.0316	P<0.05
	$\geq 1/2$	21	1.210 $\pm$ 0.041	1.0317 $\pm$ 0.0304	
Differentiation	G1-G2	39	1.231 $\pm$ 0.023	0.8280 $\pm$ 0.0305	P>0.05
	G3	9	1.254 $\pm$ 0.018	0.8336 $\pm$ 0.0325	
Figo group	I + II	39	1.280 $\pm$ 0.041	1.3266 $\pm$ 0.0320	P<0.05
	III	9	1.530 $\pm$ 0.048	1.7929 $\pm$ 0.0319	
Lymphatic metastasis	YES	36	1.574 $\pm$ 0.030	1.910 $\pm$ 0.035	P<0.05
	NO	12	1.215 $\pm$ 0.034	1.259 $\pm$ 0.022	

$P^b > 0.05$, $P^c < 0.05$, $P^d < 0.05$, $P^e < 0.05$, $P^f < 0.05$

degradation, cell proliferation, adherence and migration. The protein-mediated signal mediation is impaired and inflammatory cytokines are activated through proteolysis. In the present study, the data indicate that the expression of MMP-9 is significantly higher in atypical hyperplastic tissue and EC tissue compared with normal tissue. Furthermore, the

expression levels of MMP-9 in EC tissue of stage III were higher than those noted in stages I and II, which is consistent with the studies of Yu and colleagues (22). MMP-9 exhibits a high expression in EC, although the specific mechanism has not been fully examined. MMPs are considered an important factor in the regulation and control of tumor angiogenesis,

tumor infiltration and metastasis (23). The integrity of the basement membrane and extracellular matrix is required in order to restrict tumor infiltration and metastasis. The function of MMP-9 is to degenerate collagen IV in the basement membrane in order to promote invasion and metastasis of cancer cells. Consequently, high expression of MMP-9 exerts an increasing degenerative effect towards the extracellular matrix (24).

Cancer progression is an extremely complicated process that involves a variety of factors. The majority of the studies suggest that ARTN and MMP-9 exhibit a strong association with cancer development, infiltration and metastasis (25). In concordance with the aforementioned reports, the present study indicated that the expression levels of ARTN and MMP-9 proteins were increased in EC tissues compared with normal endometrial epithelia. Their expression levels were further increased in the clinical pathological stage. Following correlation analysis, it can be concluded that ARTN protein is positively correlated with MMP-9. Therefore, the expression products of these 2 genes are candidates for molecular markers that closely relate with the occurrence and progression of EC as well as its invasion and metastasis. These findings provide experimental evidence for the early diagnosis, adjustment of the therapeutic schedule and prognosis of EC.

In conclusion, ARTN and MMP-9 proteins play an important role in the occurrence and development of EC. A combined detection for the expression levels of the two genes may become a relatively reliable diagnostic tool for the invasion and metastasis of EC.

REFERENCES

1. Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015; 136(5):E359.
2. He X, Bao W, Li X, Chen Z, Che Q, Wang H, Wan XP. The long non-coding RNA HOTAIR is upregulated in endometrial carcinoma and correlates with poor prognosis. *Int J Mol Med* 2014; 33(2):325-32.
3. Bao W, Wang HH, Tian FJ, et al. A TrkB-STAT3-miR-204-5p regulatory circuitry controls proliferation and invasion of endometrial carcinoma cells. *Mol Cancer* 2013; 12(1):155.
4. Docea AO, Mitrut P, Grigore D, Pirici D, Călina CD, Gofiță E. Immunohistochemical expression of TGF beta (TGF- β), TGF beta receptor 1 (TGFB1), and Ki67 in intestinal variant of gastric adenocarcinomas. *Rom J Morphol Embryol* 2012; 53(3 Suppl):683-92.
5. Zlatian OM, Comănescu MV, Roșu AF, et al. Histochemical and immunohistochemical evidence of tumor heterogeneity in colorectal cancer. *Rom J Morphol Embryol* 2015; 56(1):175-81.
6. Călina D, Docea, AO, Roșu L, et al. Antimicrobial resistance development following surgical site infections. *Mol Med Rep* 2017; 15(2): 681-88.
7. Călina D, Roșu L, Roșu AF, et al. Etiological diagnosis and pharmacotherapeutic management of parapneumonic pleurisy. *Farmacia* 2016; 64 (6):946-52.
8. Tănase A, Coliță A, Ianoși G, et al. Rare case of disseminated fusariosis in a young patient with graft vs. host disease following an allogeneic transplant. *Exp Ther Med* 2016; 12(4): 2078-82.
9. Wojciechowski VV, Călina D, Tsarouhas K, et al. A guide to acquired vitamin K coagulopathy diagnosis and treatment: the Russian perspective. *DARU* 2017; 25(1):10.
10. Ciobață R, Găman A, Trașcă D, et al. Pharmacological management of non-alcoholic fatty liver disease: Atorvastatin versus pentoxifylline. *Exp Ther Med* 2017; 13(5):2375-81
11. Sani TA, Mohammadpour E, Mohammadi A, et al. Cytotoxic and apoptogenic properties of dracocephalum kotschy aerial part different fractions on calu-6 and mehr-80 lung cancer cell lines. *Farmacia* 2017; 65(2):189-99.
12. Li S, Li Z, Guo F, et al. miR-223 regulates migration and invasion by targeting Artemin in human esophageal carcinoma. *J Biomed Sci* 2011; 18(1):24.
13. Yoshida N, Kobayashi K, Yu L, et al. Inhibition of TRPA1 channel activity in sensory neurons by the glial cell line-derived neurotrophic factor family member, artemin. *Mol Pain* 2011; 7(1):41-51.
14. Zhang C, Li C, Zhu M, et al. Meta-analysis of MMP2, MMP3, and MMP9 promoter polymorphisms and head and neck cancer risk. *Plos One* 2013; 8(4):e62023.
15. Takada M, Hirata K, Ajiki T, Suzuki Y, Kuroda Y.

- Expression of receptor for advanced glycation end products (RAGE) and MMP-9 in human pancreatic cancer cells. *Hepato-Gastroenterol* 2004; 51(58):928.
16. Wang L, Wang Q, Li HL, Han LY. Expression of MiR200a, miR93, metastasis-related gene RECK and MMP2/MMP9 in human cervical carcinoma--relationship with prognosis. *Asian Pacific J Cancer Preven* 2013; 14(3):2113-18.
 17. Gao C, Cheng X, Li X, Tong B, Wu K, Liu Y. Prognostic significance of artemin and GFR α 1 expression in laryngeal squamous cell carcinoma. *Exp Ther Med* 2014; 8(3):818.
 18. Ceyhan GO, Giese NA, Erkan M, et al. The neurotrophic factor artemin promotes pancreatic cancer invasion. *Ann Surg* 2006; 244(2):274.
 19. Banerjee A, Qian P, Wu ZS, et al. Artemin stimulates radio- and chemo-resistance by promoting TWIST1-BCL-2-dependent cancer stem cell-like behavior in mammary carcinoma cells. *J Biol Chem* 2012; 287(51):42502-15.
 20. Zhang M, Zhang W, Wu Z. Artemin is hypoxia responsive and promotes oncogenicity and increased tumor initiating capacity in hepatocellular carcinoma. *Oncotarget* 2016; 7(3):3267-82.
 21. Vandooren J, Pe VDS, Opdenakker G. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9): the next decade. *Crit Rev Biochem Mol* 2013; 48(3):222.
 22. Yu F, Jiang Q, Zhou Y, et al. Abnormal expression of matrix metalloproteinase-9 (MMP9) correlates with clinical course in Chinese patients with endometrial cancer. *Dis Markers* 2012; 32(5):321-27.
 23. Sillars-Hardebol AH, Carvalho B, de Wit M, et al. Identification of key genes for carcinogenic pathways associated with colorectal adenoma-to-carcinoma progression. *Tumor Biol* 2010; 31(2):89-96.
 24. Klein T, Bischoff R. Physiology and pathophysiology of matrix metalloproteases. *Amino Acids* 2011; 41(2):271-90.
 25. Stampoliou A, Arapantoni-Dadioti P, Pavlakis K. Epigenetic mechanisms in endometrial cancer. *J BUON* 2016; 21(5):301-6.

TUMOR POTENTIAL IN RAT WOUNDS AFTER SHORT- AND LONG-TERM ADMINISTRATION OF PLATELET-RICH PLASMA

N.N. OMAR¹, A.H. EL-TAWDF², R.F. TASH³, Y. SHOUKRY³, N.A. MAHMOUD⁴ and W. EL BAKLY⁵

¹Department of Biochemistry, Faculty of Pharmacy, Modern University for Technology and Information, Cairo, Egypt; ²General surgery Department, Military Medical Academy, Cairo, Egypt; ³Department of Anatomy and Embryology, Faculty of Medicine, Ain Shams University, Cairo, Egypt; ⁴Department of Plastic, Reconstructive Surgery and Burns, Faculty of Medicine, Ain Shams University, Cairo, Egypt; ⁵Department of Pharmacology, Faculty of Medicine, Ain Shams University, Cairo, Egypt

Received August 16, 2017 – Accepted November 7, 2017

Platelet-rich plasma (PRP) has been recognized as an effective strategy for tissue regeneration, however, the safety of PRP in wound healing in terms of tumorigenicity has not yet been addressed. Therefore, the aim of this study was to examine the impact of PRP administration on the expression of the inflammatory marker, tenascin-C (TnC) and the myofibroblast markers, α -smooth muscle actin (α -SMA) and vimentin. The immune suppressive response was examined by determining the level of forkhead box protein 3 (Foxp3). PRP was administered for both long-term (two times weekly for four weeks) and short-term (for the fourth week only) post-wounding. Collagen I (col1) and lysyl oxidase (LOX) were used to indicate complete healing, after which any increase in the myofibroblast or in the inflammatory markers would suggest tumor potential. Collagen III (col3), a marker for granulation tissue, was used to remark non-healing. Quantitative real-time reverse transcriptase polymerase chain reaction (QRT-PCR) and Western blot showed that after long-term administration of PRP, the expression of TnC, α -SMA and vimentin was barely detected, while being markedly expressed in the wounded non-treated group and in the short-term administration group. Moreover, the active expression of α -SMA in the two groups was associated positively with the expression of col3 and negatively with the expression of col1. The low expression of Foxp3 after short-term administration relative to the control group indicated active immunity against tumor development. In conclusion, these findings indicate that PRP can be safely used in short- and long-term administration without tumorigenesis concern.

Cancer is among the leading causes of death worldwide with 14 million new cases every year (1). Surgical removal is one of the main approaches to manage malignant tumors besides being used exclusively in benign tumors (2). The sequel postoperative injuries disrupt skin integrity and call for wound healing, an inherent conserved restoration

strategy. However, the reported association of chronic wounds with increased risk of tumor formation could prohibit the surgical choice (3). Accordingly, speeding up the healing process by therapeutic agents is challenged by providing complete regeneration of tissues without drifting into tumorigenesis.

Cell-based regenerative strategies by using cells

Key words: platelet-rich plasma, wound, tumor, α -smooth muscle actin, tenascin-C, forkhead box protein 3

Mailing address:

Dr Nesreen Nabil Omar,
Department of Biochemistry, Faculty of Pharmacy,
Modern University for Technology and Information, Cairo, Egypt
Professional postal address: 11571
Tel.: +2 01001423075
e-mail: dr.n.nabil@gmail.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

and/or bioactive molecules has been found efficient in improving wound healing, a repair process that was parallel to an increase in platelet count (4). In this context, platelet-rich plasma (PRP) has shown success over the past two decades in enhancing wound healing in a wide range of clinical applications with cosmetic and bone grafting at one end of the spectrum and general surgery at the other end (5). However, prolonged use of PRP has been hindered by the poor knowledge of its oncogenic potential. PRP is an autologous preparation of platelets in concentrated plasma; hence it is characterized by the presence of high concentrations of various growth factors, including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and transforming growth factor (TGF). These growth factors are known to regulate the processes of inflammation, cell migration and proliferation; all are evident in wound healing (6, 7). In addition to growth factors, the prepared PRP contains catabolic mediators like matrix metalloproteinases (MMPs) which, in normal conditions of healing, catalyze the turnover of extracellular matrix macromolecules, but also have a role in abnormal conditions of tumor growth (8). Therefore, the molecular complexity of PRP preparations necessitates further study to assess the potential efficacy and safety of autologous platelet rich plasma.

Typical healing processes would begin with an inflammatory recruitment of innate and adaptive immune systems that function in removing dead cells and tissues so there is an expanse for new tissues formed by migration and proliferation of fibroblasts. Ideally, wound healing ends in remodeling where disorganized collagen type III (col3) fibers are re-organized, cross-linked by lysyl oxidase enzyme (LOXs) and aligned along tension lines to be transformed into collagen type I (col1) fibers (9). As such, the response to tissue injury is expected to transpire at a highly linked framework, but sometimes this lattice runs amok and tumor opportunistically develops.

Interestingly, tumor stroma strongly resembles the granulation tissue of healing skin wounds, however, the process in cancer tissue is not self-limiting, resulting in uncontrolled cell proliferation,

invasion and metastasis (10). The cellular and molecular mechanisms which are common between wound repair and cancer make it hard to decide on the safety of PRP. Myofibroblast and mesenchymal cells are examples of cellular phenotypes which dominate during wound healing, but are known to influence tumor progression and metastasis (11). During wound healing, transient transformation of fibroblast into myofibroblast phenotypes develops to contract the extracellular matrix (ECM) and to bring the margins of the wound together (12). On the other hand, myofibroblasts are the sovereign sub-population of cancer-associated fibroblasts in the tumor stroma, which contribute to ECM remodeling and foster mechanical cancer cell invasion through contractile actin microfilament bundles (13). Therefore, myofibroblast phenotype is a defining feature of both cell repair and growing tumors. Recognition of myofibroblasts is feasible by using α -smooth muscle actin (α -SMA) expression as a marker (14) and if found upregulated in a fully healed tissue would raise a red flag. Another remarkable shared cellular type between repair and tumor is mesenchymal cells. As the events during healing include liberating keratinocytes from adhesion, these events may be taken over in favor of cancer cell migration, a process called epithelial–mesenchymal transition (EMT) (15). Vimentin was selected as a mesenchymal marker expressed by cells when they undergo EMT (15). These cellular transitions are closely associated with the ignited inflammatory response during tissue repair or cancer progression (11). In the same manner, tenascin-C (TnC) was recognized as a highly expressed protein at the inflammatory phase of tissue repair but not detected after the repair is complete (16). Meanwhile, the active pattern of TnC expression during cancer progression presents TnC as a preponderant inflammatory mediator in tumor stroma (17). Moreover, the immune suppressive response was examined using regulatory T (Treg) cells through the expression of forkhead box protein 3 (FOXP3) mRNA. Tregs constitute a large percentage of lymphocytes that reside in the skin and, despite playing a major role in the suppression of self-harmful immune responses, they also limit eradication of tumor cells (18). Consequently, in

this study TnC expression was estimated together with FOXP3, α -SMA and vimentin as cues for the carcinogenic potential of PRP four weeks post-wounding, when it is time for the remodeling.

Since the aforementioned markers are also an integral part of incompletely healed wounds during granulation tissue formation, their remarkable expression is considered alarming only after complete healing. Therefore, verification of wound healing stage was undertaken in which completing the healing process was guaranteed using col1 and LOX upregulation while col3 was used as a marker for granulation tissue formation.

MATERIALS AND METHODS

Animals

All animal procedures and care were carried out according to the general guidelines of Research Ethics Committee of the Faculty of Medicine of Ain Shams University, which conformed to the guiding principles of the International Council on Harmonization and the Islamic Organization for Medical Sciences, the United States Office for Human Research Protections and the United States Code of Federal Regulations and operates under Federal Wide Assurance No.FWA00006444. Thirty-eight Sprague Dawely (SD) female rats (170–200 g) were selected from the Laboratory Animals Research Center in the faculty of medicine Ain Shams University. The rats were maintained under controlled temperature and 12 hours light/12 hours dark conditions for one week before the start of the experiments. They were allowed to feed on standard laboratory chow and tap water ad libitum.

Preparing platelet-rich plasma

Fourteen rats were anesthetized with ether, followed by blood collection from the rats via open chest cardiac puncture. About 100 ml of blood was collected from them after sacrifice. Collectively, the blood was then mixed with sodium citrate anticoagulant solution (3.8%) (9 parts of blood to 1 part of sodium citrate). Then, the blood was centrifuged at 1000 rpm for 15 min at 20°C for separation of platelet rich plasma. Also, the plasma was centrifuged at 3000 rpm for 10 min at 20°C to obtain platelet pellet. The platelet concentrate was dissolved in phosphate buffer saline (PBS), pooled and incubated at room temperature

for 30 min on a rotating platform to eliminate platelet agglomerates. Afterwards, autologous thrombin was prepared as previously described (19). At this step, 330 μ L of calcium gluconate (100mg/ml) was added to 10 ml of plasma and 1 ml of thrombin preparation to 4 ml of platelet concentrate and incubated for 1 h at room temperature to facilitate growth factor release. The platelet secretion was centrifuged at 4000 rpm for 5 min to reduce the presence of platelet membrane fragments. The supernatant was filtered with a 0.22 μ m pore filter, divided into aliquots, and frozen at -80°C for subsequent use (20).

Study design

The last 24 rats were randomly divided into 4 groups (n=6). Group I normal control; group II was exposed to injury as described below and injected subcutaneously with the vehicle; groups III and IV were exposed to injury and injected subcutaneously at the site of the injury with PRP at a dose of 0.5ml/kg (as 1:1 mixture with PBS) twice/week for one and four weeks, respectively. PRP dose was selected according to previous study (21).

Wound creation

Rats were anesthetized by intraperitoneal injection of ketamine and xylazine (100 mg/kg and 5 mg/kg). The hair on the back of each of the rats was removed and bare areas were disinfected using 70% alcohol, which was allowed to dry after application. The tip of a scalpel was used to make a longitudinal incision (5 x 3 cm) to a depth of 6 mm along the right portion of the back of the animal and the fragment was removed to expose the muscle and laceration of the dorsal muscle fibers to simulate a traumatic wound. 3/0 vicryl suture was used for subcutaneous closure of the wounds. Regular swiping with alcohol for disinfection was applied twice daily. All procedures were performed under clean conditions. Animals were allowed to breathe spontaneously during the surgery. A heating lamp was used to preserve body temperature at approximately 37°C (22).

At the end, the animals were anaesthetized by sodium thiopental injection (50 mg/kg) sacrificed. Wounds were excised and divided into three pieces for all animals. Two pieces were fixed into a 10% formaldehyde solution and 2.5% glutaraldehyde in 0.1M phosphate buffer for histopathological and scanning electron microscopy (SEM) examinations, respectively. The third piece was stored in -80°C until analysis.

Real-Time RT-PCR

RNA was extracted using RNeasy Plus Mini kits (Qiagen, catalog no. 52304) according to the manufacturer's instructions. RNA was eluted in 60 µL volumes. RNA yield and purity was assessed using NanoDrop 2000 (Thermo Scientific, USA) and stored at -80°C. RNA was reverse transcribed to first strand cDNA using QuantiTect Reverse Transcription Kit (Qiagen) (cat. No. 205310) and RT Primer Mix (1 µg). The expression of each gene was analyzed by RQ-PCR using QuantiTect SYBR Green PCR Kit (Cat. no. 204141) and Applied Biosystems StepOnePlus instrument. PCR initial activation step of 95°C for 15 min is required to activate HotStarTaq DNA polymerase. Standard fast thermal cycling parameters of 40 cycles of 95°C for 15 s and 60°C for 60 s were applied in accordance with the manufacturer's recommendations. All reactions were performed in triplicate, and actin beta served as an internal control. Selected primer sequences are illustrated in Table I. The results were quantified as Ct values, where Ct is defined as the threshold cycle of PCR at which the amplified product is first detected, and expressed as the ratio of target to control. The expression levels of collagen I, collagen III, lysyl oxidase, α -smooth

muscle actin, FOXP3, vimentin and tenascin-C mRNA were quantified using RT-PCR.

Western blot

Tissue protein was extracted using TRIzol reagent, and protein concentrations were estimated by the Bradford method. Twenty microgram of protein per lane were separated with 10% SDS polyacrylamide gel electrophoresis gels and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were then incubated at room temperature for 2 h with blocking solution comprised of 5% nonfat dried milk in 10 mM Tris-Cl, pH 7.5, 100 mM NaCl, and 0.1% Tween 20. Membranes were incubated overnight at 4°C with the indicated primary antibodies against col1, col3, LOX, α -smooth muscle actin, FOXP3, vimentin, tenascin-C and beta-Actin (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) and then incubated with a mouse anti-rabbit secondary monoclonal antibody conjugated to horseradish peroxidase at room temperature for 2 h. After each incubation, the membranes were washed four times with 10 mM Tris-Cl, pH 7.5, 100 mM NaCl, and 0.1% Tween 20 at room temperature. Chemiluminescence detection was performed with the

Table I. List of genes selected for RQ-RT-PCR validation.

Gene	Forward Primer	Reverse primer	Amplicon size
Col1a1	GGATCGACCCTAACCAAGGC	GATCGGAACCTTCGCTTCCA	210
Col3a1	GAAGGGCAGGGAACAACCTGA	GGGCAGTCTAGTGGCTCATC	317
LOX	CGAGAGGGAACACACTCTCAC	GGGCTGGAACGCCATAGTAA	81
α -SMA	CATCCGACCTTGCTAACGGA	GTCCAGAGCGACATAGCACA	366
Vim	GCACGTCTTGACCTTGAACG	TGAGGTCAGGCTTGGAACG	151
Tnc	GACTGCTCAGGTGGAGAGTT	CAGTATCCGTCCCATCCACG	89
FOXP3	ACAAGAACCTGATGCCCAAC	GGGGGTTCAAGGAAGAAGAG	201
ACTB	GAGGCCAAGTGTGACTTTGTG	AGGCCAGGAACCTCCCAATA	225

Col1: collagen I; col3: collagen III; LOX: lysyl oxidase; α -SMA: alpha smooth muscle actin; vim: vimentin; Tnc: tenascin-C; FOXP3: forkhead box protein 3; ACTB: actin beta

Amersham detection kit according to the manufacturer's protocols. The amount of studied protein was quantified by densitometric analysis using BioRad software, USA. Results were expressed as arbitrary units after normalization for β -actin protein expression.

Statistical analysis

Data were analyzed using a one-way ANOVA (Prism 5, GraphPad) followed by Bonferroni-corrected post hoc tests when appropriate. All results are presented as the means $\pm$ SEM. A threshold of $P < 0.05$ was considered statistically significant.

RESULTS

Early PRP treatment for 28 days increased the expression of collagen I and lysyl oxidase while decreased the expression of collagen III.

As shown in Fig. 1, after 28 days of injury without introducing any treatment, the mRNA expression of col3 was 2.4-fold higher than the control group while col1 and LOX were 7- and 3.8-fold lower than the control group, respectively ($P < 0.001$). When PRP

was applied twice weekly only for the fourth week col1, LOX and col3 mRNA expression were not significantly different from the wounded group ($P > 0.05$). When PRP was applied twice weekly for 28 days col1 and LOX mRNA expression were 7.7- and 7.4-fold higher than the wounded group while col3 expression was 4-fold lower than the wounded group ($P < 0.001$). Congruently, as shown in Fig. 3A, Western blot analysis also demonstrated that early scheduled treatment for a long period (four weeks) significantly inhibited the premature form, col3 while providing LOX enzyme in high expression to afford the mature form col1 to ensure complete transformation from granulation tissue to normal skin (23).

Early PRP treatment for 28 days decreased the expression of α -SMA and vimentin.

The mRNA of α -SMA was investigated as a marker for the preponderance of myofibroblasts in wounds when left untreated for 28 days and when treated with PRP for short- and long-term. As shown in Fig. 2, untreated wounds showed 4.7-fold increase in the mRNA of α -SMA in respect to the

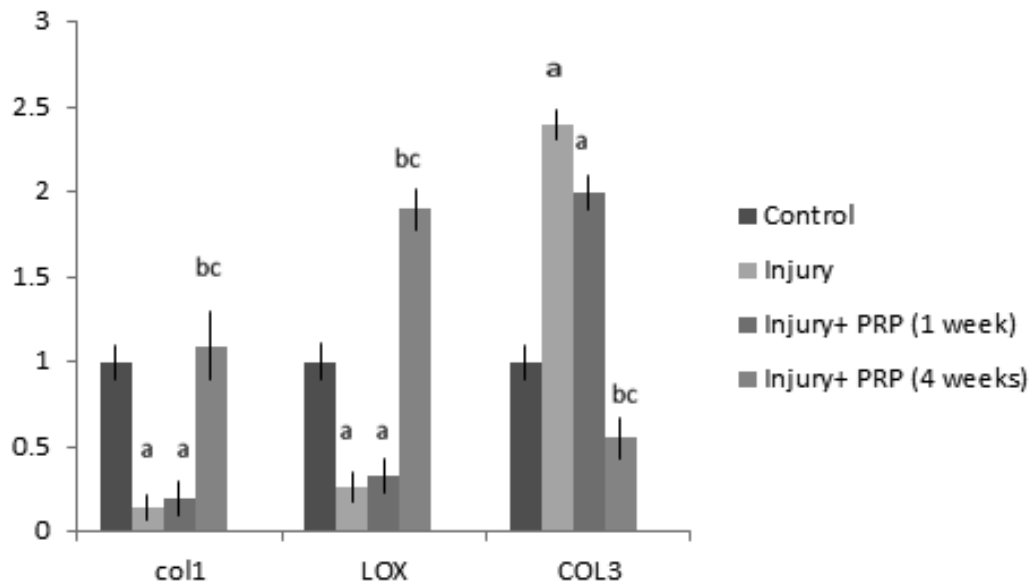


Fig. 1. Bar chart showing mRNA expression of COL-1, COL-3, LOX in rats of the control, model of skin incision, and PRP-treated groups for one week and for four weeks. Bars represent fold change and individual values are expressed mean $\pm$ S.D. COL-1, collagen I; COL-3, LOX, lysyl oxidase; a: significant ($P < 0.05$) vs control; b: significant ($P < 0.05$) vs injury group; c: significant ($P < 0.05$) vs one week PRP treatment group; one-way ANOVA followed by Bonferroni-corrected post hoc tests.

control group, despite it being as late as four weeks after wounding ($P < 0.001$). mRNA of α -SMA after short-term PRP administration was 11% lower than the non-treated group ($P = 0.023$). This result was found insignificant by post-hoc pairwise comparison ($P > 0.0083$). mRNA expression of α -SMA was positively correlated with mRNA expression of col3 and negatively correlated with mRNA expression of col1 (Table II). After long-term PRP administration, α -SMA expression was 8-fold lower than the non-treated group ($P < 0.001$).

Vimentin mRNA was activated by 6.4-fold after injury relative to the control group ($P < 0.001$). After 7 days of PRP treatment, vimentin mRNA expression was insignificant from the non-treated group as indicated by post-hoc pairwise comparison ($P > 0.0083$). After 28 days of PRP treatment, vimentin mRNA expression was 13-fold lower than its expression in the non-treated group ($P < 0.001$). In Western blot analysis, α -SMA and vimentin after 4 weeks of PRP administration were barely expressed (Fig. 3B).

Early PRP treatment for 28 days increased the expression of FOXP3 mRNA and decreased that of tenascin-C

An assertion of the inflammatory state and further verification of the tumor potential was provided by examining the expression of TnC. TnC mRNA was 2.3-fold over the control in the non-treated group. Introducing PRP for a limited time of one week decreased the expression of TnC mRNA by

17% relative to the non-treated group ($P = 0.02$). This result was found insignificant by post-hoc pairwise comparison ($P > 0.0083$). When PRP was introduced for the full time course, TnC expression was reduced by 5.9-fold relative to the non-treated group ($P < 0.001$) (Fig. 2).

As for FOXP3 mRNA, there was an 11-fold decrease of its expression in the wounded non-treated group relative to control. After one week of PRP treatment, the expression of FOXP3 was not significant compared to the wounded non-treated group. After administration of PRP for 4 weeks, FOXP3 mRNA was 9.8-fold higher than the non-treated group. Quantification of protein expression using Western analysis yielded results parallel to the PCR technique (Fig. 3B).

Sections of skin from the control group showed normal epidermis and dermis, and abundance of intact collagen fibers (Figs. 4A and 5A). On the other hand, examination of the skin of wounded rats, showed damaged dermis and fragmented collagen fibers (Figs. 4B and 5B). The skin of wounded rats treated with PRP for one week (Figs. 6C and 5C) showed histopathological changes similar to those observed in the untreated wounded group. However, morphological examination of the skin in PRP treated group for four weeks (Figs. 4D and 5D) showed similar morphology to that of the control group.

DISCUSSION

Wound healing and tumor development

Table II. Significant correlation between groups.

Parameter	Wounded gp.		One week PRP gp.	
	R	P-value	r	P-value
Col1 and α -SMA	-0.82*	P= 0.45	-0.9*	P= 0.014
Col3 and α -SMA	0.85*	P= 0.32	0.9*	P= 0.011

Col1: collagenI; α -SMA: alpha smooth muscle actin; col3: collagenIII. Correlation between the two parameters was evaluated using the Spearman correlation test. Significance level (*) $P < 0.05$

are conformists in their cellular and molecular mechanisms which drove Alexander Haddow to suggest that “tumor production is a possible overhealing” (24). Upon delving into the common phases between wound healing and tumor, it was found that similar incidents are uncontrolled in the tumor microenvironment, which turns any rejuvenating agent into a possible risk. A tumor free outcome requires a careful selection of a rejuvenating agent and introducing it at the proper time into the wound which, despite having a continuous healing process, could be deconstructed into three phases; inflammation, synthesis and remodeling (9).

An ideal therapeutic agent was described as one that would help each phase to take its course completely while forging smooth transition between phases, although too much exhortation could eventually result in tumor development. As for PRP, its proinflammatory action which is being praised for the efficient cleaning in wound repair, is, in another perspective, a font of reactive oxygen species (ROS). Consequently, overproduction of ROS that can directly damage DNA and modify the proteins involved in DNA repair and apoptosis would increase

the risk of the tumor development (6). Furthermore, growth factors in PRP, which enhance proliferation of epithelial cells and re-epithelialization of the wounded site, might also provide unchecked and mutant cancer cells with immortal traits (6).

Therefore, the current study was concerned with addressing the cellular and stromal tumorigenic potential of PRP, four weeks post-wounding. For instance, the activation of fibroblasts and their differentiation into myofibroblasts which constitute the scaffold in tumor stroma (13). Myofibroblasts participate in wound healing also through migration, proliferation and contraction necessary to restore homeostasis in damaged tissue (12). In this regard, the myofibroblast marker, alpha smooth muscle actin (14), was estimated. The current results show that alpha smooth muscle actin was barely expressed after four weeks of PRP administration. There was a multifold increase in the α -SMA expression in the non-treated group and when PRP was administered in the fourth week only. Phenotypic switching from an epithelial to mesenchymal cell (EMT) could play physiological or pathological functions according to the time window in which it occurs.

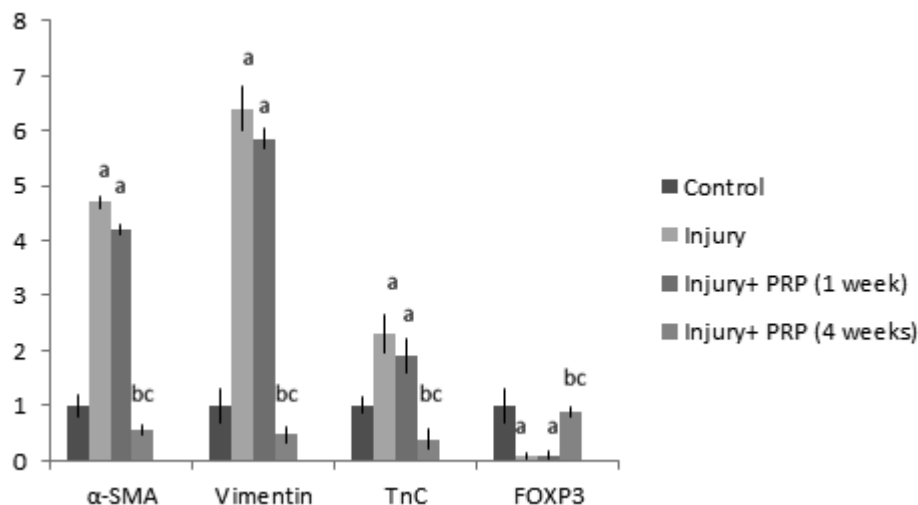


Fig. 2. Bar chart showing mRNA expression of α -SMA, vim, TnC and FOXP3 in rats of the control, model of skin incision, and PRP-treated groups for one week and for four weeks. Bars represent fold change and individual values are expressed mean $\pm$ S.D. α -SMA, α -smooth muscle actin; vim, vimentin; TnC, tenascin-C; FOXP, forkhead box protein 3; a significant ($P < 0.05$) vs control; b significant ($P < 0.05$) vs injury group; c significant ($P < 0.05$) vs one week PRP treatment group; one-way ANOVA followed by Bonferroni-corrected post hoc tests.

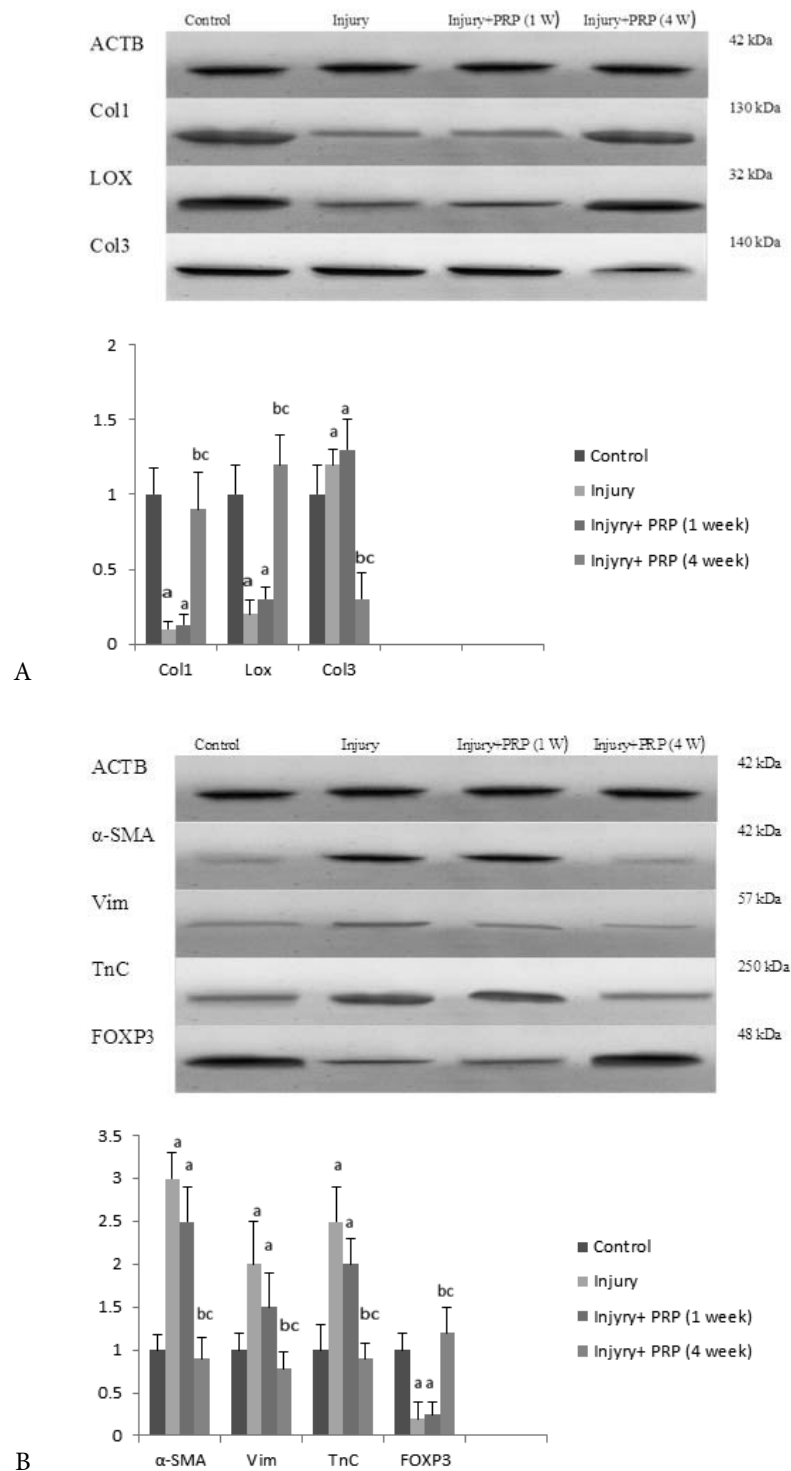


Fig. 3. Western blot analysis in control, model of skin incision, and PRP-treated rats for one week and for four weeks. **A)** Representative images and densitometric quantification of COL-1, COL-3, LOX; **(B)** Representative images and densitometric quantification of α -SMA, vim, TnC and FOXP3. Bars represent the ratio vs beta actin. Values are expressed as means $\pm$ S.D. COL-1, collagen I; COL-3, LOX, lysyl oxidase; α -SMA, α -smooth muscle actin; vim, vimentin; TnC, tenascin-C; FOXP, forkhead box protein 3; a significant ($P < 0.05$) vs control; b significant ($P < 0.05$) vs injury group; c significant ($P < 0.05$) vs one week PRP treatment group; one-way ANOVA followed by Bonferroni-corrected post hoc tests.

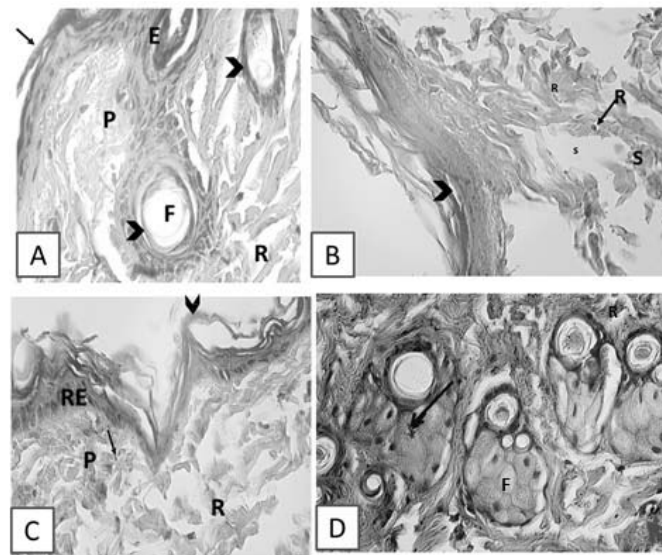


Fig. 4. A photomicrograph (H&E X 400) of sections in rat skin. **A)** Control group showing the epidermis (E) formed by multiple layers of flattened and polygonal epithelial cells, covered by a thick cellular layer of keratocytes forming the stratum corneum (arrow); the papillary dermis (P) is formed mainly of thin collagen fibers, while the reticular dermis (R) contains coarse ones. The hair follicles (F) are formed by concentric lamellae of flattened cuticle cells (arrow head). **B)** Skin injury group showing the wound scab (arrow head), the fragmented reticular dermis (R), damaged tissue spaces (S), polymorph leucocyte (arrow). **C)** One-week treated PRP group showing re-epithelization of the epidermis (RE), the discontinuous layer of keratin (arrow head), papillary dermis (P) infiltrated by some polymorphs (arrow), and branched and fragmented collagen fibers of the reticular dermis (R). **D)** Four-week treated PRP group showing hypertrophied reticular dermis with numerous newly formed hair follicles (F) that are formed of polyhedral cells with rounded central nucleus and granular basophilic cytoplasm, some of which show mitotic figures (arrow). Note no spaces between the fibers of the reticular dermis and the polymorph infiltrate (lower arrow).

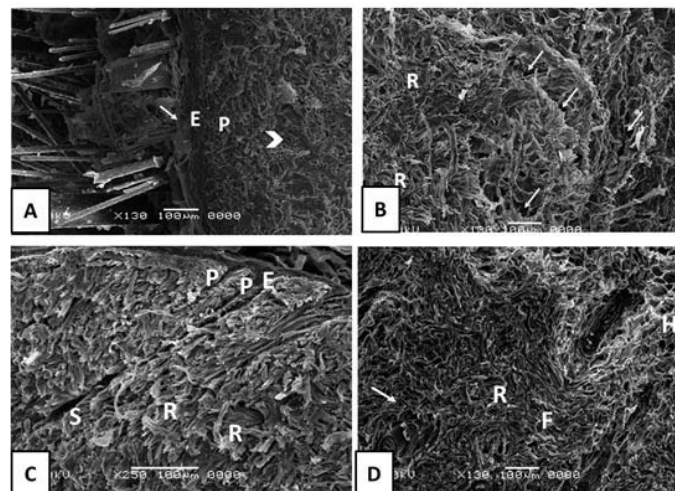


Fig. 5. A photomicrograph (SEM X 130) of sections in rat skin. **A)** Control group showing the epidermis (E), covered by a thick stratum corneum (arrow), the papillary dermis (P), hair follicles (arrow head). **B)** Skin injury group showing coarse irregular fragmented collagen fibers of the reticular dermis (R) with spaces in between (arrow). **C)** One-week treated PRP group showing the epidermis (E), dermal papillae of loose, sparse and thin collagen fibers of the papillary dermis (P), spaces (S) between the coarse collagen fibers of the reticular dermis (R). **D)** Four-week treated PRP group showing regular concentric arrangement (arrows) of the collagen fibers of the reticular dermis (R) around the newly formed hair follicles (F), follicular hyperplasia extends deep to the hypodermis (H).

In this cellular phenotype, losing adhesion and acquisition of migration is the core of this transition. EMT is remarked by activating the expression of mesenchymal genes and proteins such as vimentin. In the present study, long-time administration of PRP significantly inhibited vimentin expression compared to control rats. In addition, α -SMA mRNA levels in the short-term and non-treated rats were correlated with col3 and reversibly correlated with col1. This finding may be part of the myofibroblast cytoskeletal changes mediated for completing the wound healing. In order to further weigh the insidious tumor potential in the current study, inflammation was evaluated in wound stroma.

While self-limiting wound healing is expected to have extinguished inflammation by the end of re-epithelialization, the evolving cancer tissues would have prolonged inflammation. In this regard, TnC, an extracellular matrix glycoproteins, was evaluated. TnC modulates cell migration, proliferation and cellular signaling through induction of pro-inflammatory cytokines and oncogenic signaling molecules. Recently, it was shown to have high levels in the tumor stroma and in response to tissue injury. In the current results, expression of TnC mRNA levels in the full time course-treated rats was significantly lower than the non-treated group. This finding precludes the tumor-inducing potential of PRP and indicates inflammation-free stroma. Although previous studies reported upregulated expression of TnC in response to growth factors (25), the suppressive effect of PRP on TnC is in line with the fact that the pro-inflammatory function of TGF- β at the early stage of wound healing is dueled with an anti-inflammatory performance in the late stage (26). In the non-treated and limited time-treated groups, TnC expression was remarkably higher than in the control rats.

This continuous flared up inflammation after PRP administration in the fourth week post-wounding ignited the fears that the scant healing process would be coupled with sufficient immune suppression to start carcinogenesis. This has led us to further evaluate the immune suppressive capacity of Tregs through the expression of FOXP3. Several-fold inhibition of Tregs after seven days PRP administration compared

with marked activation in four-week-treated rats provided a cellular level affirmation for an active anti-tumor immunity even with short-time PRP administration.

In conclusion, the data we report here specifically precludes the tumorigenic threat when PRP was used for long-term in improving the process of tissue repair. α -Smooth muscle actin and vimentin-positive myofibroblasts, in association with the inflammatory marker TnC, ceased with long-term PRP administration in wounded rats and in collateral with complete healing. These findings provide evidence that using PRP in chronic wound would promote the healing process with no special considerations regarding tumor development. In the light of these data, effectiveness of long-term PRP administration together with the apparent safety should increase its potential in clinical applicability. Regarding short-term PRP administration, it seems to be non-carcinogenic, but continuity of active myofibroblast and inflammation may warrant further studies to screen for alarming markers.

REFERENCES

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; 65(2):87-108.
2. Norton JA. Surgical management of carcinoid tumors: role of debulking and surgery for patients with advanced disease. *Digestion* 1994; 55(Suppl 3):98-103.
3. Segatto I, Berton S, Sonogo M, et al. Surgery-induced wound response promotes stem-like and tumor-initiating features of breast cancer cells, via STAT3 signaling. *Oncotarget* 2014; 5(15):6267-79.
4. El-Mesallamy HO, Diab MR, Hamdy NM, Dardir SM. Cell-based regenerative strategies for treatment of diabetic skin wounds, a comparative study between human umbilical cord blood-mononuclear cells and calves' blood haemodialysate. *PLoS One* 2014; 9(3):e89853.
5. Lacci KM, Dardik A. Platelet-rich plasma: support for its use in wound healing. *Yale J Biol Med* 2010; 83(1):1-9.
6. Haubner F, Muschter D, Schuster N, Pohl F, Ahrens N, Prantl L, Gassner HG. Platelet-rich plasma stimulates

- dermal microvascular endothelial cells and adipose derived stem cells after external radiation. *Clin Hemorheol Microcirc* 2015; 61(2):279-90.
7. Park HB, Yang JH, Chung KH. Characterization of the cytokine profile of platelet rich plasma (PRP) and PRP-induced cellproliferation and migration: Upregulation of matrix metalloproteinase-1 and -9 in HaCaT cells. *Korean J Hematol* 2011; 46(4):265-73.
 8. Browning SR, Weiser AM, Woolf N, et al. Platelet rich plasma increases matrix metalloproteinases in cultures of human synovialfibroblasts. *J Bone Joint Surg Am* 2012; 94(23):e1721-7.
 9. Lorenz H, Longaker M. Wounds: Biology, Pathology and Management. New York: Springer, 2003, pp. 77-88.
 10. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* 1986; 315(26):1650-59.
 11. Rybinski B, Franco-Barraza J, Cukierman E. The wound healing, chronic fibrosis, and cancer progression triad. *Physiol Genomics* 2014; 46(7):223-44.
 12. Li B, Wang JH. Fibroblasts and myofibroblasts in wound healing: force generation and measurement. *J Tissue Viability* 2011; 20(4):108-20.
 13. Otranto M, Sarrazy V, Bonté F, Hinz B, Gabbiani G, Desmoulière A. The role of the myofibroblast in tumor stroma remodeling. *Cell Adh Migr* 2012; 6(3):203-19.
 14. Sugimoto H, Mundel TM, Kieran MW, Kalluri R. Identification of fibroblast heterogeneity in the tumor microenvironment. *Cancer Biol Ther* 2006; 5:1640-46.
 15. Clark AG, Vignjevic DM2. Modes of cancer cell invasion and the role of the microenvironment. *Curr Opin Cell Biol* 2015; 36:13-22.
 16. Chen J, Tellez G, Escobar J. Identification of biomarkers for footpad dermatitis development and wound healing. *Front Cell Infect Microbiol* 2016; 6:26.
 17. Nagaharu K, Zhang X, Yoshida T, et al. Tenascin C induces epithelial-mesenchymal transition-like change accompanied by SRC activation and focal adhesion kinase phosphorylation in human breast cancer cells. *Am J Pathol* 2011; 178(2):754-63.
 18. Nosbaum A, Prevel N, Truong HA, et al. Cutting edge: Regulatory T cells facilitate cutaneous wound healing. *J Immunol* 2016; 196(5):2010-4.
 19. Lucarelli E, Beccheroni A, Donati D, et al. Platelet-derived growth factors enhance proliferation of human stromal stem cells. *Biomaterials* 2003; 24(18):3095-100.
 20. Kazemnejad S, Allameh A, Gharehbaghian A, Soleimani M, Amirizadeh N, Jazayeri M. Efficient replacing of fetal bovine serum with human platelet releasate during propagation and differentiation of human bone-marrow-derived mesenchymal stem cells to functional hepatocyte-like cells. *Vox Sang* 2008; 95(2):149-58.
 21. Hesami Z, Jamshidzadeh A, Ayatollahi M, Geramizadeh B, Farshad O, Vahdati A. Effect of platelet-rich plasma on CCl4-induced chronic liver injury in male rats. *Int J Hepatol* 2014; 932930.
 22. Masoko P, Picard J, Eloff JN. The use of a rat model to evaluate the in vivo toxicity and wound healing activity of selected Combretum and Terminalia (Combretaceae) species extracts. *Onderstepoort J Vet Res* 2010; 77(1):E1-7.
 23. Yamauchi M, Sricholpech M. Lysine post-translational modifications of collagen. *Essays Biochem* 2012; 52:113.
 24. Haddow A. Addendum to “molecular repair, wound healing, and carcinogenesis: tumor production a possible overhealing”? *Adv Cancer Res* 1974; 20:343-66.
 25. Jinnin M, Ihn H, Asano Y, Yamane K, Trojanowska M, Tamaki K. Tenascin-C upregulation by transforming growth factor-beta in human dermal fibroblasts involves Smad3, Sp1, and Ets1. *Oncogene* 2004; 23(9):1656-67.
 26. Wang XJ, Han G, Owens P, Siddiqui Y, Li AG. Role of TGF beta-mediated inflammation in cutaneous wound healing. *J Investig Dermatol Symp Proc* 2006; 11(1):112-7.

CHARACTERIZATION OF HUMAN APICAL PAPILLA-DERIVED STEM CELLS

S. CANTORE^{1,2#}, A. BALLINI^{2,3,4#}, D. DE VITO³, F.S. MARTELLI⁵, I. GEORGAKOPOULOS^{4,6},
M. ALMASRI^{4,7}, V. DIBELLO¹, V. ALTINI¹, G. FARRONATO⁸, G. DIPALMA^{1,2#},
D. FARRONATO^{9#} and F. INCHINGOLO^{1,2,4#}

¹Department of Interdisciplinary Medicine, School of Medicine, University of Bari Aldo Moro, Bari, Italy; ²City Unity College, Athens, Greece; ³Department of Basic Medical Sciences, Neurosciences and Sense Organs, University of Bari Aldo Moro, Bari, Italy; ⁴World Academy of Growth Factors & Stem Cells in Dentistry, Athens, Greece; ⁵Microdentistry Institute, Florence, Italy; ⁶Dentist Education Institute, Athens, Greece; ⁷BPP School of Health, University Faculty of Dentistry, Birmingham, UK; ⁸Department of Biomedical, Surgical and Dental Sciences, School of Dentistry, University of Milan, Milan, Italy; ⁹Department of Morphologic and Surgical Sciences, Insubria University, Varese, Italy

Received July 5, 2017 – Accepted September 1, 2017

These Authors contributed equally to this work

Dental tissues represent an alternative and promising source of post-natal Mesenchymal stem cells (MSCs) for tissue engineering. Furthermore, dental stem cells from apical papilla (SCAPs) cells can be obtained from the wisdom tooth which is unnecessary for human masticatory function and frequently extracted for orthodontic reasons or dysodontiasis. More precisely, apical papilla is the immature, mostly uncalcified, precursor of the tooth root, therefore is composed of more undifferentiated cells than dental pulp. In addition, tooth extraction, especially by piezosurgery technique, can be considered less invasive in comparison to bone marrow or other tissues biopsy. Our work is aimed to investigate the safety of and predictable procedure on surgical immature third molar extraction and to provide new insight on SCAP research for future biomedical applications. The isolated cells were examined for stem cell properties by analyzing their colony-forming efficiency, differentiation characteristics and the expression of MSC markers.

Mesenchymal stem cells (MSCs) are adult stem cells essential for the homeostasis and the regeneration of tissues, following traumas or other kinds of pathological insult (1). MSCs were first isolated and reported about forty years ago. Such cells showed the genetic ability to differentiate into both mesodermal and non-mesodermal cell lineages, making them a useful tool in regenerative medicine (1, 2) (Fig.1).

The International Society for Cellular Therapy

(ISCT) proposed the following minimum criteria to clearly define MSCs: (i) the adherence to plastic surfaces under standard cell culture conditions; (ii) the expression of cell surface markers, such as CD90, CD73, and CD105, and the lack of expression of CD14, CD34, CD45, CD79, or CD19 and HLA-DR; and (iii) the capability to differentiate at least into chondrogenic, osteogenic, and adipogenic lineages (2).

Epithelial stem cells in dental tissues were initially reported by Harada et al. in 1999, by culturing cells

Key words: mesenchymal stem cells, piezosurgery, dental-derived stem cells, apical papilla stem cells, osteogenic differentiation

Mailing address:

Dr. Andrea Ballini,
University of Bari Aldo Moro,
P.zza Giulio Cesare, 11,
70124, Bari, Italy
Tel./fax: +3) 0805448514
e-mail: andrea.ballini@me.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

collected from the apex of incisor teeth harvested in a murine-model (3); these cells interestingly showed the characteristic to differentiate into ameloblasts able to produce enamel matrix.

To date, no information has been reported on dental-derived epithelial stem cells in humans, probably because this population may be specific to rodents, for the reason that their incisors vary from all the human teeth, since a mouse has teeth that erupt constantly during its lifetime.

Different stem cell populations have been isolated from human teeth, also known as dental-derived stem cells (D-DSCs) (Table I). The dental pulp of third molars is the most common source of dental mesenchymal stem cells, termed dental pulp stem

cells (DPSCs), able to differentiate into odontoblasts, osteoblasts, adipocytes, chondrocytes, and other cell types both *in vitro* and *in vivo* (4-6).

MSC-like cells have been identified in the other dental tissues in addition to DPSCs, such as the dental bud/follicle (DBSCs), periodontal ligament (PDLSCs), from dental pulp of exfoliated deciduous teeth (SHEDs), periapical cysts (hPCy-MSCs) and apical papilla (SCAPs) (7-13) (Fig. 2).

Stem cells from the apical papilla were found in the papilla tissue, in the apical part of the roots of developing teeth (9).

Compared with DPSCs, SCAPs show a better proliferation *in vitro* and a better regeneration of the dentin matrix when they are transplanted

Table I. Classification and characterization of dental stem cells.

Classification	Dental pulp stem cells DPSCs	Stem cells of human exfoliated deciduous teeth SHEDs	Periodontal ligament stem cells PDLSCs	Stem cells of the (dental) apical papilla SCAPs	Dental follicle stem cells DFSCs
Isolation	From dental pulp	Exfoliated deciduous teeth and coronal pulp	Root from extracted teeth	Impacted third molars	From the follicles of impacted third molars
Location	Permanent tooth pulp	Exfoliated deciduous tooth pulp	Periodontal ligament	Apical papilla of developing root	Dental follicle of developing tooth
Proliferative Index	High	High	High	High	High
Multipotentially	Odontoblast osteoblast chondrocytes myocytes neurocytes adipocytes, corneal epithelial cell, melanoma cell, induced pluripotent stem cell	Odontoblast osteoblast chondrocytes myocytes neurocytes adipocytes, induced pluripotent stem cell	Odontoblast osteoblast chondrocyte, cementoblast, neurocytes	Odontoblast Osteoblast neurocytes adipocytes, induced pluripotent stem cell	Odontoblast osteoblast neurocytes

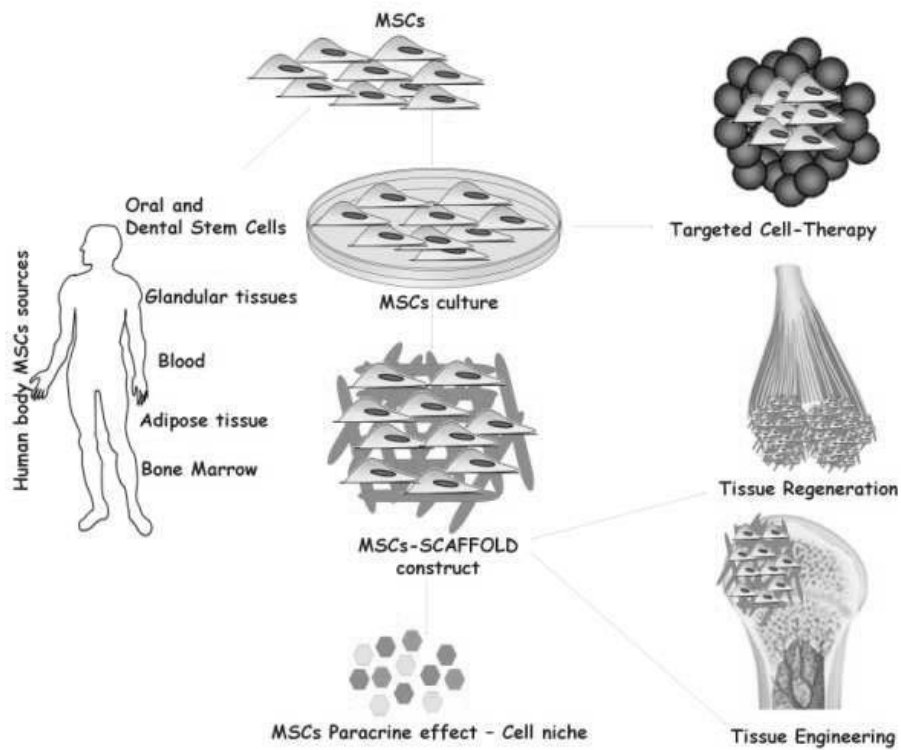


Fig. 1. Location, and current isolation approaches of the MSCs, and their applications across the breadth of regenerative medicine.

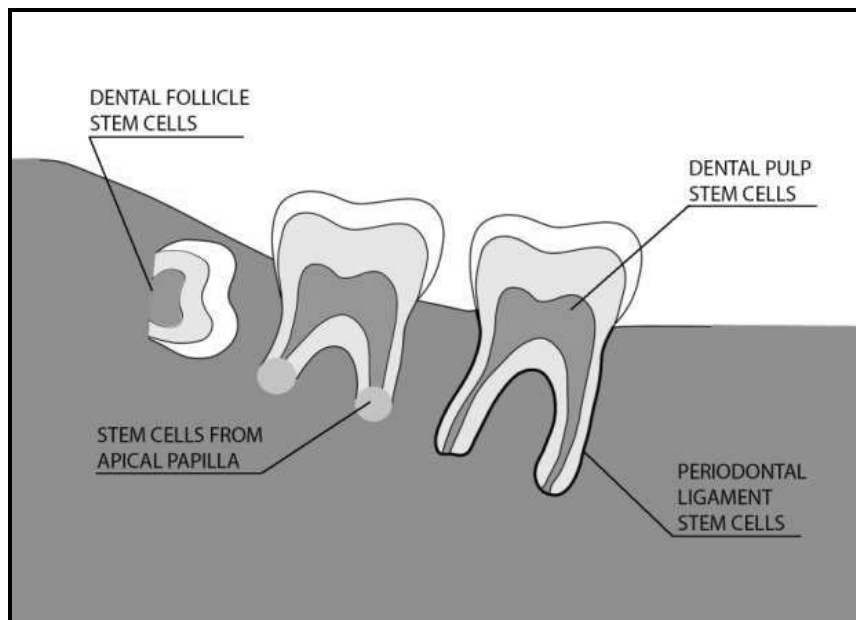


Fig. 2. In situ representation of dental-derived stem cells (D-DSCs) during tooth development.

in immunocompromised mice, suggesting that “developing” dental tissues may offer a better source for immature stem cells than “developed” dental tissues. SCAPs are capable of forming odontoblast-like cells, producing dentin *in vivo*, and are a likely cell source of primary odontoblasts for the formation of root dentin. SCAPs support apexogenesis, which can occur in infected immature permanent teeth with periradicular periodontitis or abscess. SCAPs residing in the apical papilla survive such pulp necrosis because of their proximity to the periapical tissue vasculature. Hence, even after endodontic disinfection, SCAPs can generate primary odontoblasts, which complete root formation under the influence of the surviving epithelial root sheath of Hertwig. Nevertheless, these cells are heterogeneous with various differentiation stages, as they comprise stem cells, progenitor cells and perhaps fibroblasts (9, 10).

Some Authors reports that DPSCs express the nuclear receptor nuclear receptor related 1 protein (NURR1) in basal and in osteogenic conditions (14), a surprise finding, considering that NURR1 is a member of the nuclear steroid/thyroid receptor superfamily, expressed primarily in the central nervous system, essential for the survival and development function of dopaminergic neurons of the ventral nuclei of the brain (15). Indeed the expression of NURR1 was already described in DPSCs and SHEDs, but a role for the receptor was mostly attributed during the differentiation towards a neuronal phenotype (16-18).

JunB is a proto-oncogene, a component of the activator protein-1 (AP-1) transcription factor family, which binds to the DNA of osteoblasts regulating bone remodeling and driving it towards bone formation; mice lacking JunB are osteopenic (19).

Insulin-like growth factor binding protein 5 (IGFBP-5), is a complex signal transduction protein expressed by osteoblast precursors, which stimulates osteoblast proliferation and activity acting as a growth factor, and increases bone formation (20).

NURR1 belongs to the family of orphan nuclear receptors, is induced by parathyroid hormone, and transactivates osteocalcin (OCN) expression, an osteogenic marker secreted during the late-stage differentiation process (21).

In this work, having established that SCAPs are an excellent model for studying osteoblast differentiation, we analyzed the mRNA expression of JunB, IGFBP-5, and nuclear receptor-related 1 protein NURR1, before and at the end of the differentiation process. Wherever possible we used good manufacturing practice-compliant reagents, so that the protocols were able to be directly translated to the experimental trial setting

MATERIALS AND METHODS

The present study followed the rules established in the 1975 Declaration of Helsinki, then revised in 1983. Impacted third molars (n=20) were collected from young patients (12–15 years old), who had undergone to extractions for orthodontics reasons. Before any treatments, the patients' guardians were properly informed about the advantages and disadvantages of all the planned procedures, first verbally and then with a written form for the informed consent.

Clinical procedures

After a detailed medical and dental history was obtained, orthopantomograms of the site were taken, and treatment started.

All patients were operated by the same surgeon under local anesthesia with 2% lidocaine, subsequent to a short-term antibiotic prescription. A mouth prop was put into the patient's mouth on the opposite side to ensure the mandible was adequately supported. The flap involved a sulcular incision from the mesial aspect of the 2nd mandibular molar and a distal relieving incision along the external oblique ridge to the anterior border of the ramus. After a full thickness flap was elevated, a piezosurgical device (Silfradent, Italy) was used to cut a precisely defined bony window. Cutting of bone and tooth was continuously accompanied by copious irrigation with chilled saline solution. When cutting and loosening of the alveolar bone, a curved periosteal elevator was placed on the lingual bone to improve exposure of the surgical field, to protect the lingual nerve, and to prevent the 3rd molar slipping accidentally into the lingual soft tissue. After the alveolar bone was removed by a periosteal detacher, the tooth was exposed and delivered in mesiolvestibular direction by inserting a straight elevator. No drainage was adopted in any cases.

The extraction socket was debrided and filled with colloidal silver. All extraction sockets were closed by interrupted 4/0 absorbable silk (SKD, Italy) sutures.

Laboratory procedures

The apical papilla were gently detached from the immature roots, minced and digested in a solution containing 3 mg/mL collagenase type I (Sigma, St. Louis, MO, USA) and 4 mg/mL dispase (Sigma, St. Louis, MO) for 30 min at 37°C. Purified stem cells from apical papilla (SCAPs) were seeded at 1×10^4 cells/mL into 10-cm culture dishes and cultured in alpha minimum essential medium (α -MEM, Gibco, Life Technologies, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (Gibco, USA), 100 U/mL penicillin and 100 μ g/mL streptomycin at 37°C in 5% CO₂. The fresh medium was changed every 2 days. Cells were subcultured at the ratio of 1:3 when they reached 75–85% confluence. SCAPs at 1–3 passages were used for subsequent experiments. Stem cells were routinely examined under the phase-contrast inverted microscope (Nikon 100TS). Generally, SCAPs at the same passage were used in each experiment. For differentiation towards osteogenic lineage, cell culture medium was supplemented with 10⁻⁸ M dexamethasone and 50 μ g/mL ascorbic acid (Sigma Aldrich, Milan, Italy). All the experiments were conducted in triplicate.

Reverse transcriptase polymerase chain reaction

Differentiated SCAPs were subjected to mRNA extraction using spin columns (RNeasy, QIAGEN, Hilden, Germany), according to the manufacturer's instructions. The first-strand cDNA synthesis was carried out from total RNA using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). The PCR mixture contained 0.2 mM of each dNTP, 1.5 mM MgCl₂, 0.2 mM of each primer. Briefly, an RT mixture containing 1 μ g total RNA, dNTPs (deoxyribonucleoside triphosphates), Oligo(dT), RT buffer, MgCl₂, DTT (dithiothreitol), RNaseOUT, SuperScript II RT, DEPC (diethyl pyrocarbonate) treated water to final volume of 100 μ l was prepared, according to the manufacturer's instructions. Diluted cDNA (2 μ l) was transferred into a 50 μ l PCR reaction mixture containing dNTPs, MgCl₂, primers, autoclaved distilled water, and Platinum *Taq* DNA polymerase (Invitrogen). Amplification reactions specific for the cDNAs of the housekeeping gene glyceraldehyde phosphate dehydrogenase (*GAPDH*) were carried out using *taq*/polymerase (Invitrogen).

The primer sequences were: sense NURR1 5'-GCAGTCAACcGATTTGG-3'; antisense NURR1 5'-GTGATGGGATTTCATTGAT-3', sense IGFBP-5 5'-TTGCCTCAACGAAAAGAGC-3' antisense IGFBP-5 5'-AGAATCCTTTGCGGTCACA-3' sense JunB 5'-CCAGTCCTTCCACCTCGACGTTTACAA-3' antisense JunB 5'-GACTAAGTGCCTGTTTCTTTTCCACAG-3', sense Osteocalcin 5'-CATGAGAGCCCTCACA-3', antisense Osteocalcin 5'-AGAGCGACACCCTAGAC-3'.

After amplification, 10 μ l of each reaction was analyzed by 1.5% agarose gel electrophoresis, and visualized by ethidium bromide. The resulting bands were detected by a light sensitive CCD (charge-coupled device) video system (BioDocAnalyze; Whatman Biometra, Goettingen, Germany).

Western blot analysis

SCAPs were cultured in osteoblastic-differentiation medium for 5, 10, 15 and 21 days, and solubilized by incubation on ice for 10 min in lysis buffer containing 50 mM Tris (tris(hydroxymethyl) aminomethane)-HCl (pH 8), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 1% NP40, 1 mM phenylmethylsulfonyl fluoride, and a commercial protease inhibitor mixture (Sigma Aldrich). Protein determination was performed with the Detergent Compatible (DC) Protein Assay Kit II (Bio-Rad Laboratories Inc., Richmond, CA, USA). Cell proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Hybond, Amersham Pharmacia, London, UK) using a semidry blotter (Bio-Rad Laboratories Inc.). The blots were probed overnight at 4°C with rabbit anti-Coll I, mouse anti-OCN, and mouse anti- β -actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with the appropriate fluorescent dye-conjugated secondary Ab (LI-COR Biosciences GmbH, BadHomburg, Germany), specific reactions were revealed with the LI-COR's Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE, USA).

Microarray analysis

SCAPs differentiated for 21 days and undifferentiated cells as dry pellets were used for microarray analysis. Three micrograms of RNA of each sample was processed. Microarray analysis was carried out using the Affymetrix HGU133 Plus2 GeneChip Array platform (Affymetrix,

Santa Clara, CA, USA). The samples were analyzed with GeneSpring (Agilent Technologies, Milan, Italy), using two different algorithms for normalization: MicroArray Suite 5.0 (MAS5) and GC-RobustMultiarray Average (GC-RMA). After the analysis with MAS5 or with GCRMA, probe sets regulated at least twice were selected. The results obtained were validated by reverse transcriptase–polymerase chain reaction (RT-PCR) performed on the same samples used for microarray analysis.

Flow cytometric analysis

Cells were trypsinised, resuspended, and aliquoted at 500,000/100 μ L PBS into a fluorescence-activated cell sorting (FACS) tube. Each aliquot was incubated with saturating concentrations of fluorochrome-conjugated antibodies on ice for 30 min. At the end of incubation, the tube was centrifuged at $200 \times g$ for 5 min and inverted onto a paper towel to drain the supernatant. The cells were washed twice, and 0.5 mL of PBS was added to each tube. The samples were kept on ice in the dark until used for FACS. CD73-FITC, CD90-PE, CD105-APC, CD13-PE, CD29-APC, CD44-FITC, and CD45-APC-H7 (Becton Dickinson - BD) antibodies were used for our analysis. Samples were analysed with FACSCanto II instruments (BD), and data were analysed by using FlowJo software (Tree Star).

Confocal microscopy analysis

Fixed monolayer cells were permeabilised with 0.1% Triton X-100 in PBS for 5 min. Samples were then blocked with 3% BSA in PBS for 30 min at room temperature and then incubated with fluorescein isothiocyanate (FITC)-conjugated STRO-1 (Santa Cruz) and with Phycoerythrin (PE)-conjugated CD146 (Santa Cruz) antibodies, diluted at 1:50 in PBS containing 3% BSA, for 1 h at room temperature. After washing in PBS, samples were stained with 1 mg/mL 4,6-diamidino-2-phenylindole (DAPI; Sigma) in PBS for 1 min, and then mounted with anti-fading medium (ProLong Antifade; Invitrogen) and visualised by confocal microscopy (Leica; TCS SP5). For detection, the samples were sequentially excited with the following laser wavelengths: 405 nm lines of a diode laser for DAPI and 488 nm lines of the argon laser for FITC and PE. The excitation and the detection of the samples were carried out in sequential mode to avoid overlapping of the signals. Optical sections were obtained at increments of 0.3 mm in the Z-axis, and were digitized with a scanning mode format of 1024×1024 pixels.

RESULTS

Patients were recalled on postoperative day 7 and examined for wound healing, nerve function, postoperative assessment, and major postoperative complications.

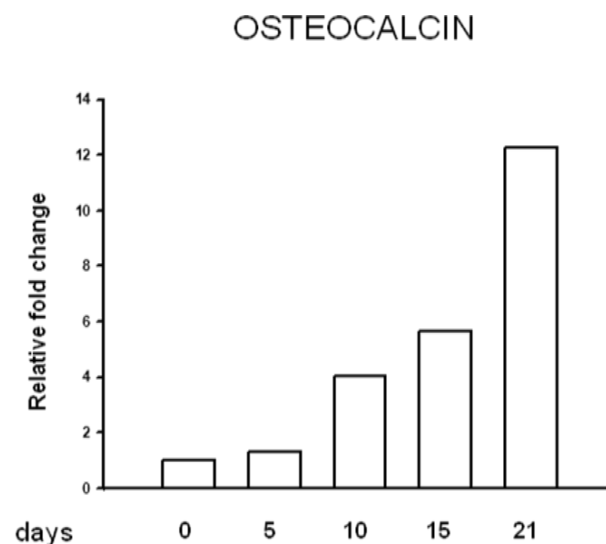


Fig. 3. Real-time PCR for *e* Osteocalcin (OCN) on SCAPs differentiated at 0-5-10-15-21 days.

From a clinical point of view, all impacted mandibular 3rd molars were successfully removed, the success rate was 100%. The average time of operation was 16.1 min (range from 10 to 32 min). No major intraoperative complication occurred during the surgical procedure.

Our results were meaningful in showing that SCAPs, already at five days of differentiation, expressed the mRNA for the noncollagenous protein OCN, known to be a specific marker of late osteoblast differentiation (Fig. 3).

To better evaluate the gene expression profile during SCAP differentiation into osteoblast-like cells, we used microarray technology. Microarray analysis showed an upregulation of NURR1, IGFBP-5 and JunB genes in SCAPs differentiated for 21 days in osteogenic conditions, respectively, 8.25-, 8.1-, and 2.8-fold more expressed than in undifferentiated cells (Fig. 4).

NURR1 was expressed in SCAPs in basal conditions and was still present when the cells differentiated into osteoblast-like cells. Detection of NURR1 protein levels were performed by Western blotting, confirming the decrease of the protein in NURR1-silenced cells. NURR1 downregulation favors the osteogenic differentiation of SCAPs. Moreover, these results confirmed the finding that NURR1 is expressed in undifferentiated SCAPs, but down levels of the receptor prompt the differentiation of the cells towards the osteoblastic lineage and mineral matrix deposition.

Throughout the immunophenotype analysis, specific cell-surface markers were selected to evaluate whether the isolated cells contained the MSC population. These experiments were carried out with the goal of isolating a population of pluripotent-like stem cells derived from human SCAPs. In order to characterise the population, we performed a flow cytometric analysis to detect the expressions of markers in cells isolated from apical papilla.

We found that the population was CD73+, CD90+, CD105+, CD13+, CD29+, CD44+, and CD45- (Fig. 5A), which indicated characteristics of MSCs (2). The cells at passage 2 expressed high levels of CD73 (99.6%), CD90 (100%), CD105 (36.6%), CD13 (100%), CD29 (100%), and CD44

(99.6%). However, they stained negatively for the hematopoietic marker CD45 (1.2%), indicating the mesenchymal lineage of these cells.

The isolated cell populations analyzed by confocal microscopy expressed the cell surface molecules STRO-1 and CD146 (Fig. 5B), two early MSC markers that are present on both DPSCs and bone marrow MSCs (6). Together, these results demonstrate that cells isolated from SCAP tissues have MSC characteristics with respect to the expressions of markers.

DISCUSSION

From a clinical point of view, piezosurgery is universally recognized as a minimally invasive and selectively cutting instrument that inertes surrounding soft tissues and important structures such as nerves, vessels, and mucosa (22-27).

The incision to approach the embedded tooth was made from the vestibular side of the anterior border of the ramus to the mesial aspect of the 2nd molar, which reduced the chance of accidental injury to the lingual nerve. In addition, using a curved periosteal elevator to retract the lingual flap could also provide protection to the lingual nerve during osteotomy, which is supported by many researchers.

From a laboratory point of view, to date NURR1 has been considered primarily involved in dopaminergic neuron differentiation and activity. Interestingly, a key role for the receptor was attributed during the differentiation of SCAPs towards a neuronal phenotype (16-18). Indeed, NURR1 is crucial for dopaminergic neurons function (28) and its malfunction has been correlated with neurological and inflammatory disease (29). By contrast, studies in the literature on the role of NURR1 in osteoblasts are controversial: studies on mice highlighted an effect in increasing the osteoblastic phenotype of primary culture and osteoblastic cell lines (21, 30), while a more recent work indicated that NURR1 down-regulated the main osteoblastic differentiation pathway, involving β -catenin, in a human osteoblastic cell line (31). In addition, some Authors report that MSCs, such as DPSCs, express NURR1 in basal and osteogenic conditions (14). Thus, NURR1 is expressed in DPSCs, with a prominent role

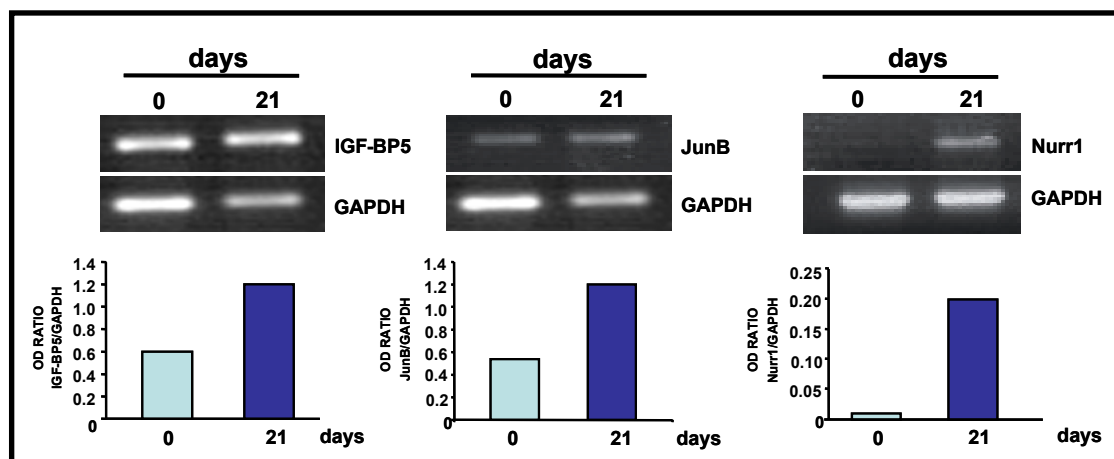
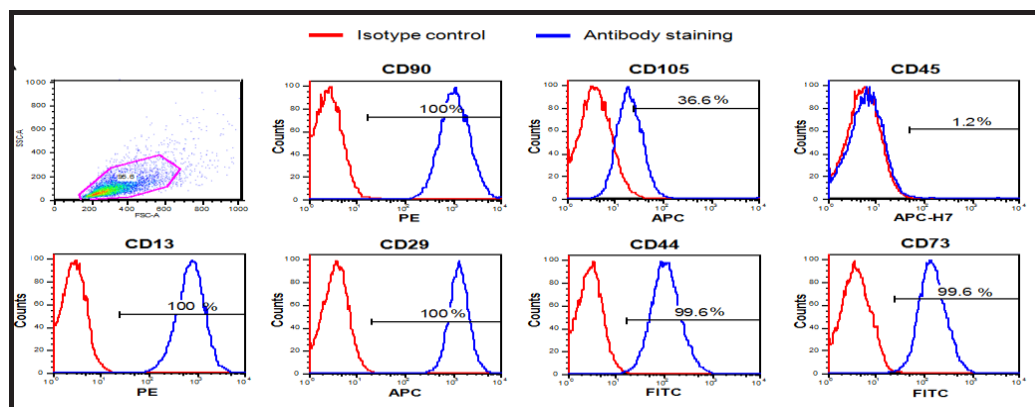


Fig. 4. *IGF-BP5*, *JunB* and *Nurr1* genes are up-regulated in SCAPs differentiated in osteoblast-like cells. Microarray gene analysis was performed on SCAPs at time 0 and after 21 days of incubation with osteogenic medium.

A



B

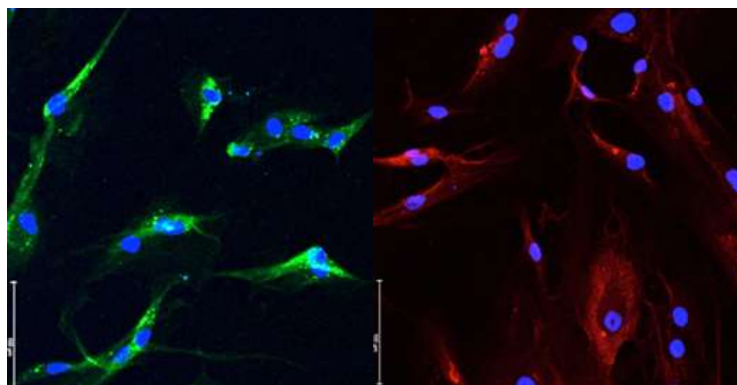


Fig. 5. Cell surface markers of SCAPs. **A)** Immunophenotype by FACS analysis of cells isolated from SCAPs. A heterogeneous, pooled population of cells at passage 4 was analysed with flow cytometry to detect the expressions of cell surface markers. Results reported forward and side scatter, and in each plot, the X-axis shows relative fluorescence and the Y-axis shows the number of events. Histograms show the isotype control (red peaks) versus specific antibody staining (blue peaks) and the percentage of cells positive for the selected molecules. **B)** Immunofluorescence analysis of STRO-1 and CD146 expressions in SCAPs. Surface antigens were stained with fluorescein isothiocyanate (FITC, green) for STRO-1 and Phycoerythrin (PE, red) for CD146, and the nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, blue); bar scale = 100 μm.

in neuronal differentiation, but its role in osteogenic differentiation needs more investigations. Consistent with the essential role played by NURR1 in nervous tissue, we speculated whether the molecule could, in some way, influence SCAP differentiation towards the osteoblastic phenotype and, more precisely, whether NURR1 inhibition could interfere with SCAP osteoblastogenesis. Tissue regeneration, based on an adult stem cell approach, is still faced with strategies directed to control and increase their differentiation capacity, thus the discovery of target molecules to modulate, in order to address the desired commitment, is still an open challenge (32). Mainly, MSC multipotency has a problem in regeneration therapy to drive cell differentiation to the correct lineage, reconstructing the expected mature tissue. The role of NURR1 in osteoblast differentiation is not yet clearly established and is intriguing, since it is expressed in MSCs during osteoblastogenesis (14, 30, 31) but it is also involved in neuronal differentiation (16, 33).

Microarray analysis showed that genes for JunB, IGFBP-5, and NURR1, which are connected with osteoblast differentiation and bone formation, increased significantly at 21 days of SCAP differentiation. These data were validated and further supported by RT-PCR analysis. The role of these genes in the SCAP osteoblastic differentiation needs to be elucidated, particularly in the case of NURR1, whose action in bone cell biology is mostly unknown.

In conclusion, using microarray and RT-PCR analysis we showed an increased expression of IGFBP-5, JunB, and NURR1, indicating that they could be involved as key genes in the differentiation process of SCAPs into osteoblasts. This represents a further step in the study of the MSC and SCAP differentiation process, leading to a more confident use of these stem cells as a source of osteoblasts to be used in regenerative and reconstructive medicine, replacing lost or damaged bone tissue.

Discovering an appropriate *in vivo* method for inhibiting NURR1 during MSC osteogenic differentiation could improve an adult stem cell-based tissue engineering, enhancing bone tissue regeneration.

ACKNOWLEDGEMENTS

The present research work is in memory of Prof.

Stefano Tetè from the University of Chieti “G. D’Annunzio”, Italy, well-regarded and distinguished national and international dental surgeon and biomedical scientist, and pioneer in international dental stem cell research.

REFERENCES

1. Ballini A, Scacco S, Coletti D, Pluchino S, Tatullo M. mesenchymal stem cells as promoters, enhancers, and playmakers of the translational regenerative medicine. *Stem Cells Int* 2017; 2017:3292810.
2. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; 8:315-17.
3. Harada H, Kettunen P, Jung HS, Mustonen T, Wang YA, Thesleff I. Localization of putative stem cells in dental epithelium and their association with Notch and FGF signalling. *J Cell Biol* 1999; 147:105-20.
4. Ballini A, Mastrangelo F, Gastaldi G, et al. Osteogenic differentiation and gene expression of dental pulp stem cells under low-level laser irradiation: a good promise for tissue engineering. *J Biol Regul Homeost Agents* 2015; 29:813-22.
5. Gronthos S, Mankani M, Brahimi J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci U S A* 2000; 97:13625-30.
6. Shi S, Gronthos S. Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *J Bone Miner Res* 2003; 18: 696-704.
7. Mori G, Ballini A, Carbone C, et al. Osteogenic differentiation of dental follicle stem cells. *Int J Med Sci* 2012; 9:480-87.
8. Di Benedetto A, Brunetti G, Posa F, et al. Osteogenic differentiation of mesenchymal stem cells from dental bud: Role of integrins and cadherins. *Stem Cell Res* 2015; 15:618-28.
9. Huang GT, Sonoyama W, Liu Y, Liu H, Wang S, Shi S. The hidden treasure in apical papilla: the potential role in pulp/dentin regeneration and bioroot engineering. *J Endod* 2008; 34:645-51.
10. Chrepa V, Henry MA, Daniel BJ, Diogenes A. Delivery of apical mesenchymal stem cells into root

- canals of mature teeth *J Dent Res* 2015; 94:1653-59.
11. Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S. SHED: stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci U S A* 2003; 100:5807-12.
 12. Wang L, Shen H, Zheng W, et al. Characterization of stem cells from alveolar periodontal ligament. *Tissue Eng Part A* 2011; 17:1015-26.
 13. Tatullo M, Falisi G, Amantea M, Rastelli C, Paduano F, Marrelli M. Dental pulp stem cells and human periapical cyst mesenchymal stem cells in bone tissue regeneration: comparison of basal and osteogenic differentiated gene expression of a newly discovered mesenchymal stem cell lineage. *J Biol Regul Homeost Agent* 2015 ;29:7131-38.
 14. Di Benedetto A, Posa F, Carbone C, et al. NURR1 Downregulation Favors Osteoblastic Differentiation of MSCs. *Stem Cells Int.* 2017; 2017:7617048.
 15. Le Grand JN, Gonzalez-Cano L, Pavlou MA, Schwamborn JC. Neural stem cells in Parkinson's disease: a role for neurogenesis defects in onset and progression. *Cell Mol Life Sci* 2015; 72:773-97.
 16. Kanafi M, Majumdar D, Bhonde R, Gupta P, Datta I. Midbrain cues dictate differentiation of human dental pulp stem cells toward functional dopaminergic neurons. *J Cell Physiol* 2014; 229:1369-77.
 17. Young FI, Telezhkin V, Youde SJ, et al. Clonal heterogeneity in the neuronal and glial differentiation of dental pulp stem/progenitor cells. *Stem Cells Int* 2016; 2016:1290561.
 18. Jarmalavičiūtė A, Tunaitis V, Strainienė E, et al. A new experimental model for neuronal and glial differentiation using stem cells derived from human exfoliated deciduous teeth. *J Mol Neurosci* 2013 Jun 26. [Epub ahead of print].
 19. Kenner L, Hoebertz A, Beil FT, et al. Mice lacking JunB are osteopenic due to cell-autonomous osteoblast and osteoclast defects. *J Cell Biol* 2004; 164:613-23.
 20. Mohan S, Baylink DJ. IGF-binding proteins are multifunctional and act via IGF-dependent and - independent mechanisms. *J Endocrinol* 2002; 175:19-31.
 21. Pirih FQ, Tang A, Ozkurt IC, Nervina JM, Tetradis S. Nuclear orphan receptor Nurr1 directly transactivates the osteocalcin gene in osteoblasts. *J Biol Chem* 2004; 279:53167-174.
 22. Labanca M, Azzola F, Vinci R, Rodella LF. Piezoelectric surgery: twenty years of use. *Br J Oral Maxillofac Surg* 2008; 46:265-69.
 23. Leung YY, Cheung LK. Risk factors of neurosensory deficits in lower third molar surgery: an literature review of prospective studies. *Int J Oral Maxillofac Surg* 2011; 40:1-10.
 24. Ballini A, Scattarella A, Crincoli V, et al. Surgical treatment of gingival overgrowth with 10 years of follow-up. *Head Face Med* 2010; 12:6-19.
 25. Piersanti L, Dilorenzo M, Monaco G, Marchetti C. Piezosurgery or conventional rotatory instruments for inferior third molar extractions?. *J Oral Maxillofac Surg* 2014; 72:1647-52.
 26. Scattarella A, Ballini A, Grassi FR, et al F. Treatment of oroantral fistula with autologous bone graft and application of a non-reabsorbable membrane. *Int J Med Sci* 2010; 7:267-71.
 27. Ballini A, Tetè S, Scattarella A, et al. The role of anti-cyclic citrullinated peptide antibody in periodontal disease. *Int J Immunopathol Pharmacol* 2010; 23:677-81.
 28. Satoh J, Kuroda Y. The constitutive and inducible expression of Nurr1, a key regulator of dopaminergic neuronal differentiation, in human neural and non-neural cell lines. *Neuropathology* 2002; 22:219-32.
 29. Vivekanantham S, Shah S, Dewji R, Dewji A, Khatri C, Ologunde R. Neuroinflammation in Parkinson's disease: role in neurodegeneration and tissue repair. *Int J Neurosci* 2015; 125:717-25.
 30. Lee MK, Choi H, Gil M, Nikodem VM. Regulation of osteoblast differentiation by Nurr1 in MC3T3-E1 cell line and mouse calvarial osteoblasts. *J Cell Biochem* 2006; 99:986-94.
 31. Rajalin AM, Aarnisalo P. Cross-talk between NR4A orphan nuclear receptors and β -catenin signaling pathway in osteoblasts. *Arch Biochem Biophys.* 2011; 509:44-51.
 32. Amini AR, Laurencin CT, Nukavarapu SP. Bone tissue engineering: recent advances and challenges. *Crit Rev Biomed Eng* 2012; 40:363-408.
 33. Long X, Olszewski M, Huang W, Kletzel M. Neural cell differentiation in vitro from adult human bone marrow mesenchymal stem cells. *Stem Cells Dev* 2005; 14:65-69.

HUMAN CARDIAC MULTIPOTENT ADULT STEM CELLS IN 3D MATRIX: NEW APPROACH OF TISSUE ENGINEERING IN CARDIAC REGENERATION POST-INFARCTION

G. DI SPIGNA¹, M. IANNONE², P. LADOGANA¹, S. SALZANO³, M. VENTRE², B. COVELLI¹,
E. DE MARINIS¹ and L. POSTIGLIONE¹

¹*Department of Translational Medical Sciences, University of Naples "Federico II", Naples, Italy;*

²*Interdisciplinary Research Centre on Biomaterials. Department of Chemical, Materials and Industrial Production Engineering, University of Naples "Federico II", Italy;* ³*Institute of Experimental Endocrinology and Oncology "G. Salvatore" (National Research Council), Naples, Italy*

Received June 22, 2017 – Accepted November 20, 2017

The first two Authors contributed equally to this paper

Myocardial infarction is the leading cause of morbidity and mortality in developed countries. It causes a left ventricular dysfunction, mainly due to the loss of functional tissue, resulting in heart failure. New therapies are being developed, using a tissue engineering approach, with the ultimate goal of restoring cardiac function by regenerating and repairing the damaged myocardium. In the present study we investigated the behaviour of a specific population of c-kit positive human cardiac stem cells, called Multipotent Adult Stem Cells (MASCs), grown within three-dimensional collagen scaffolds (3D), to establish whether they could be used in post-infarction cardiac regeneration. We also evaluated the expression levels of the Granulocyte Macrophage-Colony Stimulating Factor Receptor (GM-CSFR) and endoglin, a component of the Transforming Growth Factor beta (TGF- β) receptor complex. Finally, we also evaluated the expression of the $\alpha 2\beta 1$ integrin. MASCs cultured within 3D collagen matrices are able to proliferate and migrate even in the absence of chemotactic agents and express high levels of factors involved in cell proliferation and migration, such as GM-CSFR α chain and integrins. They therefore represent a promising approach to tissue engineering aimed to restore cardiac function. Our results also suggest a role of GM-CSF in cell proliferation, while TGF- β does not seem to be relevant.

Myocardial infarction, a clinical syndrome caused by a sudden and prolonged occlusion of a coronary arterial branch that leads to ischemic necrosis of myocardial cells, is the leading cause of death worldwide. In 90% of the cases, coronary occlusion is due to the formation of a thrombus on a pre-existing atherosclerotic plaque.

Myocardial infarction produces a left ventricular dysfunction mainly due to the death of

cardiomyocytes and the loss of functional tissue. Macrophages, monocytes and neutrophils migrate to the infarcted area and promote an inflammatory response. An important role is played by macrophage metalloproteinases that digest various structural components of the extracellular matrix (ECM). The weakening of the ECM causes a thinning of the myocardial wall and a ventricular dilatation, resulting in heart failure (1-3).

Key words: myocardial infarction, human cardiac multipotent adult stem cells, polymeric scaffolds, tissue engineering, cardiac regeneration post-infarction

Mailing address:

Prof. Postiglione Loredana,
Department of Translational Medical Sciences,
University of Naples "Federico II",
Via S. Pansini 5, Naples, Italy
Tel.: +39 0817463016 - Fax: +39 0817463321
e-mail: loredana.postiglione@unina.it

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

Heart failure can be treated pharmacologically or surgically. However, new and more efficient therapies are strongly needed, including those aimed to restore function by regenerating and repairing the damaged myocardium (4).

In our work we have approached this challenge using Tissue Engineering (TE) technology (5, 6). Essentially, many approaches have been proposed to restore cardiac function by repairing and regenerating the damaged myocardium: i) the use of networks (meshes) made with different polymers to prevent the expansion of the left ventricle; ii) tissue engineering *in vitro*, consisting in a first phase of cell culture on biodegradable scaffolds followed by *in vivo* implantation; iii) tissue engineering *in situ*, consisting in the injection of either the biomaterial alone or the scaffold as a vehicle for the release of cells or therapeutic agents, such as genes or proteins (7-11).

For TE, the choice of cells is central (12). To be clinically useful, engineered cardiac tissue should consist of autologous or immune-tolerated, phenotypically stable, cardiac cell populations (myocytes, fibroblasts, vascular cells) within a native-like tissue matrix. The size and thickness of an engineered graft should have a clinically relevant size, a requirement that cannot be met without vascularization. Electromechanical coupling of the cells with the host myocardium with synchronous contractile activity and generation of a contractile force are all necessary for the reestablishment of heart function. Many cell types potentially able to support angiogenesis, myogenesis, or both, have been taken into consideration.

Myoblasts, derived from skeletal muscle satellite cells, were among the first to be utilized in cardiac cell transplantation experiments (13). These cells are normally found under the basal membrane of muscle fibres in a quiescent state and, in response to injury, have the potential to re-enter the cell cycle, divide, and differentiate into functional skeletal muscle. The advantages of skeletal myoblasts include their availability from autologous sources, ability to proliferate and be expanded *ex vivo* and a higher resistance to ischemia as compared with cardiomyocytes. It was initially hoped that skeletal myoblasts, cells committed to a contractile tissue

phenotype (14), could trans-differentiate into cardiomyocytes following injection. However, it was found that skeletal myoblasts remain committed to form mature skeletal myotubes in the heart. Furthermore, mature myotubes as well as unfused skeletal myoblasts appear to remain mechanically and electrically isolated from the recipient myocardium (15-17).

Other studies have been focused on stem cell-derived cardiomyocytes. In particular, Embryonic Stem Cells (ESC) seem to be the ideal cell type for cardiac repair because of their pluripotency. In fact, they can form any cell in the heart with consequent extensive regenerative potential. There are, however, several problems, including the immunological incompatibility with the host myocardium and the tendency to form teratomas (18-20).

By manipulating the expression of transcription factors it is possible to transform somatic cells, such as fibroblasts, into induced pluripotent stem cells (iPSC) which are indistinguishable from ESC (21). Differentiation *in vitro* of iPSC cells into functional cardiomyocytes is possible and their use for treatment of injured myocardium is promising.

The discovery of circulating endothelial progenitor cells, their mobilization in response to ischemia, and their isolation from human bone marrow have stimulated studies on bone marrow-derived elements to regenerate ischemic myocardium (22-24).

The bone marrow compartment can be subdivided into two interdependent spaces: the hematopoietic cell compartment and the stroma containing Mesenchymal Stem Cells (MSCs). MSCs represent between 0.01% and 0.001% of all nucleated cells in adult human bone marrow, but can be readily expanded in culture (25). Normally, MSCs can differentiate into osteocytes, chondrocytes and adipocytes but, under certain conditions, they can differentiate into other cell types, including cardiomyocytes (26). MSCs can also evade immune detection due to a direct inhibition of inflammation as well as to a lack of cell-surface costimulatory molecules (27). These properties, combined with the ability to rapidly expand in culture, raised the possibility of employing MSCs for myocardial revascularization. However, clinical trials showed

that the injections of bone marrow-derived cell populations caused only a marginal increases in the ejection fraction (0-5%) (28).

A subset of adult bone marrow cells (BMCs) shares with embryonic stem cells a degree of developmental plasticity; in fact, c-kit-positive BMCs implanted in the infarcted heart lose the hematopoietic commitment and integrate with the host myocardium, thus establishing a microenvironment necessary for the engrafted cells to adopt the cardiac destiny and form *de novo* myocardium (29). However, the mechanism by which BMCs improve the outcome of acute myocardial infarction and chronic ischemic cardiomyopathy in humans remains unclear. It has been suggested that BMC therapy of the infarcted heart exerts its beneficial effects by activation of resident progenitor cells. According to this hypothesis, BMCs may contribute indirectly to cardiac regeneration by releasing a variety of peptides, exerting a paracrine action on the myocardium and its resident cells (28).

Traditionally, the heart has been viewed as a static organ unable to repair damage. According to this paradigm, the number of myocytes is established at birth and this population of terminally differentiated myocytes is irreplaceable throughout life. The static view of the myocardium implies that myocyte death and regeneration have a scant role in cardiac homeostasis. However, the existence of cycling ventricular myocytes in the normal and pathological heart has been reported (30).

These data raised questions about two apparently contradictory lines of evidence: the early and irreversible withdrawal of cardiac myocytes from the cell cycle and the presence of cycling myocytes undergoing mitosis and cytokinesis. These results therefore raised the issue of the origin of dividing myocytes and their increase in response to acute or chronic overload, suggesting the presence of a Cardiac Stem Cell (CSC) population (30). Recently, CSCs have been defined as undifferentiated cells expressing on the membrane the stem cell-related antigens, MDR1, c-kit, and Sca-1, in variable combinations. CSCs sorted separately for each of the stem cell antigens appear to have comparable growth and differentiation behaviour *in vitro*. Whether the

three classes of CSCs have the same potential *in vivo* is at present unclear (31).

The aim of our study was to investigate the behaviour of a specific population of human stem cells, called Multipotent Adult Stem Cells (MASCs), grown on three-dimensional matrix (3D), in order to establish whether they can be used in post-infarction cardiac regeneration.

MASCs, obtained from adult human heart, are c-kit positive, express several pluripotent state specific transcription factors, such as OCT4, Nanog, Sox2, Rex1, and display high levels of telomerase activity (32). MSCs, obtained from bone marrow, were used as a control. MSCs and MASCs were cultured 2D on petri dishes or within 3D collagen matrix that closely mimics the physiological environment of cells.

In our study we evaluated the effects of 3D collagen matrix on cell proliferation and migration. Moreover, since previous studies (33) have shown a potential role of growth factors in cardiac tissue regeneration, the expression levels of Granulocyte Macrophage-Colony Stimulating Factor Receptor (GM-CSFR) and endoglin, an accessory component of the Transforming Growth Factor beta (TGF- β) receptor complex, were also evaluated at different times of culture (3 and 7 days).

An increase in serum levels of GM-CSF after an ischemic damage, suggesting its possible role in stem cells homing, proliferation, migration and differentiation, has been reported (34). Moreover, it has been recently demonstrated that GM-CSF is able to mobilize circulating stem cells, leading to more rapid tissue growth and differentiation into cardiomyocytes on membranes implanted in the left ventricles (33). Other studies showed that endoglin is involved in angiogenesis and represents a powerful marker of neovascularization (35).

Since integrins regulate cell-to-cell and cell-matrix adhesion, we also evaluated their expression levels; in particular, we studied $\alpha 2$ and $\beta 1$ subunit expression, components of a specific heterodimeric transmembrane receptor for type I Collagen (36-37).

Cell proliferation on 3D scaffolds was evaluated on different days by alamarBlue assay, while cell migration was measured by time-lapse microscopy.

GM-CSFR and endoglin expression levels were analyzed by Western blotting assays and, finally, the expression levels of the $\alpha 2\beta 1$ integrin were evaluated by Flow Cytometry.

MATERIALS AND METHODS

Cell culture

Human cardiac MSCs, obtained from human cardiac tissue of explanted hearts, kindly provided by the Department of Pathology, University of Udine, were employed.

Stem cells were plated onto human fibronectin (10 ng/mL) (Sigma-Aldrich, St. Louis, Missouri, USA) coated dishes, in a medium composed as follows: 60% low-glucose Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY, USA), 40% MCDB-201 (Sigma), 1 X insulin-transferrin-sodium selenite (Sigma), 1 mg/mL linoleic acid-BSA (Sigma), 10⁻⁹ M dexamethasone (Sigma), 10⁻⁴ M ascorbic acid 2-phosphate (Sigma), 100 U penicillin and 1000 U streptomycin (Gibco), 2% fetal calf serum (FCS) (Gibco). This medium was supplemented with 10 ng/mL hEGF (Peprotech, London, UK) and 10 ng/mL hPDGF-BB (Peprotech). Cells were seeded at the density of 5-10×10³ cells/cm² and incubated at 37°C, with 5% CO₂. Medium was replaced every 3-4 days. Once adherent stem cells reached more than 50% confluence, they were detached with 0.25% trypsin-EDTA (Sigma) and re-plated at a 1:4 dilution, under the same culture conditions. MSCs, derived from bone marrow, were used as a control. These cells were cultured in α -Minimal Essential Medium (α -MEM) (Gibco), supplemented with 10% foetal bovine serum (FBS) (Gibco), 2 mM L-glutamine (Gibco), 100 U ml⁻¹ penicillin and 100 ml ml⁻¹ streptomycin (Gibco).

For cultures in 3D collagen lattices, MSCs and MSCs were cultivated in reconstituted bovine collagen gel (Sigma, St. Louis, MO, USA) prepared following the manufacturer's instructions. Briefly, 1 mL of 10X DMEM (Gibco, Grand Island, NY, USA) was added to 8 mL collagen (stock solution: 3 mg/mL), 1 mL of 0.1 M NaOH was added and pH 7.2 was reached by adding 0.1 M HCl. The resulting collagen solution (2.4 mg/mL) was gently mixed with the cells and allowed to gelify for approximately 40 min at 37°C, 5% CO₂. The medium was replaced every 3 days. All experiments in this work were performed two times.

alamarBlue assay

Cell proliferation was evaluated at different times (24h, 48h, 72h, 96h, 7 days, 10 days, 12 days) by the alamarBlue assay, an assay designed to measure quantitatively the proliferation of various human and animal cell lines. The assay incorporates a fluorometric/colorimetric growth indicator, detecting metabolic activity. Specifically, the system incorporates an oxidation-reduction (REDOX) indicator (alamarBlu), minimally toxic to living cells, that both fluoresces and changes colour in response to chemical reduction of the culture medium during cell growth. In fact, as cells being tested grow, innate metabolic activity results in a chemical reduction of the REDOX indicator. This growth-related reduction causes alamarBlu to switch from the oxidized (non-fluorescent, blue) form to the reduced (fluorescent, red) one.

alamarBlue assay of MSCs and MSCs, cultured on petri dish (2D) or within collagen matrix (3D), was studied and cells were seeded at the density of 5-10×10³ cells/mL in a full α -MEM.

In each culture well, 2 ml of DMEM medium (HyClone, UK) containing 10% (v/v) alamarBlue (AbD Serotec Ltd, UK) were added, followed by the incubation for 4 h at 37°C and 5% CO₂. The solution was subsequently removed from the wells and the culture plates were analyzed by a microplate reader (1420 Victor, Perkin Elmer, Italy) at wavelengths of 570 and 595 nm. Cell proliferation was finally determined by comparing the percentage (%) of reduction resulting at different times with those of the calibration curve obtained by correlating known cell numbers with the corresponding % of reduction values.

Cell migration assay

Migration of MSCs and MSCs, cultured on petri dishes or within collagen matrix, was studied. Cell migration experiments were performed with an Olympus IX 50 optical inverted microscope equipped with a mini incubator on a motorized stage (PRIOR). Experimental settings comprised migration both in 2D and in 3D, i.e. collagen gel. MSCs or MSCs were plated (2×10⁴cell/mL) or encapsulated at the density of (4×10⁴cell/mL) in the gels. Images of the samples were collected with a 10X magnification objective with a digital camera (CoolSnap Camera, Photometrics). Grayscale digital images were acquired every 10 min for 24 h. Cell trajectories were

extracted from the time lapse videos analyzed with Metamorph (Molecular Devices). The migration parameters are: Speed (S), i.e. the average speed of all cells analysed, and Persistence time (P), indicating the average time spent by the cells without encountering significant directional changes. Speed and Persistence time were extracted from the mean square displacement evaluated by each trajectory according to Iannone et al. (38).

Western blotting analysis

The expression of GM-CSFR (CD116) and endoglin (CD 105) was analyzed by Western blotting. Cells were washed three times and then lysed by lysis buffer (1% Triton X-100 in PBS). Cell lysates were collected by scraping and then centrifuged. Protein concentration in cell lysates was evaluated by Protein assay (Biorad, Richmond, CO, USA). The same amount of proteins, 40 µg/µL, was incubated in Laemmli sample buffer. Proteins were separated by 7.5% SDS-PAGE and then transferred on PVDF filters (Millipore, Bedford, MA). Non-specific reactive sites of membranes were blocked by 5% non-fat dried milk, 1% ovalbumin, 5% FCS and 7.5% glycine in PBS for 1 h at room temperature. Membranes were then incubated at 4°C overnight with the primary monoclonal antibodies: rabbit polyclonal anti human CD105 (dilution 1:200) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse polyclonal anti human GM-CSF (dilution 1:100) (Serotec, Oxford, UK).

After three washes, membranes were incubated for 1 h at room temperature with the respective secondary antibodies (anti-Mouse, anti-Rabbit) (BioRad) diluted 1:2000 and 1:3000. After three final washes, membranes were stained with an enhanced chemo-luminescence (ECL) detection kit (Amersham, Little Chalfont, England). Membranes were normalized with an anti-β-actin antibody (dilution 1:1000) (Sigma). A quantitative analysis was performed with a Scanner Densytometre (Snapscan 1212, AGFA).

Flow cytometry

In order to analyze integrin subunit expression, cells were washed twice with PBS 1X; cells in two-dimensional culture were then disaggregated with PBS/EDTA 2 mM, those in three-dimensional culture with 2mg/ml collagenase, for 10' at 37°C. Subsequently, cells were fixed in paraformaldehyde (PFA) 4% for 10 min at

37°C. After washing with PBS/BSA, 1mg/ml, cells were incubated with primary monoclonal mouse antibodies against α2 and β1 integrins (Chemicon, Temecula, CA, USA) for 40 min at 4°C. After washing again with PBS/BSA, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody for 20 min at 4°C. Finally, cells were suspended in PBS/BSA and analysed by flow cytometry (FACScan, Beckton Dickinson, Mountain View, CA, USA). Non-specific IgGs of the same isotype were used as a negative control. Results were reported as fold of expression, as compared to the negative control.

The stemness of the cells at each step was verified by the evaluation of CD90 expression after incubation with R-phycoerythrin (R-PE)-conjugated mouse anti-human monoclonal antibody (BD Biosciences).

Statistical analysis

Statistical analysis for normally distributed parametric data was performed using the Student's *t*-test, and the differences were considered statistically significant with *p*-values <0.05. Statistical analysis on non-parametric data were performed running a Kruskal-Wallis written in Matlab (The Matworks).

RESULTS

Proliferation on 3D scaffolds

Cell proliferation was evaluated by the alamarBlue assay on different days (1, 2, 3, 4, 7, 10, 12). No differences in proliferation rate were observed between MSCs cultured with or without a collagen matrix. In fact, in both cases their number duplicated in 12 days (Fig. 1a).

On the contrary, the proliferation rate of MSCs cultured on scaffold was lower as compared to cells cultured without matrix. In fact, while MSCs cultured in 3D conditions did not behave in the same way as MSCs, duplicating their number in 12 days, MSCs cultured in 2D conditions showed a 9-fold increase in their number on day 12 (Fig. 1b).

Migration on 3D scaffolds

In order to analyse migratory behaviour of MSCs and MSCs cultured on either petri dishes or within 3D collagen matrix, we evaluated relevant parameters of cell motility i.e Random Speed and

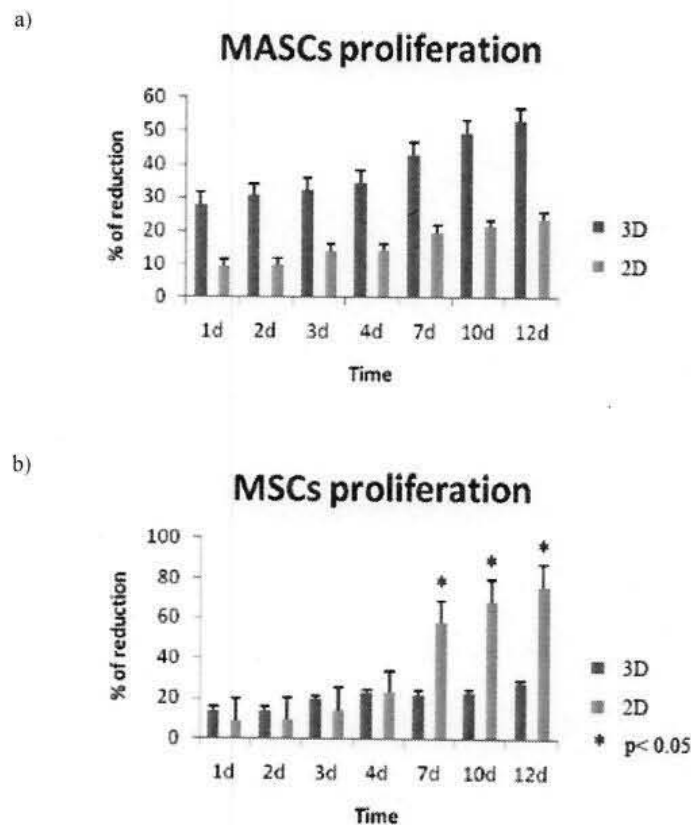


Fig. 1. Cell proliferation measured by alamarBlue assay during culture under two- or three-dimensional conditions. **a)** MASCs proliferation rates; **b)** MSCs proliferation rates. Proliferation rate of MSCs cultured in 3D matrix was significantly lower in respect to 2D condition (MSC 2D vs MSC 3D - * $p < 0.05$). (Analysis of Student's *t*-test was applied).

Persistence time. Fig. 2a shows that MASC cells cultured on petri dishes possess high random speed, whereas, when seeded within collagen gel, they show a migratory behaviour similar to MSCs cultured both in 2D and 3D environment. MASC cells cultured within collagen gel show a higher persistence time of migration that denotes a tendency for the cells to not change their direction of movement in a frequent manner. This behaviour is different to a significantly lower persistence of MSC both in 2D and in 3D conditions (Fig. 2b).

Expression of GM-CSFR α chain and endoglin

In order to analyze the GM-CSFR α chain and endoglin expressions, whole lysates obtained from MASCs and MSCs, cultured with and without

the collagen scaffold, were analyzed by Western blotting, as described above. Bands with the expected molecular weight of about 84 kDa, for GM-CSFR α chain, and of about 95 kDa, for endoglin, were present, with variable intensities (Fig. 3a). Expression levels of GM-CSFR α chain in MASCs cultured on 3D scaffold, on day 3 were 36% lower than the levels present in the same cells cultured without collagen matrix. From day 3 to day 7 the expression increased in both culture conditions but a more marked expression was observed in cells grown in 3D conditions. In fact, while in this case there was a 74% increase, in the population cultured in 2D conditions there was only a 33% increase.

Interestingly, even though MSCs cultured without collagen matrix showed the highest GM-

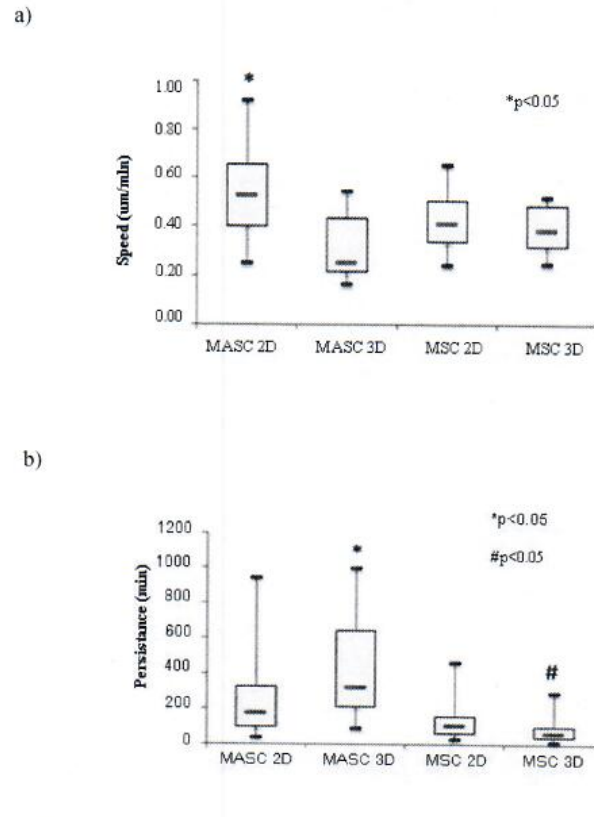


Fig. 2. Box plots of speed (a) and persistence time (b). a) Denotes significantly higher values (MASC 2D vs MASC 3D - * $p<0.05$); b) Denotes significantly lower values (MASC 2D vs MASC 3D - * $p<0.05$) and (MSC 3D vs MSC 2D - # $p<0.05$). (Analysis of Kruskal-Wallis was applied).

CSFR α chain levels, both on days 3 and 7, with a small, not statistically significant decrease (6%) on day 7, on 3D scaffolds MSCs did not express GM-CSFR α chain at all.

Endoglin was not present in either MASCs or MSCs cultured on scaffolds; by contrast, its expression could be observed in both cell lines cultured without matrix, although it was more pronounced in MASCs. Endoglin expression decreased with time. The decrease on day 7 was more pronounced in MASCs (62%), while it was not statistically significant in MSCs (17%). A quantitative analysis of the bands is shown in Fig. 3b.

Expression of $\alpha 2\beta 1$ integrin

In order to analyze the $\alpha 2$ and $\beta 1$ integrin

subunits expression, MASCs and MSCs, cultured with and without collagen scaffold, were analyzed by flow cytometry. Seven days after seeding, the expression levels were higher in MASCs cultured in 3D conditions, but there was not a statistically significant difference between MASCs and MSCs in 2D and 3D conditions (Fig. 4).

DISCUSSION

Myocardial infarction, a leading cause of death worldwide, causes a left ventricular dysfunction mainly due to a loss of functional tissue, resulting in heart failure. It can be treated pharmacologically or surgically, by heart transplantation. New and more efficient heart failure therapies are strongly needed,

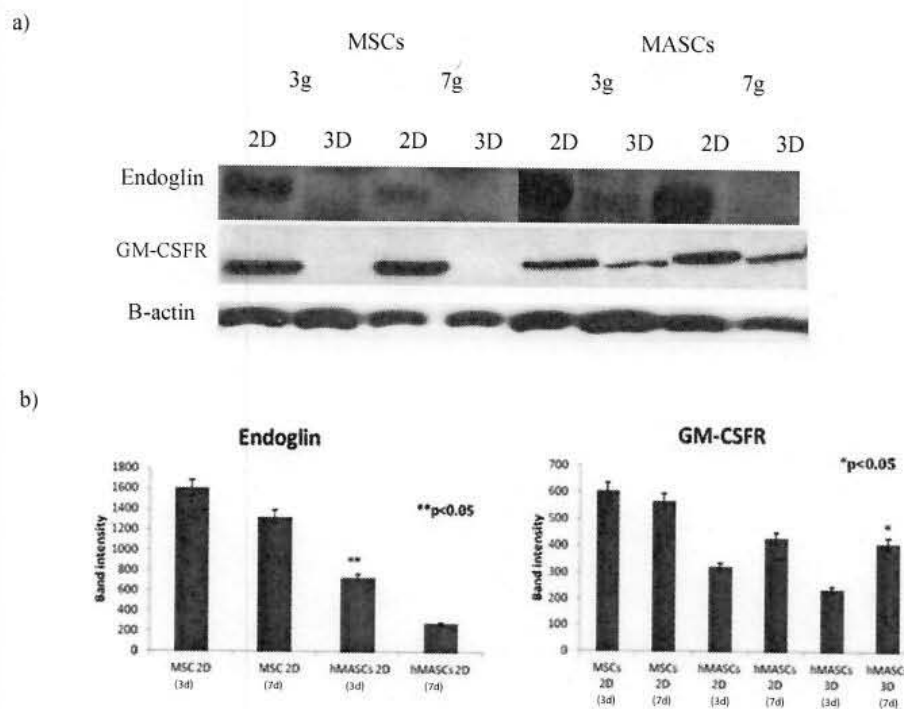


Fig. 3. Western blotting analyses of the expression of GM-CSFR α chain and endoglin in cells cultured on two- and three dimensional collagen-based gel matrices for 3 and 7 days. **a)** Expression of GM-CSFR α chain (84 kDa) and endoglin (95 kDa). For normalization b-actin was used as loading control. **b)** Quantification of the band intensity. Differences were considered statistically significant if: (MASC 3D 7gg vs MASC 3D 3gg - * $p < 0.05$) or (MASC 2D 3gg vs MASC 2D 7gg - ** $p < 0.05$). (Analysis of Student's test was applied).

including those aimed at restoring function by regenerating and repairing the damaged myocardium. Tissue engineering seems to be a promising approach to restore cardiac function.

Traditionally, the heart has been viewed as a static organ unable to repair damage; however, cycling ventricular myocytes have been described in the normal and pathological heart, indicating the presence of CSCs). CSCs express on the membrane the stem cell-related antigens, MDR1, c-kit, and Sca-1, in variable combinations. The growth potential *in vitro* of c-kit-positive cells seems to be greater than that of Sca-1-like or MDR1-positive ones.

The aim of this study was to investigate the behaviour of a specific population of human CSCs, the so-called MASCs, grown on three-dimensional collagen scaffolds (3D), in order to establish whether they could be used in post-infarction cardiac regeneration. MSC, obtained from bone marrow,

were used as a control.

In this study we first evaluated the effects of collagen matrix on cell proliferation and migration. MASCs showed a good proliferation rate, with a doubling time of about 12 days, even if there was a small but not significant difference between MASCs cultured in 2D and 3D conditions.

MASCs also showed a very good mobility on the 3D scaffolds, even in the absence of chemotactic agents. The control MSCs were much less mobile.

Cell migration is a central process in the development and maintenance of multicellular organisms; it is also of extreme importance in tissue engineering because it could be directed to a specific site in order to achieve repair and regeneration of an organ or a tissue. Moreover, cell migration may promote the colonization of a synthetic scaffold by endogenous cells within the patient's body, without requiring implants of cells cultured *in vitro*.

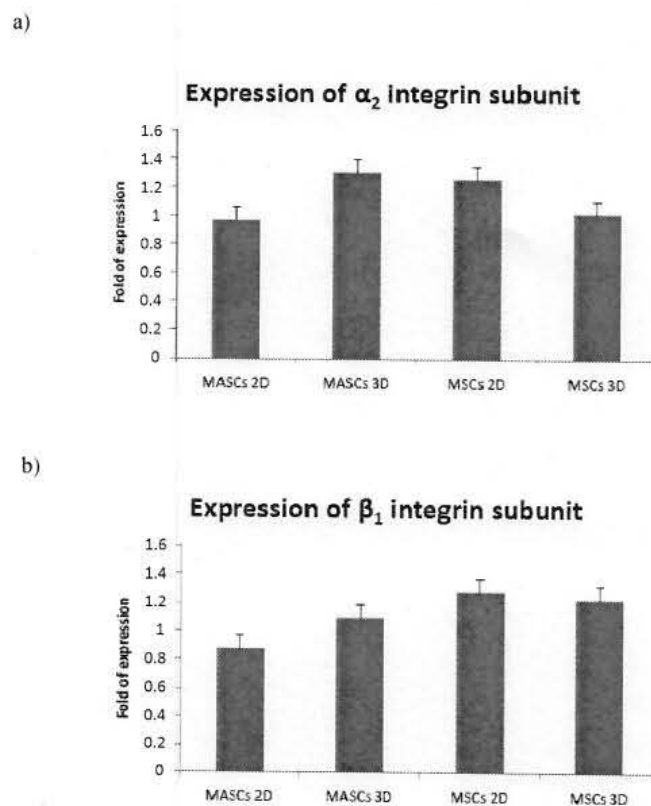


Fig. 4. Analysis of integrin subunits expression by flow cytometry. *a)* Expression of the α_2 integrin subunit. *b)* Expression of the β_1 integrin subunit. (Analysis of Student's *t*-test was applied).

Therefore, cell functionality and differentiation capacity, which can be lost *in vitro*, could be better preserved.

Since previous studies (39) showed a potential role of growth factors in cardiac tissue regeneration, we also evaluated, by Western blotting, at different time points (3 and 7 days), the expression levels of GM-CSFR and endoglin, an accessory component of the TGF- β receptor complex.

After an ischemic damage, an increase of serum levels of GM-CSF has been observed, suggesting a possible role of this factor in stem cells homing, proliferation, migration and differentiation (32). It has also been demonstrated that GM-CSF is able to mobilize circulating stem cells leading to more rapid tissue growth and differentiation into cardiomyocytes on membranes implanted in the left ventricles (33).

After 3 days in culture, we observed the highest expression levels of GM-CSFR α chain in MASCs

cultured without matrix. Levels strongly increased from day 3 to 7; the increase was more pronounced in cells cultured on scaffolds. As shown in Fig. 1, cells start to proliferate after two-three days. It is therefore possible that cells grown on scaffolds need to adapt to the new environment, before starting to proliferate.

On the basis of these results, we suggest a possible role of GM-CSFR α chain on cell proliferation. Further support to this hypothesis was given by the observation that the highest GM-CSFR α chain levels were observed in cells that showed the highest proliferative capacity, i.e. the control MSCs cultured without scaffolds. Curiously, MSCs cultured on scaffolds, did not express the GM-CSFR α chain at all.

Endoglin was strongly expressed only by MASCs and MSCs grown without the collagen matrix. Endoglin levels tend to decrease between day 3 and

7. These results indicate that TGF- β is probably not involved in proliferation and neoangiogenesis of cells cultured on scaffolds.

As integrins regulate not only cell-to-cell and cell-matrix adhesion, but also cellular differentiation, proliferation, motility and signal transduction (35), we also evaluated the $\alpha 2$ and $\beta 1$ integrin subunit expression by flow cytometry. The results obtained showed a good integrin subunits expression by both MSCs and MSCs cultured with and without collagen scaffolds.

The mechanisms by which a cell can be induced to migrate toward a specific site need further investigation. Our next step will be to evaluate cell migration in 3D collagen scaffolds enriched with proteins derived from the extra-cellular matrix, such as Fibronectin, Laminin and Vitronectin. Finally, we plan to investigate the ability of MSCs to differentiate into cardiomyocytes when cultured on a 3D matrix, by analyzing the presence of specific markers of cardiac differentiation, such as GATA-4.

In conclusion, MSCs grow well on 3D scaffolds and maintain a good mobility as compared to MSCs and express factors involved in cell proliferation and migration, such as GM-CSFR α chain and integrins. They therefore represent a promising approach to tissue engineering aimed to restore cardiac function.

REFERENCES

- Mizuno Y, Jacob RF, Mason RP. Inflammation and the development of atherosclerosis: effects of lipid-lowering therapy. *J Atheroscler Thromb* 2011; 18(5):351-58.
- Zipes D, Braunwald E. *Braunwald's Heart Disease: A Textbook of Cardiovascular Medicine*. Saunders W B CO, Philadelphia (USA) 2004.
- Katz AM. Heart failure in 2001: a prophecy revisited. *Am J Cardiol* 2001; 87(12):1383-86.
- Rosenstrauch D, Poglajen G, Zidar N, Gregoric ID. Stem cell therapy for ischemic heart failure. *Tex Heart Inst J* 2005; 32(3):339-47.
- Mikos AG, Herring SW, Ocharcon P, et al. *Tissue Eng* 2006; 12:3307-39.
- Ikada Y. Challenges in tissue engineering. *J R Soc Interface* 2006; 3:589-601.
- Shimizu T, Yamato M, Kikuchi A, Okano T. Cell sheet engineering for myocardial tissue reconstruction. *Biomaterials* 2003; (24):2309-16.
- Masuda S, Shimizu T, Yamato M, Okano T. Cell sheet engineering for heart tissue repair. *Adv Drug Deliv Rev* 2008; 60(2):277-85.
- Shimizu T, Sekine H, Yamato M, Okano T. Cell sheet-based myocardial tissue engineering: new hope for damaged heart rescue. *Curr Pharm Des* 2009; 15(24):2807-14.
- Haraguchi Y, Shimizu T, Yamato M, Okano T. Concise review: cell therapy and tissue engineering for cardiovascular disease. *Stem Cells Transl Med* 2012; 1(2):136-41.
- Haraguchi Y, Shimizu T, Matsuura K, et al. Cell sheet technology for cardiac tissue engineering. *Methods Mol Biol* 2014; 1181:139-55.
- Vunjak-Novakovic G, Tandon N, Godier A, Maidhof R, Marsano A, Martens TP, Radisic M. Challenges in cardiac tissue engineering. *Tissue Eng Part B Rev* 2010; 16(2):169-87.
- Taylor DA, Atkins BZ, Hungspreugs P, et al. Regenerating functional myocardium: improved performance after skeletal myoblast transplantation. *Nat Med* 1998; 4:929-33.
- Collins CA, Olsen I, Zammit PS, et al. Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. *Cell* 2005; 122:289-301.
- Le'obon B, Garcin I, Menasche P, Vilquin JT, Audinat E, Charpak S. Myoblasts transplanted into rat infarcted myocardium are functionally isolated from their host. *Proc Natl Acad Sci USA* 2003; 100(13):7808-11.
- Rubart M, Soonpaa MH, Nakajima H, et al. Spontaneous and evoked intracellular calcium transients in donor-derived myocytes following intracardiac myoblast transplantation. *J Clin Invest* 2004; 114:775-83.
- Roell W, Lewalter T, Sasse P, et al. Engraftment of connexin 43-expressing cells prevents post-infarct arrhythmia. *Nature* 2007; 450:819-24.
- Boheler KR, Czyz J, Tweedie D, Yang HT, Anisimov SV, Wobus AM. Differentiation of pluripotent embryonic stem cells into cardiomyocytes. *Circ Res* 2002; 91:189-201.

19. Orlic D, Hill JM, Arai AE. Stem cells for myocardial regeneration. *Circ Res* 2002; 91:1092-102.
20. Nussbaum J, Minami E, Laflamme MA, et al. Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. *FASEB J* 2007; 21:1345-57.
21. Park IH, Zhao R, West JA, et al. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* 2008; 451:141-46.
22. Asahara T, Murohara T, Sullivan A, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997; 275:964-67.
23. Takahashi T, Kalka C, Masuda H, et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med* 1999; 5(4):434-38.
24. Kocher AA, Schuster MD, Szabolcs MJ, et al. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med* 2001; 7(4): 430-36.
25. Pittenger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac therapeutics. *Circ Res* 2004; 95(1):9-20.
26. Cesselli D, Beltrami AP, Rigo S, et al. Multipotent progenitor cells are present in human peripheral blood. *Circ Res* 2009; 104:1225-34.
27. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005; 105(4):1815-22.
28. Rota M, Kajstura J, Hosoda T, et al. Bone marrow cells adopt the cardiomyogenic fate *in vivo*. *Proc Natl Acad Sci USA* 2007; 104(45):17783-88.
29. Orlic D, Kajstura J, Chimenti S, et al. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. *PNAS* 2001; 98(28):10344-49.
30. Leri A, Kajstura J, Anversa P. Cardiac stem cells and mechanisms of myocardial regeneration. *Physiol Rev* 2005; 85(4):1373-416.
31. Linke A, Müller P, Nurzynska D, et al. Cardiac stem cells in the dog heart regenerate infarcted myocardium improving cardiac performance. *Proc Natl Acad Sci USA* 2005; 102:8966-71.
32. Beltrami AP, Cesselli D, Bergamin N, et al. Multipotent cells can be generated *in vitro* from several adult human organs (heart, liver, and bone marrow). *Blood* 2007; 110(9):3438-46.
33. Parissis JT, Adamopoulos S, Venetsanou KF, Mentziko DG, Karas SM, Kremastinos D. Clinical and neurohormonal correlates of circulating granulocyte-macrophage colony stimulating factor in severe heart failure secondary to ischemic or idiopathic dilated cardiomyopathy. *AM J Cardiol* 2000; 86(6):707-10.
34. Möllmann H, Nef HM, Voss S, et al. Stem cell-mediated natural tissue engineering. *J Cell Mol Med* 2011; 15(1):52-62.
35. Duff SE, Li C, Garland JM, Kumar S. CD105 is important for angiogenesis: evidence and potential applications. *FASEB J* 2003; 17(9):984-92.
36. Danen EH, Sonnenberg A. Integrins in regulation of tissue development and function. *J Pathol* 2003; 200:471-80.
37. Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell* 2002; 110(6):673-87.
38. Iannone M, Ventre M, Pagano G, Giannoni P, Quarto R, Netti PA. Defining an optimal Stromal Derived Factor-1 presentation for effective recruitment of mesenchymal stem cells in 3D. *Biotechnol Bioeng* 2014; 111(11):2303-16.
39. Postiglione L, Montagnani S, Ladogana P, et al. Granulocyte macrophage-colony stimulating factor receptor expression on human cardiomyocytes from end-stage heart failure patients. *Eur J Heart Fail* 2006; 8(6):564-70.

LETTER TO THE EDITOR

AUTOPHAGY-RELATED GENE EXPRESSION IN COLORECTAL CANCER PATIENTS

M. BEDNARCZYK^{1,2}, M. MUC-WIERZGOŃ², D. WANICZEK³, E. FATYGA²,
K. KLAKLA², U. MAZUREK¹ and J. WIERZGOŃ⁴

¹School of Pharmacy with the Division of Laboratory Medicine in Sosnowiec, Medical University of Silesia, Department of Molecular Biology, Sosnowiec, Poland; ²School of Public Health, Medical University of Silesia, Department of Internal Medicine, Bytom, Poland; ³SHS in Katowice, Department of Propedeutics Surgery, Chair of General, Colorectal and Polytrauma Surgery, Medical University of Silesia, Bytom, Poland; ⁴Department of Oncological and Reconstructive Surgery, Maria Skłodowska-Curie Memorial Cancer Centre and Institute of Oncology, Gliwice Branch, Gliwice, Poland

Received June 1, 2017 – Accepted August 30, 2017

There is evidence that autophagy can play a dual role in tumor cells – as a tumor suppressor, and a process involved in tumor cell survival. The aim of this work was to assess the expression of the genes engaged in the autophagy process in biopsies taken from the colon, confirmed as adenocarcinoma, and normal tissue and to relate them to the clinical stage of the tumor. A total of 20 pairs of surgically removed tumors and healthy (marginal) tissue samples from colorectal cancer patients at clinical stages (CS) I-IV were analyzed. Gene expression profile analysis was performed using HG-U133A microarrays. Differentially expressed genes were identified, using the PL-Grid Infrastructure. Only for CSI, there were two specific genes: *FOXO1* and *BNIP1*; further in CSII – *LAMP2*, *MET* and *BCL2L*, in CSIII – *HIF1A* and 2 ID mRNAs for *HGF* and 18 genes were specific for CSIV in comparison to controls. *PINK1* is the only gene that differentiates all transcriptome groups from controls. Furthermore, examination of the expression of genes associated with the autophagy process may allow for better knowledge and understanding of the processes occurring during the development of colon cancer. The presented genes may be used as prognostic markers of clinical stages of colorectal cancer, contributing to the development of new lines of therapy focused on reducing metastasis of the primary tumor.

To the Editor,

Autophagy is the highly conservative, self-destructive mechanism of the cell, responsible for removal of long-term damaged proteins, organelles and proteins disassembled during biosynthesis. It maintains intracellular homeostasis, allowing for cell survival in conditions of stress. Autophagy participates in the regulation of basic cellular functions such as growth, differentiation, and death.

It has been shown that autophagy also regulates the lifespan of organisms (1-3). There are four basic types of autophagy, depending on the pathway of transport to the lysosomes: macroautophagy, microautophagy, chaperone-mediated autophagy and specific autophagy (for example, mitophagy) (4, 5). Different factors are responsible for induction of autophagy: lack of nutrients, hypoxia, cytokines, hormones, DNA damage. The role of autophagy in

Keywords: autophagy, autophagy-related genes, colorectal cancer, healthy tissues, clinical stages

Mailing address:

Prof. Małgorzata Muc-Wierzoń,
School of Public Health, Medical University of Silesia,
Department of Internal Medicine, 41-902 Bytom,
Żeromskiego 7 st, Poland
Tel./Fax: +48 32 2812122
e-mail: mwierzgon@sum.edu.plm

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

cancer has not been elucidated, and can depend on many factors, such as clinical stage or mutations present in a particular type of a tumor. There is evidence that autophagy can play a dual role in tumor cells - as a tumor suppressor and as a process involved in tumor cell survival. During early stages of the disease, autophagy acts as a tumor suppressor by inducing apoptosis, thus protecting from further tumor development (6). In the next stages, it promotes oncogenesis and potentially leads to the development of resistance to treatment. It also induces tumor development by supporting access to nutrients and promotes metastasis at late stages by promoting cell survival, despite the lack of extracellular matrix (7, 8). Experimental, genetic, epidemiologic and socioeconomic studies have suggested that CRC results from complex interactions between inherited susceptibility, clinical conditions, and environmental/lifestyle-related risk factors. Many studies concerning autophagy in CRC illustrated the multifaceted nature of this process (9, 10).

The aim of this work was to assess expression of the genes involved in the autophagy process in biopsies taken from the colon, confirmed as adenocarcinoma, and normal tissue, and to relate this to the clinical stage of the tumor.

MATERIALS AND METHODS

Patients

A total of 20 pairs of surgically removed tumor and healthy (marginal) tissue samples from colorectal cancer (CRC) patients at clinical stages (CS) I- IV (according to the 7th edition of the Union for International Cancer Control/American Joint Committee on Cancer) were analyzed. Healthy control tissue specimens (marked C) were obtained from an area of 10 mm outside of the histologically negative margin –Table I. The study protocol was approved by the Bioethical Committee of the Medical University of Silesia (KNW/0022/KB1/42/14), and informed consent was obtained from all patients.

Before molecular analysis, the colon tissues were placed in sterile tubes with RNeasy Lysis Buffer (Sigma) in the proportion 10 μ l for 1 mg of tissue (200 μ l RNeasy Lysis Buffer for 20 mg of tissue). The samples were incubated at room temperature for 24 h and then maintained at -80°C until

further analysis.

RNA extraction and quantitative and qualitative RNA analysis

The first step was a homogenization of tissue material using a Polytron homogenizer (Kinematica, AG, Switzerland). Total RNA was then isolated using TRIzol® (Invitrogen Life Technologies, California, USA), according to the manufacturer's protocol. Isolated RNA was purified with RNeasy Mini Kit (Qiagen) with DNase I digestion. Qualitative RNA analysis was carried out with Gene Quant II spectrophotometer at 260 nm. The purity of isolated RNA was defined with A260/A280 ratio. Electrophoresis in 1% agarose gel followed by ethidium bromide staining was used to analyze RNA quantitatively.

Oligonucleotide microarrays

Transcriptional activity of autophagy-related genes was determined with Human Genome U133A Array (Affymetrix). Double-stranded DNA was synthesized using 8 μ g RNA (SuperScript Choice system; Invitrogen Life Technologies, CA, USA). Biotinylated cRNA was prepared with BioArray HighYield RNA Transcript Labeling Kit (Enzo Life Sciences, New York, USA). Sample Cleanup Module Kit (Qiagen GmbH, Germany) was used to fragment cRNA. Washing, staining with streptavidin-phycoerythrin complex and microarray scanning were performed in GeneArray (Agilent), according to the Affymetrix Gene Expression Analysis Technical Manual.

Statistical analysis

The results obtained were analyzed statistically using PL-Grid infrastructure (<http://www.plgrid.pl/>) and Statistica 12.0 software. The results were considered as statistically significant with $p(\alpha) < 0.05$. The diversity of gene expression was determined using one-way ANOVA test and post hoc TUKEY with Benjamini-Hochberg correction.

RESULTS

The experimental part of this work started by defining 40 transcriptomes (each 22283 mRNA) of colorectal cancer and the colon tissue defined as normal by histopathology using the microarray technique (HGU 133 A – Affymetrix). By molecular

and histopathological tests, transcriptomes were divided into five groups: a control group (C) and the others depending on the clinical stage of cancer: CSI, CSII, CSIII, and CSIV.

The next stage involved a comparative analysis of concentration profiles of 435 ID mRNAs involved in autophagy between transcriptomes of CSI, CSII, CSIII and, CSIV and the controls. The result of the comparative analysis was a basis to define ID mRNAs, which differentiate the compared groups of the colon tissues. By using PL-Grid infrastructure (<http://www.plgrid.pl/>), heatmaps were defined to compare transcriptional activities of selected genes with the mean transcriptional activity of the colon cells. On the basis of the fluorescence signal intensity, 50 ID mRNAs were typed for autophagy-related genes in transcript groups from the colon. One-way ANOVA test with Benjamini-Hochberg correction, then post hoc Tukey with Benjamini-Hochberg correction were used to analyze statistical significance of the

observed changes in the concentration levels for 50 ID mRNAs, depending on the clinical stage.

When analyzing the obtained results in CSI, CSII, CSIII, CSIV groups vs control (C) group, from the groups of 50 ID mRNAs, the following ones were typed: for CSI vs C – 4 ID mRNAs, for CSII vs C – 9 ID mRNAs, for CSIII vs C – 12 ID mRNAs and for CSIV vs C – 29 ID mRNAs. Venna diagram was used to assess the specificity of ID mRNAs in the differentiation of four groups of transcriptomes of colon adenocarcinoma in comparison to controls. The results are shown in Fig. 1.

Only for CSI, there are two specific genes: *FOXO1* and *BNIP1*, in CSII – *LAMP2*, *MET* and *BCL2L*, in CSIII – *HIF1A* and 2 ID mRNAs for *HGF* in comparison to controls. From the group of 29 ID mRNAs differentiating fourth clinical stage, only 18 genes were specific for CSIV: *TMEM59*, *LAMP1*, *NPC1*, *BCL2*, *ABL2*, *PSEN1*, *PTPN22*, *SH3GLB1*, *CDKN2A*, *ANXA7*, *HGF*, *CLEC16A*,

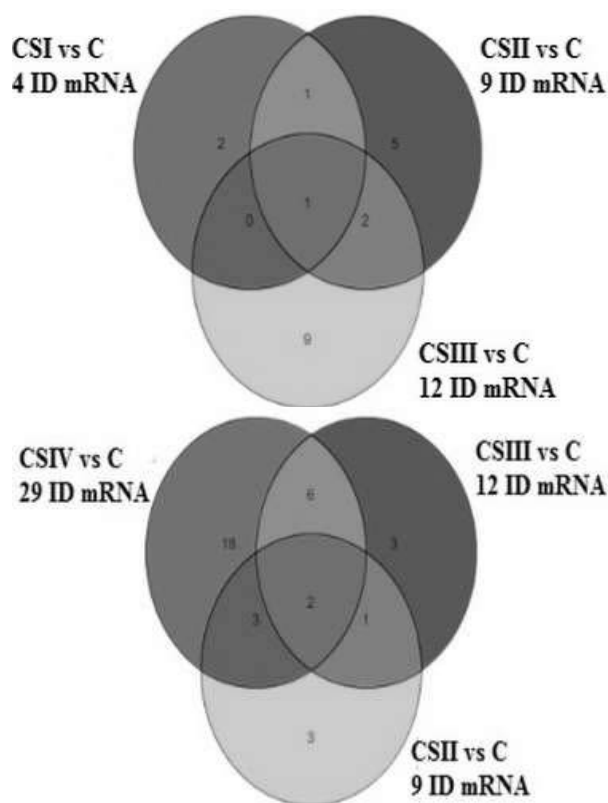


Fig. 1. Venna diagram illustrating ID mRNAs in differentiation of four groups of transcriptomes of colon adenocarcinoma in comparison to control.

USP33, *TOMM22*, *KRCC1*, *DRAM1*, *MTMR8*, and *LZTS1*. *PINK1* is the only gene that differentiates all transcriptome groups (CSI, CSII, CSIII, CSIV) from the controls. All colon cancer specimens show decreased expression of the tested gene with the lowest one found in the fourth stage and the highest expression of *PINK1* in the third clinical stage.

DISCUSSION

Currently, different panels of genes and mRNA are being proposed to improve the prognosis of CRC and treatment selection (11).

The results of our study indicate autophagy-related genes specific for the clinical stage of colorectal cancer. The higher the clinical stage, the more differentially expressed are the genes in colorectal

cancer in comparison to controls, namely, the CSI – CSIV genes. There are no similar studies examining autophagy-related genes/proteins representative for a particular stage of colorectal cancer in the available literature. Most commonly, the authors discuss the activity of the key genes involved in autophagy - beclin-1 (*BECN1*) and light chain 3 *LC3* (11-13). The only gene whose expression changes observed for each clinical stage was PTEN-induced putative kinase 1 (*PINK1*) - considered to be the main mitochondrial quality control protein involved in regulation of mitochondrial fission and fusion (14). *PINK1* is essential at G2/M and G0/G1 cell cycle checkpoints, necessary for cell division, growth, and resistance to stress. Deletion of this gene inhibits proliferation, colony formation, migration and invasive potential of tumor cells (15). Moreover, it is believed that *PINK1*

Table I. Characteristics of colorectal cancer patients.

	Clinical stages (CS)	Sex	Age (yrs)	Anatomical location of the tumour	Tumor grades
1.	CS_I	F	39	sigmoid	G1
2.	CS_I	F	45	sigmoid	G1
3.	CS_I	M	49	sigmoid	G2
4.	CS_I	M	70	sigmoid	G2
5.	CS_I	F	43	caecum	G2
6.	CS_II	F	58	sigmoid	G2
7.	CS_II	F	70	caecum	G1
8.	CS_II	M	81	sigmoid	G1
9.	CS_II	F	54	ascending colon	G1
10.	CS_II	M	57	descending colon	G2
11.	CS_III	F	86	sigmoid	G1
12.	CS_III	M	61	ascending colon	G3
13.	CS_III	M	57	descending colon	G2
14.	CS_III	F	58	transverse	G2
15.	CS_III	M	69	descending colon	G2
16.	CS_III	M	61	ascending colon	G3
17.	CS_IV	F	71	sigmoid	G2
18.	CS_IV	M	69	sigmoid	G3
19.	CS_IV	F	73	caecum	G1
20.	CS_IV	F	70	caecum	G1

G1-G3 histopathological tumor grading: G1 – well differentiated; G2- moderately differentiated; G3- poorly differentiated

also plays a role in stress-induced autophagy. Loss of this protein causes decreased expression of the main genes involved in autophagy, including *BECN1* and *LC3*. Recent studies show that decrease in *PINK1* expression correlates with the decreased activity of *LC3-II*, whereas overexpression of *PINK1* increases the level of *LC3-II* (15). To date, there has been little data on the involvement of the *PINK1* protein in the development of colorectal cancer. However, it is known that it may contribute to the induction of many cancer types. Our results suggest that *PINK1* is silenced in adenocarcinoma in comparison to control, and its expression is only slightly dependent on the disease stage, wherein its transcriptional activity is higher in the early stages of the disease and the lowest in CSIV. Reduction of the mRNA level for this gene may suggest that with malignant transformation, cells lose their ability for mitophagy.

The presented studies are preliminary, requiring a greater number of patients, which would allow to find specific genes, depending on gender, age, clinical stage of cancer, its anatomic location, and treatment. At the same time, further studies are needed at the molecular level, to help to determine the relationship between individual genes/proteins and regression or progression of cancer and to identify specific signaling pathways which, in turn, can be used as an essential element of the anti-cancer treatment strategy.

Further examination of the expression of genes associated with the autophagy process may allow for better knowledge and understanding of the processes occurring during the development of colon cancer. Presented genes may be used as prognostic markers of clinical stages of colorectal cancer, contributing to the development of new lines of therapy focused on reducing metastasis of the primary tumor.

REFERENCES

1. Badadani M. Autophagy mechanism, regulation, functions, and disorders. *Cell Biol* 2012; 1-11. doi: 10.5402/2012/927064.
2. Lockshin RA, Zakeri Z. Apoptosis, autophagy, and more. *Int J Biochem Cell Biol* 2004; 2405-19.
3. Baehrecke EH. Autophagy: dual roles in life and death? *Nature Reviews Mol Cell Biol* 2005; 6:505-10.
4. Feng Y, He D, Yao Z, Kilonsky D. The machinery of macroautophagy. *Cell Research* 2014; 24:24-41. doi:10.1038/cr.2013.168.
5. Groulx JF, Khalfaoui T, Benoit YD. Autophagy is active in normal colon mucosa. *Autophagy* 2012; 8(6):893-902.
6. Nagelkerke A, Sweep FC, Geurts-Moespot A, Bussink J, Span PN. Therapeutic targeting of autophagy in cancer. Part I: molecular pathways controlling autophagy. *Semin Cancer Biol* 2015; 31:89-98.
7. Rosenfeldt MT, Ryan KM. The multiple roles of autophagy in cancer. *Carcinogenesis* 2011; 32(7):955-63.
8. Gil J, Pesz K, Sasiadek M. May autophagy be a novel biomarker and antitumor target in colorectal cancer? *Biomark Med* 2016, doi: 10.2217/bmm-2016-0083.
9. Brackowski R, Romanowski W, Danikiewicz A, Muc-Wierzoń M, Zubelewicz B. Decrease erythropoietin level by human TNF factor in patients with advanced cancer. *J Biol Regul Homeost Agents* 2001; 15(4):366-69.
10. Ye LC, Jiang C, Bai J, Jiang J, Hong HF, Qiu LS. Knockdown of casein kinase 1 ϵ inhibits cell proliferation and invasion of colorectal cancer cells via inhibition of the wnt/ β -catenin signaling. *J Biol Regul Homeost Agents* 2015; 9(2):307-15.
11. Chen Z, Li Y, Zhang C. Downregulation of Beclin 1 and impairment of autophagy in a small population of colorectal cancer. *Dig. Dis Sci* 2013; 58(10):2887-94.
12. Guo GF, Jiang WQ, Zhang B. Autophagy-related proteins Beclin-1 and LC3 predict cetuximab efficacy in advanced colorectal cancer. *World J Gastroenterol* 2011; 17(43):4779-86.
13. Koukourakis MI, Giatromanolaki A, Sivridis E, Pitiakoudis M, Gatter KC, Harris AL. Beclin 1 over- and underexpression in colorectal cancer: distinct patterns relate to prognosis and tumour hypoxia. *Br J Cancer* 2010; 103(8):1209-14.
14. Arena G, Gelmetti V, Torosantucci L, Lamorte G, de Rosa P, Cilia E, Jonas A. PINK1 protects against cell death induced by mitochondrial depolarization, by phosphorylating Bcl-xL and impairing its pro-apoptotic cleavage. *Cell Death Differ* 2013; 20:920-30.
15. Ivankovic D, Chau KY, Schapira AH, Gegg ME. Mitochondrial and lysosomal biogenesis are activated following PINK1/parkin-mediated mitophagy. *J Neurochem* 2016; 136(2):388-402. doi: 10.1111/jnc.13412.

LETTER TO THE EDITOR

**SERUM LEVELS OF ADAM10, ADAM12, ADAM17 AND ADAM28
IN COLORECTAL CANCER PATIENTS**

K. WALKIEWICZ¹, E. NOWAKOWSKA-ZAJDEL², J. STRZELCZYK³,
S. DZIĘGIELEWSKA-GĘSIĄK¹ and M. MUC-WIERZGOŃ¹

¹Department of Internal Medicine, School of Public Health in Bytom, Medical University of Silesia, Katowice, Poland; ²Department of Nutrition Related Disease Prevention, School of Public Health in Bytom, Medical University of Silesia, Katowice, Poland; ³Department of Medical and Molecular Biology, School of Medicine with the Division of Dentistry in Zabrze, Medical University of Silesia, Katowice, Poland

Received June 11, 2017 - Accepted August 29, 2017

Colorectal cancer is the third most common cancer in the world. Our study analyzed the potential significance of serum levels of selected adamalysines (ADAM10, ADAM12, ADAM17, ADAM28) in colorectal cancer patients. The study was performed on a group of 85 colorectal cancer patients (48 men, 37 women). Serum protein concentrations were measured by ELISA. The ADAMs serum level changes were analyzed according to selected clinical parameters (BMI, sex, age, clinical stage of disease). The following ranges of concentration of analyzed proteins were obtained: ADAM10 min=1.7, max=321.8 [ng/ml]; ADAM12 min=0.6, max=26.7 [ng/ml]; ADAM17 min=0.4, max=9.8 [ng/ml]; ADAM28 min=17.1, max=1545.8 [ng/ml]. In addition, it was stated that there is a relationship between the serum level of ADAM28 and the degree of the clinical stage ($p<0.04$). The obtained results could be the starting point for further research into the role of adamalysines in the development of colorectal cancer, as well as the potential predictive and prognostic value of these proteins.

To the Editor,

Colorectal cancer (CRC) is the third most common cancer in the world. Although the role of obesity, insulin-resistance and high levels of insulin-like growth factors (IGF) is generally acknowledged, because IGFs are strong mutagens, pathogenesis of CRC is still not fully understood. One of the biochemical pathways potentially involved in development of CRC, and closely associated with other growth factors, is the pathway related to adamalysines (ADAMs).

ADAMs belong to the family of metalloproteinases and this is a group of transmembrane glycoproteins

containing metalloproteinase and disintegrin domain. In humans about 20 members of this group were identified. The main role of these proteins is their involvement in the processes of migration, adhesion and cell proliferation, hence their importance in the process of carcinogenesis. Moreover, adamalysines regulate bioavailability of some growth factors, such as IGF, transforming growth factor alpha (TGF α) and epidermal growth factor (EGF) (1-4). Additionally, the role of adamalysines, especially ADAM9, ADAM10, and ADAM17, in the process of formation of CRC metastasis is especially interesting. The major cause of cancer metastasis

Key words: colorectal cancer, adamalysines, carcinogenesis, serological markers, biomarkers

Mailing address:

Ewa Nowakowska-Zajdel,
Department of Nutrition Related Disease Prevention,
School of Public Health in Bytom,
Medical University of Silesia, Katowice, Poland
41-902 Bytom, ul. Piekarska 18, Poland
email: ewanz@onet.eu

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
**DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF
INTEREST RELEVANT TO THIS ARTICLE.**

is lowering the intracellular integrity. Study of the CRC cell line (HT 29) showed that the ADAM9-transmembrane form overexpression is related to cell adhesion via E-cadherin degradation and can promote cancer metastasis (5). Similar observations apply to overexpression of ADAM10 on the CRC cell surface (6). Moreover, the clinical study with the use of CRC tissue samples showed that ADAM17-gene overexpression correlated with staging of CRC and could be associated with the cell migration process (7).

Despite many previous reports about the potential role of adamalysines in CRC, there are no data on translating this knowledge into practice. On the contrary, our study focuses on presenting the results of serum levels of selected ADAM proteins in CRC patients.

MATERIALS AND METHODS

Patients

The study included 85 adult colorectal cancer patients (48 men, 37 women) hospitalized in the Department of Internal Medicine. All subjects signed informed consent to participate in the study. Serum samples [3cm³] were collected from all the examined patients, anthropometric measurements were conducted, and clinical data were analyzed. To minimize the risk of methodological errors, patients having undergone cancer treatment, or with significant immune-deficiency, inter alia with HCV, HBV and HIV infection or acute infection were excluded from the study.

Patients were referred to surgery to determine the clinical stage of the disease and the histological type of colorectal cancer. In fourteen patients the value of histopathological grading (G) was not specified.

Because of the limited number of patients participating in the study, they were divided into two groups according to the clinical stage of colorectal cancer: (i) Patients with locally advanced disease (TNM I-IIIc) - without distant metastases; and (ii) patients with metastatic disease (TNM IVa,b). Anthropometric and clinical features are presented in Table I and II.

Methods

Serum for testing was frozen at -80°C. Assays were

performed using KIT-tests Cloud - Clone Corporation by ELISA according to the manufacturer's instructions. In the present study sets of plates with the wells coated with an antibody specific for ADAM10, ADAM12, ADAM17, ADAM28 were used. The manufacturer based the test on the reaction of the biotin-avidin with horseradish peroxidase. Changes in the color of the test solution were measured spectrophotometrically using light of wavelength 450nm±10nm. The ADAMs concentrations of the tested samples were then determined by comparing the result obtained to the result of a standard sample. The obtained data were compiled in the STATISTICA 12 - the normality of value distribution was checked by Shapiro-Wilks test. Then a nonparametric Mann-Whitney U test was used to assess the differences between the studied groups. Bivariate correlations were expressed as Pearson's correlation coefficient. A $p < 0.05$ was considered to reflect a significant difference and correlations. The study was approved by the Bioethics Committee of the Medical University of Silesia in Katowice (KNW/0022/KB1/42/14).

RESULTS

In the examined group of colorectal cancer patients the results showed a significant indicator of abnormal body weight (overweight/obesity), which affected up to 60% of patients (regardless of gender). Diabetes mellitus coexisted in 41.5% of the subjects.

Histological analysis showed adenocarcinoma including subtypes such as *mucinosum*, *male differentiatum* and *necroticans*. The most frequent cancer location was the colon: ascending colon-36%, descending colon-52%, and transverse colon-12%. Moreover, in men, quite often a location in the rectum was found (total in 24.7%, males in 33.3%).

The obtained results of ADAM protein serum level in gender-related study groups are presented in Table III.

In the next step, the relationship between the concentrations of ADAMs and the concentrations of ADAMs and the clinical and anthropometric parameters was analyzed. A significant dependence between serum levels of ADAM28 and the clinical stage of cancer was discovered. In patients with locally advanced disease (TNM I-IIIc) there

Table I. Anthropometric features of the study group

Gender	Age [years]			Weight [kg]			Height [m]			BMI [kg/m ²]		
	<i>min</i>	<i>max</i>	<i>mean</i>	<i>min</i>	<i>max</i>	<i>mean</i>	<i>min</i>	<i>max</i>	<i>mean</i>	<i>min</i>	<i>max</i>	<i>mean</i>
Female (n=37)	47	86	69.7	43	94	66.1	1.43	1.76	1.58	19.5	34.3	26.4
Male (n=48)	32	88	68.1	49.5	114	78.4	1.6	1.88	1.72	18.2	35.9	26.4

Table II. Clinical data of the study group.

Gender	TNM I-IIIc	TNM IVA,B	Histological grading			Histological type	Location	
			G1	G2	G3		Colon	Rectum
Female (n)	29	8	6	19	5	<i>adenocarcinoma</i>	32	5
Male (n)	32	16	6	28	7	<i>adenocarcinoma</i>	32	16
Total (n)	61	24	12	47	12	<i>adenocarcinoma</i>	64	21

Table III. ADAM protein serum levels.

	ADAM10 [ng/ml]			ADAM12 [ng/ml]			ADAM17 [ng/ml]			ADAM28 [ng/ml]		
	<i>min</i>	<i>max</i>	<i>mean</i>	<i>min</i>	<i>max</i>	<i>mean</i>	<i>min</i>	<i>max</i>	<i>mean</i>	<i>min</i>	<i>max</i>	<i>mean</i>
Female	1.8	223.8	87.8	0.8	10.7	4.6	0.4	4.1	1.8	18.1	1251.8	742.5
Male	1.7	321.8	133.9	0.6	26.7	5.3	0.7	9.8	2.6	17.1	1545.8	893.6
Total	1.7	321.8	112.4	0.6	26.7	5.0	0.4	9.8	2.3	17.1	1545.8	831.2

was a higher serum concentration of ADAM28 compared to patients with metastatic disease ($p < 0.04$) (Fig. 1). However, there was no significant relationship between concentrations of ADAMs and anthropometric parameters (BMI, weight and height, gender and age).

Spearman correlation analysis was also carried

out and resulted that serum levels of ADAM10, ADAM12, ADAM17 and ADAM28 were correlated, especially significant being ADAM12 and ADAM17 ($R = 0.91$; $p < 0.05$).

A correlation was also found between serum levels of ADAM10 and weight of the patients with early stage of CRC ($R = 0.53$; $p < 0.05$).

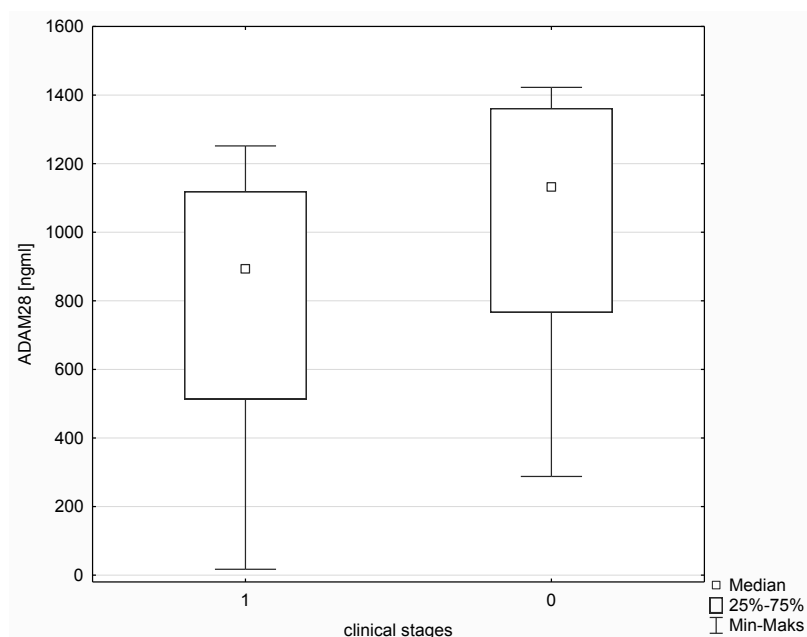


Fig. 1. Serum levels of ADAM 28, depending on clinical stage (1-TNM IVA,B; 0-TNM I-III).

DISCUSSION

Although serum levels of ADAMs are examined as the potential markers in prenatal diagnostics (8, 9) their role in cancer diagnostics has not been a field of frequent study. However, in 2004, it was found that serum levels of ADAM 8 correlated with clinical stage of non-small cell lung cancer (NSCLC), and were more sensitive tumor marker than CEA (63% vs 57%) (10). Furthermore, high expression of ADAM 8 in tissue of NSCLC samples adversely affects the prognosis. In turn, in 2010, the role was examined of ADAM 28 for NSCLC. The serum level of ADAM28 was significantly higher in the NSCLC patients than in the control group ($p < 0.001$) and increased with progress of tumor stage ($p < 0.001$) (10). In light of these considerations, the study by Alvarez-Fernandez et al. was very interesting as it presented the results of measuring serum levels of anti-ADAM10 auto-Abs. That study showed that anti-ADAM10 auto-Abs were significantly induced in a large group of colon cancer patients, in particular in patients in stage II and III of CRC. Interestingly, in patients in stage III of CRC, serum levels of anti-ADAM10

auto-Abs was associated with a better prognosis and improved PFS (progression free survival) (12).

The role of ADAM28 activation in cancer development was described in the study of Abe, et al. (13) which demonstrated that oncogene transformants, v-src-transformed MDCK (Madin-Darby canine kidney) cells selectively induce ADAM28 by activation of Src and its signaling pathways. Transformation of the cell lines by v-src induces significant cellular changes which are related to malignant phenotypes. A crucial role in the activation of this pathway is played by ADAM28. It has been found that the use of anti-ADAM28 antibody suppressed the tumor growth (13). The distinctness of ADAM-related carcinogenesis was analyzed in our previous research. We found that change of ADAM28 and IGFBP-3 gene expression was observed in the healthy colon tissue of overweight patients with colorectal cancer. High levels of ADAM28 can lead to increase of IGF1 activity, therefore promoting cell growth. Moreover, our study showed that the microscopic margin in a group of obese patients was not equal to the molecular margin, because in “healthy” colon tissue

in the area of CRC of overweight patients, a change of genes expression was also observed (14).

The ADAMs may play a crucial role in the regulation of bioavailability of adiponectins, which was postulated for ADAM10 (15). Other studies confirmed that serum levels of ADAM10 were higher in patients with type 1 diabetes, and correlated with activity of soluble receptor for advanced glycation end products (soluble RAGE). These data suggest that ADAM10 is involved in the shedding of RAGE. Serum levels of ADAM10 were increased in type 1 diabetes and were a determinant of circulating cRAGE (16).

A correlation between ADAM10 and bodyweight is interesting because it was detected in a group of early CRC but does not occur in advanced cancer. This dependence may suggest that ADAM10 is involved in cancer development especially in overweight patients, and with the progress of cancer the relationship disappears.

Moreover, in cases of advanced CRC, ADAM10 is engaged in the development of chemotherapy resistance with programs with 5-FU (fluorouracyl) (17). Similar results on CRC lines for ADAM17 protein were achieved in the study by Kyula et al. (18).

In summary, ADAM proteins seem to be a highly interesting subject of research, not only in understanding the mechanisms of carcinogenesis, but also, in the longer term, in clinical practice. An issue of particular interest is the connection of the ADAM system with metabolic disorders. The presented results could be a starting point for further projects.

REFERENCES

1. Seals DF, Courtneidge SA. The ADAMs family of metalloproteases: multidomain proteins with multiple functions. *Dev Genes Evol* 2003; 17:7-30.
2. Duffy MJ, McKiernan E, O'Donovan N. Role of ADAMs in cancer formation and progression. *Clin Cancer Res* 2009; 15:1140-44.
3. Walkiewicz K, Gętek M, Muc-Wierzgoń M, Kokot T, Nowakowska-Zajdel E. The importance of ADAM family proteins in malignant tumors. *Postep Hig Med Dosw* 2015; 70:67-73.
4. Miller MA, Sullivan RJ, Lauffenburger DA. Molecular pathways: receptor ectodomain shedding in treatment, resistance and monitoring of cancer. *Clin Cancer Res* 2016; 23(3):1-7.
5. Hirao T, Nanba D, Tanaka M, et al. Overexpression of ADAM 9 enhances growth factor-mediated recycling of E-cadherin in human colon cancer cell line HT29 cells. *Exp Cell Res* 2006; 312(3):331-39.
6. Gavert N, Scheffer M, Raveh S, et al. Expression of L1-CAM and ADAM 10 in human colon cancer cells induces metastasis. *Cancer Res* 2007; DOI: 10.1158/0008-5472.CAN-07-0991.
7. Walkiewicz K, Koziel P, Bednarczyk M, Błażelonis A, Mazurek U, Muc-Wierzgoń M. Expression of migration-related genes in human colorectal cancer and activity of a disintegrin and metalloproteinase 17. *Biomed Res Int* 2016; doi: 10.1155/2016/8208904.
8. Poon LCY, Chelemen T, Granvillano O, Pendeva I, Nicolaides K. First-trimester maternal serum a disintegrin and metalloproteinase-12 (ADAM 12) and adverse pregnancy outcomes. *Obstet Gynecol* 2008; 112(5):1082-90.
9. Goetzinger KR, Zhong Y, Cahill AG, Odibo L, Macones GA, Odibo A. Efficiency of first-trimester utery artery Doppler, a-disintegrin and metalloproteinase 12, pregnancy- associated plasma protein A, and maternal characteristics in the prediction of preeclampsia. *J Ultrasound* 2013; 32(9):1593-600.
10. Ishikawa N, Daigo Y, Yasui W, et al. ADAM 8 as a novel serological and histochemical marker of lung cancer. *Clin Cancer Res* 2004; 10(24):8363-70.
11. Kuroda H, Mochizuki S, Shimoda M, et al. ADAM 28 is a serological and histochemical marker for non-small-cell lung cancers. *Int J Cancer* 2010; 127(8):1844-1856.
12. Alvarez-Fernandez SM, Barbariga M, Cannizzaro L, et al. Serological immune response against ADAM10 pro-domain is associated with favourable prognosis in stage III colorectal cancer patients. *Oncotarget* 2016; doi: 10.18632/oncotarget.11181.
13. Abe H, Mochizuki S, Ohara K, et al. Src plays a key role in ADAM28 expression in v-src- transformed epithelial cells and human carcinoma cells. *Am J Pathol* 2013; 183(5):1667-78.
14. Nowakowska-Zajdel E, Mazurek U, Wierzgoń J, et al. Expression of ADAM 28 and IGFBP-3 genes in patients with colorectal cancer - a preliminary report.

- Int J Immunopathol Pharmacol 2013; 26(1):223-28.
15. Schaab M, Kratzsch J. The soluble leptin receptor. Clin Endocrin Metabol 2015; 29(5):661-70.
 16. Lee ACH, Lam JKY, Shiu SWM, Wong Y, Betteridge J, Tan KCB. Serum level of soluble receptor for advanced glycation end products is associated with a disintegrin and metalloproteinase 10 in type 1 diabetes. PLoS ONE 2015; 10(9) doi:10.1371/journal.pone.0137330.
 17. Carloni V, Mazzocca A, Mello T, Galli A, Capaccioli S. Cell fusion promotes chemoresistance in metastatic colon carcinoma. Oncogene 2013; 32:2649-60.
 18. Kyula JN, van Schaeybroeck S, Doherty J. Chemotherapy-induced activation of ADAM-17: a novel mechanism of drug resistance in colorectal cancer. Clin Cancer Res 2010; 16:3378-89.

LETTER TO THE EDITOR

BIOINFORMATIC ANALYSIS OF PIVOTAL GENES ASSOCIATED
WITH SEPTIC SHOCKS.Y. LIU¹, L. ZHANG², Y. ZHANG³, Y. ZHEN⁴ and Y.F. WU⁵

¹Department of Neurosurgery, Second Hospital, Jilin University, Changchun, China; ²Department of Neurology, Second Hospital, Jilin University, Changchun, China; ³Department of Pediatrics, First Hospital, Jilin University, Changchun, China; ⁴Department of Neurosurgery, Northern Jiangsu People's Hospital, Yangzhou, China; ⁵Department of Pneumology, Second Hospital of Jilin University, Changchun, China

Received April 7, 2017 – Accepted September 1, 2017

The first two Authors contributed equally to this work

We aimed to identify important genes associated with septic shock and then explore the possibly significant mechanisms of this disease. We downloaded GSE26440 expression data of samples from 98 children with septic shock and 32 normal controls from the Gene Expression Omnibus (GEO) database. Differentially expressed genes (DEGs) in samples from patients with septic shock were analyzed in comparison with those in samples from normal controls using a limma package. Functional enrichment analysis for DEGs was performed using DAVID, and a protein–protein interaction (PPI) network was constructed. Upstream transcription factors for DEGs were predicted using the CHIPBase database, and a transcriptional regulation network was constructed. A total of 383 significantly DEGs, including 141 downregulated and 242 upregulated genes, were obtained in the sepsis shock group compared with the normal group. The top five nodes in the PPI network were lysine (K)-specific demethylase 6B (*KDM6B*), histone deacetylase 2 (*HDAC2*), V-Myc avian myelocytomatosis viral oncogene homolog (*MYC*), heat-shock protein 90 kDa alpha (cytosolic), class B member 1 (*HSP90AB1*), and poly (A)-binding protein, cytoplasmic 1 (*PABPC1*). Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) was the transcription factor targeted by most genes, and it regulated the expression of *KDM6B*, *HDAC2*, *MYC*, *HSP90AB1*, and *PABPC1*. In conclusion, *KDM6B*, *HDAC2*, *MYC*, *HSP90AB1*, and *PABPC1* may play important roles in the development of septic shock. Furthermore, NFκB may be involved in septic shock by regulating the expression of *KDM6B*, *HDAC2*, *MYC*, *HSP90AB1*, and *PABPC1*.

To the Editor,

Septic shock, an infection-based disease, is a heterogeneous syndrome with a wide range of biological and physiological manifestations (1), and the risk of death remains high despite decreasing mortality in recent years (2). There is therefore an

urgent need to identify effective therapies for this condition as the most effective ones are currently unknown. Understanding underlying molecular mechanisms of septic shock may provide a route for treatment. Berghe et al. indicated that combined anti-interleukin 1 (*IL-1*) and *IL-18* treatment might

Key words: septic shock, differentially expressed genes, protein–protein interaction network, transcriptional regulation network, upstream transcription factor

Mailing address:

Dr Yanfeng Wu,
Department of Pneumology,
Second Hospital of Jilin University,
Changchun 130041, China
Tel.: +86 0431 88796820
e-mail: yanfengwu2016@hotmail.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
**DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF
INTEREST RELEVANT TO THIS ARTICLE.**

be a better approach for treating septic shock (3). Glucocorticoid receptor dimerization is required for the survival of patients by suppressing *IL-1* in macrophages (4). Tumor necrosis factor- α (*TNF α*) is a primary mediator in the pathophysiology (5). Although many genes associated with septic shock have been identified, this has proved inadequate in clarifying underlying molecular mechanisms of this condition because of the complexity of such mechanisms. More research on molecular mechanisms of septic shock is needed.

In this study, GSE26440 was used to analyze differentially expressed genes (DEGs) in samples from patients with septic shock and normal controls. Functional enrichment analysis for DEGs was performed, and a protein–protein interaction (PPI) network was constructed. A transcriptional regulation network for upstream transcription factors was also analyzed. We aimed to identify important genes associated with septic shock and then explore the possibly significant mechanisms of this disease.

MATERIALS AND METHODS

Expression data

Expression data of GSE26440 of samples from 98 children (≤ 10 years) with septic shock and 32 normal controls (≤ 10 years) were downloaded from the Gene Expression Omnibus (GEO) database. Expression data were based on the GPL570 platform [Affymetrix Human Genome U133 Plus 2.0 Array (HG-U133_Plus_2)]. Whole blood samples were used for this analysis.

Data preprocessing

CEL data were transformed to an expression value matrix using the robust multi-array average of affy package in R statistical software program. The probe number was transformed to the gene name using the annotation function in R/Bioconductor and the GPL570 platform, and the mean value was set as the final expression value of this gene when several probes mapped to a single gene symbol.

DEG analysis

DEGs in samples from patients with septic shock and from normal controls were analyzed using a limma

package in R. p-values of DEGs were calculated using *t*-test provided by limma package, and p-values were adjusted by the Benjamini-Hochberg procedure. The adjusted $p < 0.05$ was regarded as the cut-off criterion for DEGs. $FC > 1$ was regarded as indicative of significantly upregulated DEGs, and $FC < 1$ was regarded as indicative of significantly downregulated DEGs.

Gene ontology and pathway enrichment analyses

Gene ontology (GO) was used in this study, which is a tool for annotating genes with a defined, structured, and controlled vocabulary. Kyoto Encyclopedia of Genes and Genomes (KEGG) was also applied, which can be used to allocate related gene sets into each pathway. Finally, this study used DAVID, which is an integrated data-mining environment used for gene list analysis (6).

GO annotation and KEGG pathway enrichment analyses for upregulated and downregulated genes were performed using DAVID. A p-value < 0.05 was considered to indicate statistical significance. At the same time, an association network for different terms was constructed using overlapping genes among different terms; this association network was graphically presented using the Enrichment Map plugin in Cytoscape.

PPI network analysis

The Search Tool for the Retrieval of Interacting Genes (STRING) database can provide information for the integration and prediction of protein interactions. The PPI network was constructed by screening PPI pairs that had a combined score of > 0.4 with STRING database, and Cytoscape was used for presentation.

Transcriptional regulation network analysis

Transcription factors upstream of DEGs were predicted using the CHIPBase database (7), and a transcriptional regulation network was constructed.

RESULTS

Data preprocessing

Expression data for a total of 20,545 genes in 130 samples were obtained after data preprocessing. After normalization, the median line of the boxplot remained at the same level, indicating that all data had been successfully normalized.

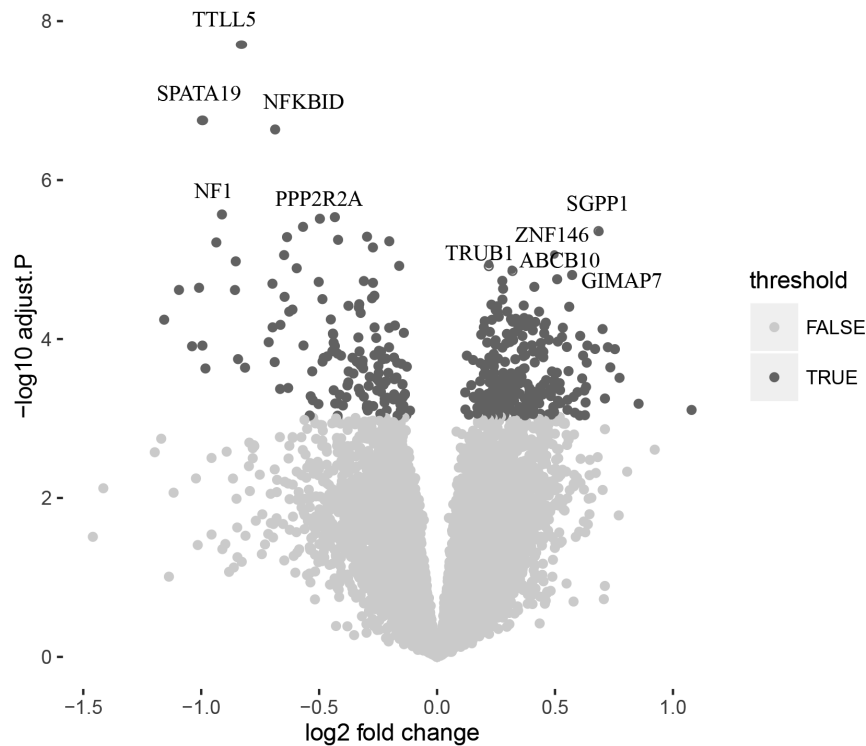


Fig. 1. The Volcano plot for differentially expressed genes (DEGs). Light color nodes: DEGs. The top 5 upregulated DEGs and top 5 downregulated DEGs are marked with specific gene names.

DEG analysis

A total of 383 significant DEGs, including 141 downregulated and 242 upregulated genes, were obtained in the sepsis shock group compared with the normal group. The Volcano plot is shown in Fig. 1, and information for all DEGs is shown in Table I. Additionally, bidirectional hierarchical clustering was drawn based on gene expression.

GO and pathway enrichment analyses

GO and KEGG pathway analyses for upregulated and downregulated DEGs were performed. GO terms of upregulated DEGs were mainly enriched during RNA processing, mRNA metabolic process, and RNA splicing (Table IA). GO terms of downregulated DEGs were mainly enriched in response to wounding and inflammatory response (Table IB).

PPI network analysis

The PPI network for significantly DEGs is shown in Fig. 2. In total, 211 nodes and 450 edges,

involving 161 significantly upregulated genes and 50 significantly downregulated ones, were included in the PPI network. The node degree indicates the number of other nodes connected to this particular node. The degree of each node in the network was calculated, and the top 5 nodes were lysine (K)-specific demethylase 6B (*KDM6B*, degree = 25), histone deacetylase 2 (*HDAC2*, degree = 21), V-Myc avian myelocytomatosis viral oncogene homolog (*MYC*, degree = 20), heat-shock protein 90 kDa alpha (Cytosolic), class B member 1 (*HSP90AB1*, degree = 18) and poly (A)-binding protein, cytoplasmic 1 (*PABPC1*, degree = 15).

Transcriptional regulation network analysis

A total of 117 upstream transcription factors were predicted for DEGs, and a transcriptional regulation network including 419 nodes and 13,708 edges was obtained (Fig. 3). The top five transcription factors (those with the highest number of target genes) were nuclear factor kappa-light-chain-enhancer of

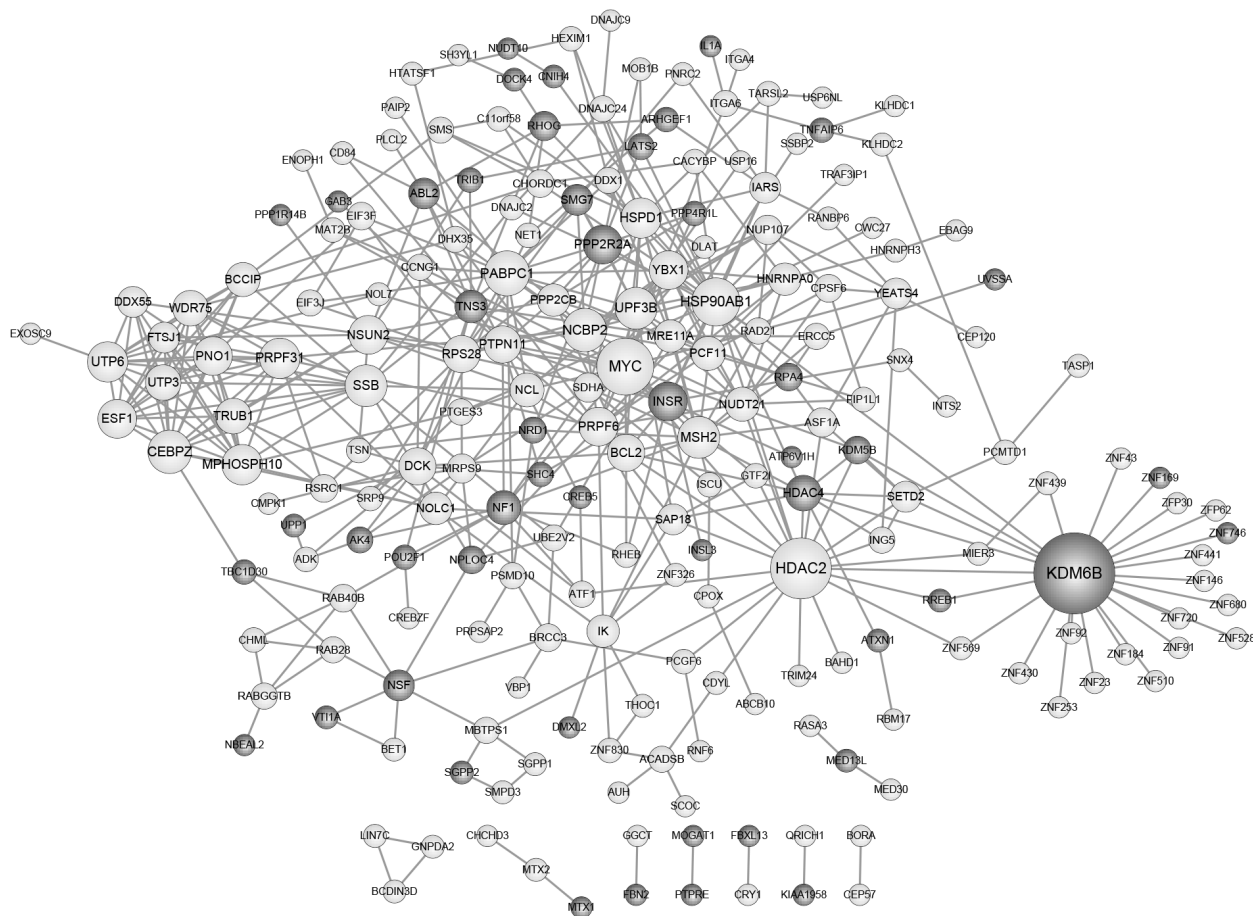


Fig. 2. The PPI network for differentially expressed genes (DEGs). Light color nodes: upregulated genes; dark-colored nodes: downregulated genes; the node size is proportional to the node degree.

activated B cells (NFkB, number of target genes = 289), hepatocyte nuclear factor 4, alpha (HNF4A, number of target genes = 285), E2F transcription factor 4, P107/P130-binding (E2F4, number of target genes = 280), caudal type homeobox 2 (CDX2, number of target genes = 280), and TAF1 RNA polymerase II, TATA box-binding protein-associated factor (TAF1, number of target genes = 277). In addition, these five transcription factors regulated the expression of *KDM6B*, *HDAC2*, *MYC*, *HSP90AB1*, and *PABPC1*.

DISCUSSION

The present study showed that the top 5 nodes in the PPI network were *KDM6B*, *HDAC2*,

MYC, *HSP90AB1*, and *PABPC1*. NFkB was the transcription factor targeted by most genes, and it regulated the expression of *KDM6B*, *HDAC2*, *MYC*, *HSP90AB1*, and *PABPC1*.

Excessive inflammatory response to infectious agents could result in septic shock (8). *KDM6B* can modulate the inflammatory response and is a key regulator of the inflammatory gene (9). Inhibition of *HDAC2* can enhance the expression of pro-inflammatory genes and *HDAC2* may be able to restore the inflammatory response (10). *Myc* plays a central role in inflammation and is a positive effector of tissue inflammation (11). HSP90-targeted agents may be helpful for treating inflammatory diseases associated with inflammation (12). *PABPC1* is

Table I. Functional enrichment analysis for differentially expressed genes (DEGs).

Term	Description	Count	PValue
A: Up-regulated			
GOTERM_BP	GO:0006396~RNA processing	30	2.94E-11
GOTERM_BP	GO:0016071~mRNA metabolic process	21	3.31E-08
GOTERM_BP	GO:0008380~RNA splicing	17	4.91E-07
GOTERM_BP	GO:0006397~mRNA processing	18	5.06E-07
GOTERM_BP	GO:0034660~ncRNA metabolic process	14	5.84E-06
GOTERM_CC	GO:0031974~membrane-enclosed lumen	44	5.35E-08
GOTERM_CC	GO:0070013~intracellular organelle lumen	42	1.46E-07
GOTERM_CC	GO:0031981~nuclear lumen	37	1.82E-07
GOTERM_CC	GO:0043233~organelle lumen	42	2.73E-07
GOTERM_CC	GO:0030529~ribonucleoprotein complex	19	4.03E-06
GOTERM_MF	GO:0003723~RNA binding	27	4.00E-07
GOTERM_MF	GO:0043566~structure-specific DNA binding	9	3.61E-04
GOTERM_MF	GO:0003690~double-stranded DNA binding	7	1.10E-03
GOTERM_MF	GO:0003677~DNA binding	44	1.88E-03
GOTERM_MF	GO:0003697~single-stranded DNA binding	5	4.25E-03
KEGG_PATHWAY	hsa03040:Spliceosome	5	4.39E-02
B: Down-regulated			
GOTERM_BP	GO:0009611~response to wounding	9	7.57E-03
GOTERM_BP	GO:0006954~inflammatory response	7	8.11E-03
GOTERM_BP	GO:0006469~negative regulation of protein kinase activity	4	1.16E-02
GOTERM_BP	GO:0051056~regulation of small GTPase mediated signal transduction	6	1.16E-02
GOTERM_BP	GO:0033673~negative regulation of kinase activity	4	1.27E-02
GOTERM_MF	GO:0005083~small GTPase regulator activity	7	2.63E-03
GOTERM_MF	GO:0030695~GTPase regulator activity	8	4.30E-03
GOTERM_MF	GO:0060589~nucleoside-triphosphatase regulator activity	8	4.85E-03
GOTERM_MF	GO:0005096~GTPase activator activity	6	5.16E-03
GOTERM_MF	GO:0008047~enzyme activator activity	6	2.75E-02

BP represents biological process; CC represents cellular component; MF represents molecular function; Description represents the identification number of gene ontology (GO) or kyoto encyclopedia of genes and genomes (KEGG) term and the names of GO or KEGG term; Counts represent the number of genes enriched in GO or KEGG terms.

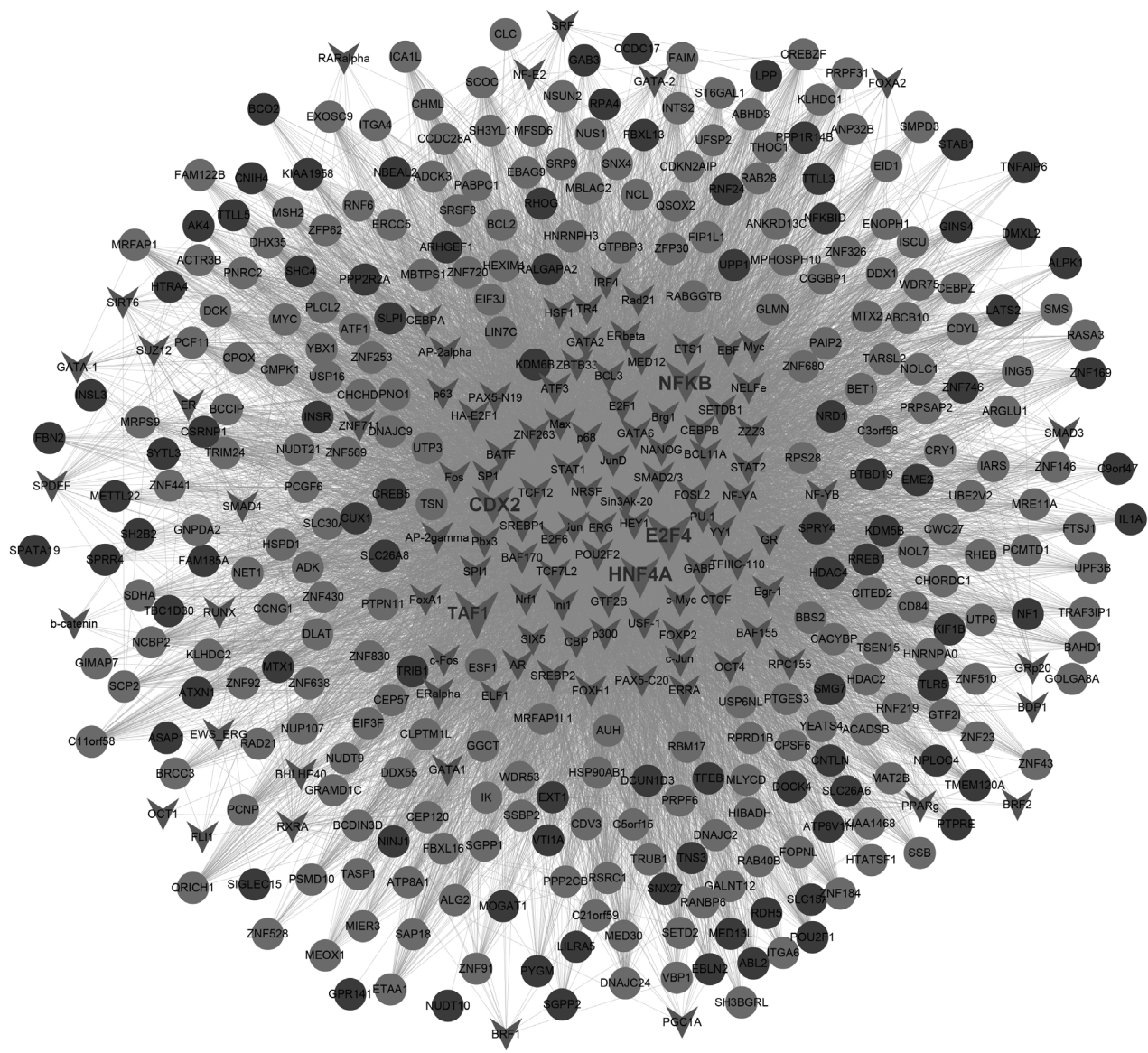


Fig. 3. The transcriptional regulation network. Arrow: transcription factors; light color round nodes: upregulated genes; dark-colored round nodes: downregulated genes. The top five transcription factors are highlighted with a larger font.

required for translation initiation by interacting with eukaryotic translation initiation factor 4 gamma, 1, which is important in inflammatory breast cancer (13). NFkB activation plays a central role in the pathophysiology of septic shock (14).

In conclusion, *KDM6B*, *HDAC2*, *MYC*, *HSP90AB1*, and *PABPC1* may play important roles in the development of septic shock. Furthermore, NFkB may be involved in septic shock by regulating the expression of *KDM6B*, *HDAC2*, *MYC*, *HSP90AB1*,

and *PABPC1*. However, the present study is limited by the lack of experimental verification for the candidate genes, which should be obtained in future research.

REFERENCES

1. Hotchkiss RS, Monneret G, Payen D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. *Nat Rev Immunol* 2013; 13:862-74.
2. Kaukonen K-M, Bailey M, Suzuki S, Pilcher D,

- Bellomo R. Mortality related to severe sepsis and septic shock among critically ill patients in Australia and New Zealand, 2000-2012. *Jama* 2014; 311:1308-16.
3. Berghe TV, Demon D, Bogaert P, et al. Simultaneous targeting of IL-1 and IL-18 is required for protection against inflammatory and septic shock. *Am J Respir Crit Care Med* 2014; 189:282-91.
 4. Kleiman A, Hübner S, Parkitna JMR, et al. Glucocorticoid receptor dimerization is required for survival in septic shock via suppression of interleukin-1 in macrophages. *FASEB J* 2012; 26:722-29.
 5. Lorente J, Marshall JC. Neutralization of tumor necrosis factor in preclinical models of sepsis. *Shock* 2005; 24:107-19.
 6. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 2009; 4:44.
 7. Yang J-H, Li J-H, Jiang S, Zhou H, Qu L-H. ChIPBase: a database for decoding the transcriptional regulation of long non-coding RNA and microRNA genes from ChIP-Seq data. *Nucleic Acids Res* 2013; 41:D177-D187.
 8. Stearns-Kurosawa DJ, Osuchowski MF, Valentine C, Kurosawa S, Remick DG. The pathogenesis of sepsis. *Annu Rev Pathol* 2011; 6:19.
 9. Wei Y, Jia Y, Zheng H, Yang H, et al. Pharmacological inhibition of histone demethylase JMJD3 reduces leukemia cell survival and represses production of the cytokine CCL2 in MDS/AML. *Blood* 2015; 126:5219-19.
 10. Meja KK, Rajendrasozhan S, Adenuga D, et al. Curcumin restores corticosteroid function in monocytes exposed to oxidants by maintaining HDAC2. *Am J Respir Cell Mol Biol* 2008; 39:312-23.
 11. McKeown MR, Bradner JE. Therapeutic strategies to inhibit MYC. *Cold Spring Harb Perspect Med* 2014; 4:a014266.
 12. Rice JW, Veal JM, Fadden RP, et al. Small molecule inhibitors of Hsp90 potentially affect inflammatory disease pathways and exhibit activity in models of rheumatoid arthritis. *Arthritis Rheum* 2008; 58:3765-75.
 13. Silvera D, Arju R, Darvishian F, et al. Essential role for eIF4GI overexpression in the pathogenesis of inflammatory breast cancer. *Nat. Cell Biol* 2009; 11:903-8.
 14. Liu SF, Malik AB. NF- κ B activation as a pathological mechanism of septic shock and inflammation. *Am J Physiol Lung Cell Mol Physiol* 2006; 290:L622-L645.

LETTER TO THE EDITOR

PROPOLIS NASAL SPRAY EFFECTIVELY IMPROVES RECOVERY FROM INFECTIOUS ACUTE RHINITIS AND COMMON COLD SYMPTOMS IN CHILDREN: A PILOT STUDYJ. MARTI¹, F. LÓPEZ², I. GASCÓN¹ and J. JULVE³¹*Pediatric Service Primary Care, La Marina Primary Care Center, Barcelona, Spain;*²*Pediatric Service Primary Care, El Clot Primary Care Center, Barcelona, Spain;* ³*Pharmalink, S.L., Parc Tecnològic del Vallès, Cerdanyola, Spain**Received April 26, 2017 – Accepted September 5, 2017*

Currently, treatment for acute rhinitis (AR) is symptomatic but no clear agreement exists to control its development. Propolis extract may appear as a promising natural treatment for AR, but its beneficial effects have not yet been fully tested. Forty children suffering from AR and common cold symptoms aged between 2-12 years were enrolled in a prospective epidemiological multicentre study. A 7-day treatment with propolis nasal spray (3 times/day) was applied and a comparison of symptomatology, subjective global improvement and quality of life (QoL) between baseline (day 1) and final (day 7) visits were performed. The main goal was to evaluate the changes in symptom intensity using the Jackson's scoring test. After 7 days of treatment there was a significant decrease of symptoms both in the total score ($p < 0.0001$) and in regard to each AR symptom ($p < 0.01$). On the whole, the sample reported no symptoms by day 7, and the resolution of symptoms occurred approximately at day 4. Furthermore, there was no need for supplementary treatment. Both the subjective global improvement impression and the QoL of patients appeared to significantly improve after treatment. No adverse events (AEs) were found globally. It can be concluded that propolis nasal spray effectively improves recovery from infectious AR and common cold symptoms in children and is an optimal alternative in the treatment of this disease without need for any adjuvant treatment.

To the Editor,

Nonallergic acute rhinitis (AR) is a very common condition in adults and children that can affect all types of populations regardless of gender, age, or ethnic background (6). It appears as a very frustrating disorder characterized by a number of disabling symptoms, ranging from mild to debilitating, such as profuse watery rhinorrhea, nasal congestion,

sneezing and/or itching. Untreated or uncontrolled symptoms of AR can considerably affect the QoL, leading to systemic symptoms of fatigue, headache, sleep disturbance and cognitive impairment. This is especially relevant for the child population, as it is associated with interrupted sleep patterns, daytime drowsiness, irritability and poor concentration (1, 2). Its most common cause is an infectious external

*Key words: propolis, acute rhinitis, cold, effectiveness, quality of life, children**Mailing address:*

Dr Jordi Julve,
PHARMALINK S.L.
Av. Universitat Autònoma,
13, Parc Tecnològic del Vallès, 08290 Cerdanyola del Vallès, Spain
Tel.: +34 666 570401 - Fax: +34 93 592 3143
e-mail address: jjulve@pharmalink.es

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.
Unauthorized reproduction may result in financial and other penalties

agent, normally a virus, and the diagnosis must be made through clinical data.

Treatment for AR is symptomatic. First-generation antihistamines, anticholinergics, like the ipratropium bromide (topic use), and alfa-adrenergic agonists have demonstrated their effectiveness in reducing rhinorrhea and nasal congestion, joining the nasal lavage with a saline solution (3), however, they can produce rebound effects.

In recent years, propolis has appeared as a new option to control AR symptoms. Propolis is a natural resin with a complex nature produced by honey bees (20) that provides beneficial effects on the health (4). It presents antibacterial, antiviral and anti-inflammatory properties, among others (5-11), and diverse studies have highlighted the beneficial effects of propolis on the acute and chronic inflammatory processes of the upper respiratory tract (12), demonstrating that it reduces the symptomatic, viral and microbial charge of the upper respiratory tract. This is the first study to identify the benefits of a propolis nasal treatment in spray (Child Free Nose Propolis) in relation to childhood infectious AR.

MATERIALS AND METHODS

Design

A prospective epidemiological multicenter study was conducted in two Pediatric Primary Care services of Spain (CAP La Marina and CAP El Clot, Barcelona, Spain) in order to evaluate the effects of propolis nasal spray on a child population suffering from AR and common cold symptoms. Patients underwent two visits (baseline and final visit, separated by a 7-day period). At the baseline visit, information on the sociodemographic, clinical and symptomatic characteristics of patients was collected. At the end of the baseline visit, the first dose of the propolis was administered (1 spray into each nostril, 3 times/day). During the following 7 days, the symptoms and their intensity were registered through a patient's daily diary by the caregivers and/or the parents. On the final visit, different aspects regarding the symptom evolution, status of health, subjective global improvement of the symptomatology and the QoL were collected. Frequency of night waking episodes by parents/caregivers was also requested (see secondary outcomes below). All data was

compiled by researchers in a case record form (CRF) summarizing the information of the clinical medical records, the patient's daily diary and questions conducted during the study visits.

Study population

Patients of both sexes aged between 2 and 12 years suffering from AR and with the occurrence of at least one of the common cold symptoms (nasal obstruction or secretion, sneezing or sore throat) within the 36 hours of symptomatology presence were consecutively included from two Spanish Pediatric Primary Care services (CAP La Marina and CAP El Clot, Barcelona, Spain). Children with previous diagnosis of asthma, a non-productive cough, fever, or other bacterial-related symptoms, those undergoing antibacterial concomitant treatment, or with hypersensitivity to any component of the study drug or with any other clinical or therapeutic relevant data were excluded. Patients without a baseline or final score in the Jackson's symptom scale (13, 14) were also excluded (see variables below).

The study was conducted in accordance with the Helsinki Declaration version 2013 and was approved by a local clinical research ethics committee (CEIC IDIAP Jordi Gol, Barcelona, Spain). An informed patient consent form was obtained from all children's parents or legal guardians.

Study and dosage of medication

The 'Child Free Nose Propolis' is a Medical Device Class I designed for nostril hygiene by targeting pathological processes such as rhinitis, excessive secretions and flu. It is also indicated for nostril hygiene in case of contamination, tobacco and dry atmospheres. The composition contains isotonic saline solution, propolis extract, methyl p-hydroxybenzoate sodium, potassium sorbate, hydrogen chloride and thyme extract. It is packaged in a 20-ml spray bottle with a specialized applicator (without propellant gas). The study was performed according to the prescribed information of the product. During the baseline visit, all of the children received treatment with the Child Free Nose Propolis nasal spray. The treatment started at the end of the baseline visit, after shaking the puffer, by 1 spraying time into each nostril, with a slight massage 3 times/day. This treatment was repeated and self-administered for 7 days in the patient's home.

Evaluated variables

Sociodemographic, physical and clinical characteristics: All patients underwent a first baseline visit in order to obtain information about different sociodemographic (age, gender, home surroundings), physical (body mass index, temperature, time from when symptoms appeared) and clinical variables (concomitant treatments, relevant antecedents, antecedents of respiratory diseases, smoking habits of parents).

Effectiveness outcome: Over half a century ago, Jackson et al. (1958) (57) developed a symptom scoring system to differentiate adults with viral upper respiratory tract infections from those with other conditions. This system is based on a 4-point Likert scale (from 0 to 3: 0= absence of symptoms, 1= slight symptoms, 2= moderate symptoms, 3= severe symptoms) to evaluate the intensity of the following 8 symptoms: sneezing, headache, global physical discomfort, shivering, nasal obstruction and secretion, sore throat and coughing. This scale has been used in the current study to obtain a main effectiveness variable consisting of the difference between the baseline and the final score of the symptoms' intensity after the propolis treatment.

Secondary outcomes: The following variables were also collected across the study in order to evaluate the subjective global improvement of the symptomatology and the quality of life: follow-up characteristics, nasal and secretion obstruction 1 hour after the 1st, 2nd and 3rd doses, symptoms' duration, subjective global improvement impression in relation to AR by the investigator and by the parents/caregivers (evaluated through a scale of 0 to 10, where 10 indicates the best possible health state globally for the patient), the frequency of night waking episodes as evaluated by parents/caregivers, QoL of children (including daytime activities and quality of the sleep). A Visual Analogue Scale (VAS) was also used to evaluate the state of health at the specific moment of the visit (from 0-100 points, where 100 indicates the best possible state of health).

Safety evaluation: The safety assessment consisted of vigilance and recording of all adverse events (AEs) and serious AEs (SAEs) related to the treatment. The adverse reactions were coded according to the AEs classification of the World Health Organization (WHO) (MedDRA version 15.0) and were collected by the System Organ Class (SOC) and Preferred Terms (PT), including the

duration, intensity, seriousness, causality, action taken and resolution.

Statistical analysis

Data description and statistical analysis were performed using the Statistical Analysis System (SAS) software package (version 9.3; SAS Institute, Cary, North Carolina, USA). A descriptive analysis was conducted, describing categorical variables using numbers and percentages, and quantitative variables using means, standard deviation (SD) and ranges.

For the baseline and final quantitative variables comparison, paired *t*-tests were used. All statistical tests were considered significant at $p < 0.05$.

RESULTS

Characteristics of the sample

A total sample of 40 children was finally included from an initial sample of 48 (8 exclusions: age outside the inclusion criteria range (2), patients with a previous diagnosis of asthma (3), and patients with missing values in the Jackson's symptoms scale (5)). Table I shows the main sociodemographic and clinical characteristics of the patients included in the sample.

Effectiveness outcome

The Jackson's symptoms scoring results showed a significant decrease both in the total score and in relation to each of the individual symptoms separately, both before and after the propolis treatment (Table II).

Secondary outcomes

Nasal obstruction and secretion 1 hour after the doses of the propolis treatment: The evaluation of the nasal obstruction and secretion 1 hour after the 1st, 2nd and 3rd doses showed a significant reduction in both symptoms across the 7 days for each dosing time. Interestingly, there was a reduction in the number of symptoms when comparing the different intakes within the same day, e.g. day 1 for nasal obstruction showed significant differences between dosing times (after 1st dose – after 2nd dose, difference of 0.1696, $p=0.0397$; after 1st dose – after 3rd dose, difference of

Table I. *Baseline sociodemographic and clinical characteristics of patients in the study group.*

	N=40
Gender (male), N (%)	16 (40)
Age (years), mean $\pm$ SD	5.3 $\pm$ 3
Body Mass Index (Kg/m ²), mean $\pm$ SD	17.4 $\pm$ 3.5
Home surrounding, N (%)	
Urban	35 (87.5)
Metropolitan	4 (10)
Rural	1 (2.5)
Clinical characteristics,	
Body temperature (°C), mean $\pm$ SD	36.2 $\pm$ 0.7
Time since the symptoms appeared (weeks), mean $\pm$ SD	0.5 $\pm$ 0.5
Concomitant treatment, N (%)	7 (17.5)
Relevant medical antecedents*, N (%)	3 (7.5)
Previous respiratory antecedents**, N (%)	11 (27.5)
Smoking habit in parents, N (%)	
Smoker parents (yes)	13 (32.5)
Father	5 (38.5)
Mother	5 (38.5)
Both	3 (23.1)
They smoke at home (yes), N (%)	5 (38.5)
Years with the smoking habit, mean $\pm$ SD	
Father	16.1 $\pm$ 4.6
Mother	10.5 $\pm$ 6.7

** A patient can present more than one antecedent [adenoidal hypertrophy 1 (33.3%), otitis media 1 (33.3%), obesity 1 (33.3%), epilepsy 1 (33.3%)]

** A patient can present more than one antecedent [allergic rhinitis 1 (12.5%), bronchitis 7 (87.5%), pharyngitis 1 (12.5%)] ; three missing data (percent calculated over the no-missing data).

SD: standard deviation.

Table II. Jackson's symptoms scoring.

N=40	Mean±SD	CI 95%	Range	p-value
Total Score				
Baseline	8.6±3.2		3-17	
Final	0.6±1.0		0-4	
Difference*	8.0±3.6	6.9-9.1	2-17	<.0001
Sneezes				
Baseline	1.1±1.0		0-3	
Final	0.1±0.2		0-1	
Difference*	1.1±1.0	0.7-1.4	0-3	<.0001
Nasal obstruction				
Baseline	2.2±0.7		0-3	
Final	0.2±0.5		0-2	
Difference*	2.0±0.8	1.7-2.2	0-3	<.0001
Nasal secretion				
Baseline	2.3±0.7		0-3	
Final	0.3±0.4		0-1	
Difference*	2.1±0.8	1.8-2.3	0-3	<.0001
Cough				
Baseline	1.3±0.8		0-3	
Final	0.1±0.2		0-1	
Difference*	1.3±0.9	1.0-1.6	0-3	<.0001
Global discomfort				
Baseline	0.6±0.8		0-2	
Final	0.0±0.0		0	
Difference*	0.6±0.8	0.4-0.9	0-2	<.0001
Shivers				
Baseline	0.2±0.5		0-2	
Final	0.0±0.0		0	
Difference*	0.2±0.5	0.1-0.3	0-2	0.0096
Headache				
Baseline	0.3±0.5		0-2	
Final	0.0±0.0		0	
Difference*	0.3±0.5	0.1-0.4	0-2	0.0059
Sore throat				
Baseline	0.6±0.9		0-3	
Final	0.0±0.0		0	
Difference*	0.6±0.9	0.3-0.9	0-3	0.0004

*: final-baseline difference; CI: confidence interval; SD: standard deviation.

0.3448, $p=0.0069$); the data regarding nasal secretion was not significant at this time point (see Fig. 1).

Duration of the symptoms across the study: From the total evaluated sample ($N=40$) most of patients had no symptomatology at day 7 (final visit); only three cases (7.5%) showed some symptoms at the final visit. The evaluation of the patients without symptomatology at the final visit demonstrated that they suffered from symptoms approximately half of the days of treatment, with a mean of 3.8 ± 1.4 days. It is important to note that from the entire sample, only 2 cases required additional treatment to treat symptoms. In general, there was no need for any supplementary treatment.

Subjective global improvement impression: We found statistically significant differences in the subjective global improvement impression regarding AR by both the investigator and the parents/caregivers (Table III). Compared to the baseline impression, at the end of the study both populations reported that patients showed an improvement after 7 days.

Frequency of night waking episodes by parents/caregivers: The mean frequency of night waking episodes by parents/caregivers was 0.7 ± 1.2 episodes the first day and 0.3 ± 0.8 episodes the last day of treatment. A reduction in these episodes was evident after one week of treatment.

QoL changes related to daytime activities and the quality of sleep of the children: The VAS scores regarding the global evaluation of the quality of life related to daily activities and the sleep quality of children showed a significant improvement in these aspects after one week of treatment. There were statistically significant differences ($p<0.0001$) between baseline and final values, with a significant increase in the VAS value at day 7 vs day 1 (87.3 ± 12.4 vs 64.5 ± 16.7 , respectively; difference of 22.8, 95% confidence interval (CI) (15.8-29.8).

Safety analyses

Propolis treatment for 1 week showed a very good safety profile. There was hardly any AE. During the study only 1 patient suffered 2 mild AEs (pain and reaction in the subcutaneous skin) related to the treatment (4 days of duration; which was solved without any action taken).

DISCUSSION

Propolis nasal spray treatment for 7 days in children with AR and common cold symptoms was associated with a significant improvement of their symptoms and QoL related to daily activities and sleep characteristics from the beginning,

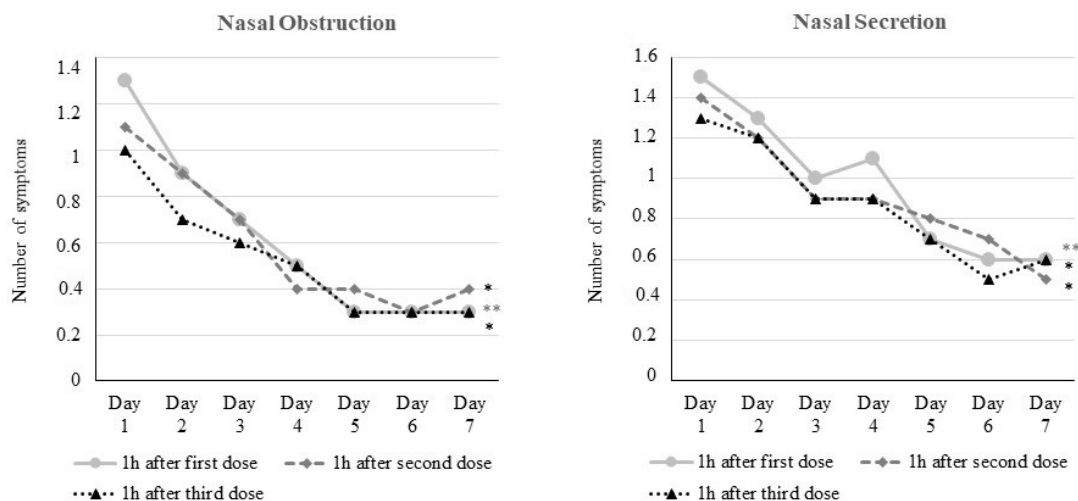


Fig. 1. Nasal obstruction and secretion 1 hour after the 1st, 2nd and 3rd propolis doses: evaluation of the number of symptoms by doses. ** p value <0.001 differences between day 1 and day 7 for first dose; * p value <0.05 differences between day 1 and day 7 for the second and third doses.

Table III. *Global evaluation of the patient's health.*

N=40	Mean±SD	CI 95%	Range	p-value
Researcher				
Baseline	6.8±1.3		4-9	
Final	9.2±0.9		7-10	
Difference*	2.3±1.8	1.7-2.9	(-)-2-6	<.0001
Caregivers/parents				
Baseline	6.4±1.5		3-9	
Final	8.9±1.2		6-10	
Difference*	2.5±2.2	1.8-3.2	(-)-2-7	<.0001

*: *final-baseline difference; CI: confidence interval; SD: standard deviation.*

being most evident the first day.

There is only one older previous trial from Crisan et al. (1995) evaluating the effects of a periodical aqueous propolis treatment in children with rhinopharyngitis (15) and their results are in agreement with the symptomatologic improvement found here, in addition to a decrease of the viral-microbial flora carriage in the upper airways. Nevertheless, a difference is that they used a prolonged treatment.

An important piece of data is that most of the patients completely resolved symptoms by mid-treatment (within the 4 first days) without AEs or the need of any supplementary treatment. Interestingly, some attempts to evaluate the effects of other treatments used in rhinitis, such as corticosteroids (16), antibiotics (17) or antihistamines, (18) failed in finding significant improvement of symptoms over a short period of time (i.e. 3 days), even after longer periods with adjuvant complementary symptomatic treatments (17).

This study is not without limitations. The main one is not having a control group to compare the treatment with propolis spray to traditional treatment (i.e. just saline solution), or even administered as an adjuvant together with the standard treatment, but this is the first study featuring the Child Free Nose Propolis and the results are preliminary, therefore

further research should be carried out to corroborate our data. The use of an adult's scale rather than specific child's scale is another limitation, since it might not be the most appropriate.

In summary, treatment with propolis nasal spray in childhood patients with AR and common colds resulted in clear beneficial effects both of symptoms and the QoL in a short time period and showed its quick effectiveness without supplementary treatments. Moreover, the natural characteristics of this product make it especially interesting for treating infectious AR.

ACKNOWLEDGEMENTS

Pharmalink funded this work.

DISCLOSURE: J. Julve works for Pharmalink. The other Authors report no conflicts of interest relevant to this article.

REFERENCES

1. Hellgren J. Quality of life in nonallergic rhinitis. *Clin Allergy Immunol* 2007; 19:383-87.
2. Chang HY, Seo JH, Kim HY, et al. Allergic diseases in preschoolers are associated with psychological and behavioural problems. *Allergy Asthma Immunol*

- Res 2013; 5:315-21.
3. Fashner J, Ericson K, Werner S. Treatment of the common cold in children and adults. *Am Fam Physician* 2012; 86:153-59.
 4. Farooqui T, Farooqui AA. Beneficial effects of propolis on human health and neurological diseases. *Front Biosci (Elite Ed)* 2012; 4:779-93.
 5. Kujumgiev A, Tsvetkova I, Serkedjieva Y, Bankova V, Christov R, Popov S. Antibacterial, antifungal and antiviral activity of propolis of different geographic origin. *J Ethnopharmacol* 1999; 64:235-40.
 6. Kilic A, Baysallar M, Besirbellioglu B, Salih B, Sorkun K, Tanyuksel M. In vitro antimicrobial activity of propolis against methicillin-resistant *Staphylococcus aureus*. *Ann Microbiol* 2005; 55:113-17.
 7. Falcão SI, Vale N, Cos P, et al. In vitro evaluation of Portuguese propolis and floral sources for antiprotozoal, antibacterial and antifungal activity. *Phytother Res* 2014; 28:437-43.
 8. Huleihel M, Isanu V. Anti-herpes simplex virus effect of an aqueous extract of propolis. *Isr Med Assoc J* 2002; 4(Suppl 11):923-27.
 9. Wang K, Zhang J, Ping S, et al. Anti-inflammatory effects of ethanol extracts of Chinese propolis and buds from poplar (*Populus×canadensis*). *J Ethnopharmacol* 2014; 155:300-11.
 10. Banskota AH, Tezuka Y, Kadota S. Recent progress in pharmacological research of propolis. *Phytother Res* 2001; 15:561-71.
 11. De Groot AC. Propolis: a review of properties, applications, chemical composition, contact allergy, and other adverse effects. *Dermatitis* 2013; 24:263-82.
 12. Speciale A, Costanzo R, Puglisi S, et al. Antibacterial activity of propolis and its active principles alone and in combination with macrolides, beta-lactams and fluoroquinolones against microorganisms responsible for respiratory infections. *J Chemother* 2006; 18:164-71.
 13. Jackson GG, Dowling HF, Spiesman IG, Boand AV. Transmission of the common cold to volunteers under controlled conditions. I. The common cold as a clinical entity. *AMA Arch Intern Med* 1958; 101:267-78.
 14. Taylor JA, Weber WJ, Martin ET, McCarty RL, Englund JA. Development of a symptom score for clinical studies to identify children with a documented viral upper respiratory tract infection. *Pediatr Res* 2010; 68:252-57.
 15. Crișan I, Zaharia CN, Popovici F, et al. Natural propolis extract NIVCRISOL in the treatment of acute and chronic rhinopharyngitis in children. *Rom J Virol* 1995; 46:115-33.
 16. Jacobs R, Lieberman P, Kent E, Silvey M, Locantore N, Philpot EE. Weather/temperature-sensitive vasomotor rhinitis may be refractory to intranasal corticosteroid treatment. *Allergy Asthma Proc* 2009; 30:120-27.
 17. Garbutt JM, Banister C, Spitznagel E, Piccirillo JF. Amoxicillin for acute rhinosinusitis: a randomized controlled trial. *JAMA* 2012; 307:685-92.
 18. Banov CH, Lieberman P, Vasomotor Rhinitis Study Groups. Efficacy of azelastine nasal spray in the treatment of vasomotor (perennial nonallergic) rhinitis. *Ann Allergy Asthma Immunol* 2001; 86:28-35.

LETTER TO THE EDITOR

ALTERATIONS IN TRANSAMINASE ACTIVITY AND SERUM LEVEL OF LEPTIN AND HEPCIDIN INDUCED BY HIGH FAT DIET IN ALBINO RATS

M.B. KHAWAR¹ and N. SHEIKH^{1,2}¹Cell and Molecular Biology Laboratory, Department of Zoology, University of the Punjab, Lahore-Pakistan; ²Centre for Applied Molecular Biology, University of the Punjab, Lahore-Pakistan*Received April 17, 2017 – Accepted September 7, 2017*

Obesity is a commonly growing life-threatening problem of the modern world. The present study was aimed to assess alterations in transaminase levels as well as leptin and hepcidin levels of sera through ELISA after high fat diet consumption for sixteen weeks by albino rats (n=5). Three groups were established: experimental groups 1 and 2 and a control group. Group 1 was fed on a high fat diet having a composition of 33% rat chow +33% sucrose +33% milk powder. Similarly, group 2 was fed with another high fat diet with a mixture of rat chow and milk powder *ad libitum*. The control group was fed on normal rat chow and water *ad libitum*. After sixteen weeks, the rats were euthanized and blood was collected for serum separation. Serum levels of alanine aminotransferase showed a positive significant increase ($P=0.0325$) while a significant negative change ($P=0.0006$) was noted in aspartate aminotransferase levels in both the experimental groups compared to the control group. Serum leptin levels were found to be increased up to 10.06-fold in Group 1 and 6.11-fold in Group 2 when compared to controls. On the other hand, serum hepcidin levels showed up to 1-fold and 2.59-fold changes in Group 1 and Group 2, respectively, compared to controls. Taken together, from these results it can be concluded that a high fat diet not only disturbs normal metabolism, but it also leads to liver inflammation which is obvious by the changes in transaminase activity as well as leptin and hepcidin levels.

To the Editor,

Obesity is a severe and widespread metabolic problem around the globe. This condition develops when energy intake exceeds the energy output regarding body weight and glucose metabolism (1). As the expenditure of energy during obesity decreases, the extra energy is piled up in adipose tissue. Adipocytes are a storage house of extra energy and easily congest with fat; the growth of adipose tissue due to accumulation of fats results in a change in morphology of these tissues as well as in whole organism (2). According to one

study in US, around 69% of adults are overweight with $BMI \geq 25 \text{ kg/m}^2$, and 36% are grouped as obese with $BMI \geq 30 \text{ kg/m}^2$ (3).

In the past few years, the idea that there is a positive correlation between obesity and inflammation is becoming popular. Hotamisligil et al. developed a link between obesity and inflammation for the first time. According to this concept, there is a direct relationship between adipose mass and tumor necrosis factor- α gene (a pro-inflammatory gene) (4). In later studies, this link was further supplemented by an enhanced level of

Key words: alanine aminotransferase, aspartate aminotransferases, hepcidin, high fat diet, leptin, liver, obesity, transaminases

Mailing address:

Dr Nadeem Sheikh,
Cell & Molecular Biology Lab,
Department of Zoology,
University of the Punjab, Lahore, Pakistan
Tel.: +92 3224222036
e-mail: s_nadeem77@yahoo.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

various other inflammatory markers in the plasma of obese individuals (5, 6). These observations have led to the view that obesity results in chronic low-grade inflammation characterized by changes in the shape and structure of adipose tissue.

The liver is one of the vital and most important organs of the body as it is the main site of metabolism and is involved in multiple tasks, including storage of a number of biological molecules, bile production, heat formation and as a basic line of defense against foreign particles or toxic species (7, 8). Liver is perhaps one of the major organs of the body on which the effects of obesity due to adipose mass and pro-inflammatory substances are most obvious. The aim of the present study was therefore to assess alterations in transaminase levels as well as leptin and hepcidin levels of sera through ELISA after high fat diet consumption for sixteen weeks in albino rats.

MATERIALS AND METHODS

Animal colonies

Colonies of healthy *Rattus norvegicus* were reared in an animal room situated in the Department of the Zoology, University of the Punjab, Lahore, Pakistan. Rats were kept in setoff conditions characterized with 12h light dark-cycle and at room temperature. Weaning rats of 30 ± 10 g were selected for the study. These weaning rats were further sub-divided into three groups ($n=5$).

Experimental design

Three groups, each composed of 5 rats, were named as groups I and II, and a control group. Group I was fed on a fat rich diet of 33% Rat chow +33% Sucrose + 33% commercially available Milk Powder. Group II received a fat rich diet of rat chow *ad libitum* + Milk Powder *ad libitum*. The control group was fed on regular rat chow throughout the experimental study period. The rats were reared with water and food available *ad libitum* for sixteen weeks.

Sample collection

Rats were fasted overnight before dissections and were anesthetized by injecting ketamin (1.5 ml/kg body weight) (1:1) diluted with injection water intraperitoneally. Sharp scissors were used to cut the muscular abdominal layer to expose the internal organs.

Blood of the animals was drawn through direct cardiac puncture into serum gel tubes.

Evaluation of serum indices

Blood was allowed to stand overnight at 4°C and centrifuged for 20 min at 2000 g to collect sera and was used to analyze the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) using ready to-use kits.

Enzyme-linked immunosorbent assay (ELISA)

All the assessments were carried out according to the instructions provided by the manufacturer. Quantification of the reactions was determined by the optical density using an automated ELISA reader (Biorad-680 Microplate reader, USA) at 450 nm. The magnitude of the absorbance for this developed color is proportional to the amount of hepcidin and leptin.

Statistical analysis

Data were statistically analyzed using Prism Graph pad 5 software (San Diego, CA, USA). Statistical significance was calculated by using one way ANOVA with *P* value of less than 0.05 considered significant.

RESULTS

Aminotransferases

Serum level of ALT showed a significant increase ($P=0.0325$) of 31.58% in both Group 1 and Group 2 when compared to control.s On the other hand, an opposite trend was found in the level of AST, with a significant negative change ($P=0.0006$) of about 26.93% and 30.91% in Group 1 and Group 2, respectively, compared to the control group (Fig. 1).

Alkaline phosphatases

ALP level depicted an overall increase of about 28.68% and 33.42% in the sera of Group 1 and Group 2, respectively, although these changes were not significant when compared to the control group (Fig. 2).

ELISA

Serum leptin concentration was increased up to 10.06-fold in Group 1 and 6.11-fold in Group

2 fed on fat rich diet when compared with the control group (Fig. 3).

Serum hepcidin level was increased up to 1- and 2.59-fold, in rats fed on a fat rich diet when compared with the control group (Fig. 4).

DISCUSSION

Obesity is a growing global health problem, the prevalence of which has risen significantly during the last few decades (9). Worldwide, approximately

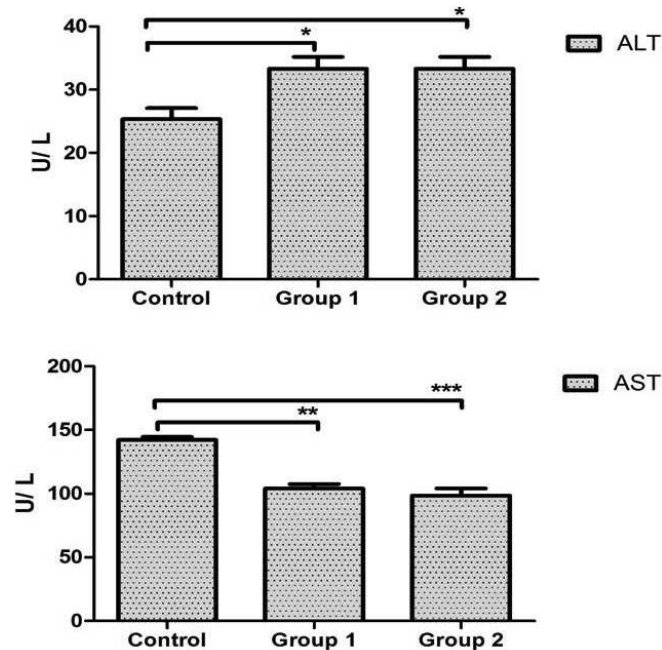


Fig. 1. Level of aminotransferases (ALT+ AST) in sera. The levels of ALT showed a trend of significant positive increase in both groups while AST levels were found to be decreased in both the studied experimental groups. The results are representative of five animals per group (mean $\pm$ S.E.M.). Statistically significant changes are marked with asterisks (* P <0.05, ** P <0.01, *** P <0.001).

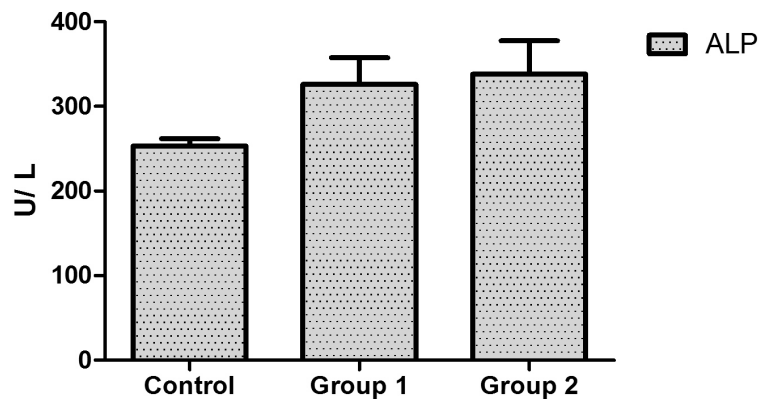


Fig. 2. Alkaline phosphatases (ALP) level in sera. The levels of ALP showed a trend of positive increase in both experimental groups. The results are representative of five animals per group (mean $\pm$ S.E.M.). Statistically significant changes are marked with asterisks (* P <0.05, ** P <0.01, *** P <0.001).

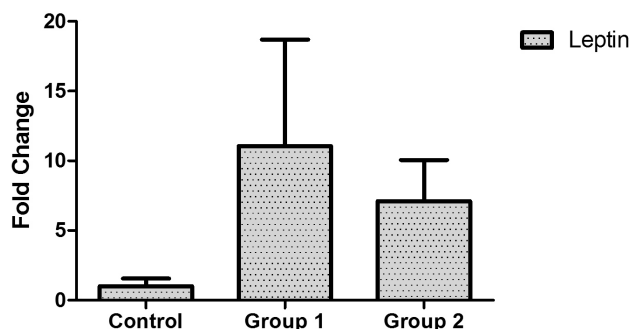


Fig. 3. ELISA for serum leptin level. Leptin levels showed an overall increase in the sera of Group 1 and Group 2 compared to controls. The results are shown in fold change.

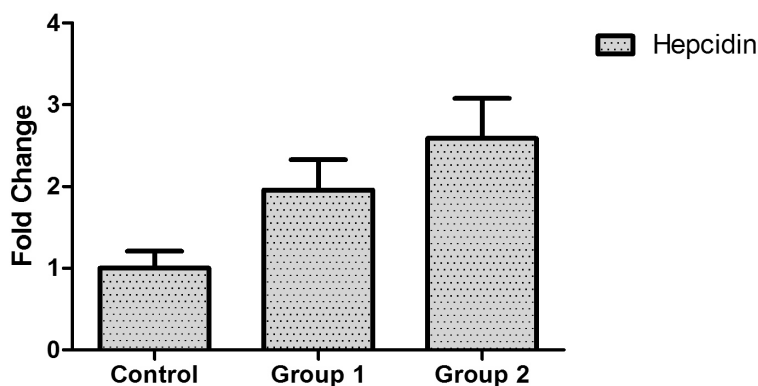


Fig. 4. ELISA for serum Hepcidin level. Hepcidin levels showed an overall increase in the sera of Group 1 and Group 2 compared to controls. The results are shown in fold change.

500 million adults are obese and 1.5 billion are overweight (10). Overweight is characterized by the presence of enlarged adipose mass and defined as an increase in body weight that results in extreme fat accumulation. The present study was therefore aimed to evaluate the alterations in transaminase activity and changes in leptin and hepcidin levels of sera after high fat diet induction in albino rats.

Hepcidin is a 25 amino acid long peptide involved in iron metabolism. It results in internalization and lysosomal degradation of ferroportin by binding to it (11, 12). Hepcidin level was found to be upregulated by a number of factors, i.e. iron overload, endoplasmic stress and inflammation. In contrast, its level is down-regulated in certain conditions i.e. hypoxia, anemia and oxidative stress (13, 14).

These findings are concurrent with the findings of our study in which hepcidin levels were found to be increased up to 1- and 2.59-fold in both Group 1 and 2, respectively.

Leptin, secreted by adipocytes, is involved in inhibiting food intake by signaling the brain, hence involved in reducing weight (15, 16). Its level has been reported to be decreased during fasting, characterized by significant changes in hormonal levels and energy balance (16, 17). Increase in serum leptin concentration is in accordance with these findings as an increase of 10.06-fold in Group 1 and 6.11-fold in Group 2 fed on fat rich diet was noted compared to the control group.

ALT and AST estimation are key markers to assess liver damage (18). Cell damage and necrosis

increase the permeability of membrane, hence their levels rise in sera, indicating a degree of liver injury (19, 20). The increased levels of ALT in the present study in both groups I and II are in accordance with the above findings, and are clearly reflective of liver injury. Similarly, a decrease in AST level is also an indication of disturbed liver function. ALP expression has recently been identified in adipose tissues where it is involved in the regulation of adipogenesis (21). ALP has also been previously reported to increase in obesity (22). Serum levels of ALP have been reported to be higher in obese versus non-obese subjects in another study (23). These findings are concurrent with the findings of our study.

Taking into account all the above findings, it can be concluded that a high fat diet induces considerable variations in serum hepcidin, leptin and transaminase levels. Changes in these parameters are clear reflective of disturbance in hepatic function.

ACKNOWLEDGEMENTS

The current study was supported by the Higher Education Commission (HEC) funded project No. 20-2115. The Authors are grateful to HEC for providing financial support. The authors thank the Vice Chancellor, University of the Punjab, Lahore, Pakistan for providing financial support to publish the article.

REFERENCES

1. Akiyama T, Tachibana I, Shirohara H, Watanabe N, Otsuki M. High-fat hypercaloric diet induces obesity, glucose intolerance and hyperlipidemia in normal adult male Wistar rat. *Diabetes Res Clin Pr* 1996; 31:27-35.
2. Jernas M, Palming J, Sjöholm K, et al. Separation of human adipocytes by size: hypertrophic fat cells display distinct gene expression. *FASEB J* 2006; 20:1540-42.
3. Flegal KM, Carroll MD, Kit BK, Ogden CL. Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999-2010. *Jama* 2012; 307:491-97.
4. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor- α : direct role in obesity-linked insulin resistance. *Science* 1993; 259:87-91.
5. Trayhurn P, Wood IS. Signalling role of adipose tissue: adipokines and inflammation in obesity. *Biochem Soc T* 2005; 33:1078-81.
6. Florez H, Castillo-Florez S, Mendez A, Casanova-Romero P, Larreal-Urdaneta C, Lee D, Goldberg R. C-reactive protein is elevated in obese patients with the metabolic syndrome. *Diabetes Res Clin Pr* 2006; 71:92-100.
7. Nakatani K, Kaneda K, Seki S, Nakajima Y. Pit cells as liver-associated natural killer cells: morphology and function. *Med Electron Microsc* 2004; 37:29-36.
8. Malarkey DE, Johnson K, Ryan L, Boorman G, Maronpot RR. New insights into functional aspects of liver morphology. *Toxicol Pathol* 2005; 33:27-34.
9. Rodriguez-Hernandez H, Simental-Mendia LE, Rodriguez-Ramirez G, Reyes-Romero MA. Obesity and inflammation: epidemiology, risk factors, and markers of inflammation. *Int J Endocrinol* 2013; 2013:678159.
10. Danaei G, Finucane MM, Lu Y, et al. National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2+ 7 million participants. *The Lancet* 2011; 378:31-40.
11. Nemeth E. Hepcidin biology and therapeutic applications. *Expert Rev Hematol* 2010; 3:153-55.
12. Nemeth E, Valore EV, Territo M, Schiller G, Lichtenstein A, Ganz T. Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. *Blood* 2003; 101:2461-63.
13. Viatte L, Vaulont S. Hepcidin, the iron watcher. *Biochimie* 2009; 91:1223-28.
14. Miura K, Taura K, Kodama Y, Schnabl B, Brenner DA. Hepatitis C virus-induced oxidative stress suppresses hepcidin expression through increased histone deacetylase activity. *Hepatology* 2008; 48:1420-29.
15. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994; 372:425-32.
16. Ahima RS, Prabakaran D, Mantzoros C, Qu D,

- Lowell B, Maratos-Flier E, Flier JS. Role of leptin in the neuroendocrine response to fasting. *Nature* 1996; 382:250-52.
17. Flier JS: Whats in a Name? In Search of Leptins Physiologic Role 1. *J Clin Endocr Metab* 1998; 83:1407-13.
18. Balamurugan M, Parthasarathi K, Cooper EL, Ranganathan LS. Earthworm paste (*Lampito mauritii*, Kinberg) alters inflammatory, oxidative, haematological and serum biochemical indices of inflamed rat. *Eur Rev Med Pharmacol* 2007; 11:77.
19. Center SA. Interpretation of liver enzymes. *Vet Clin N Am-Small* 2007; 37:297-333.
20. Friedman SL. The cellular basis of hepatic fibrosis-mechanisms and treatment strategies. *New Engl J Med* 1993; 328:1828-35.
21. Ali AT, Ferris WF, Penny CB, Van der Merwe M-T, Jacobson BF, Paiker JE, Crowther NJ. Lipid accumulation and alkaline phosphatase activity in human preadipocytes isolated from different body fat depots. *J Endocrinol Metabol Diabetes SA* 2013; 18:58-64.
22. Khan AR, Awan FR, Najam S, Islam M, Siddique T, Zain M. Elevated serum level of human alkaline phosphatase in obesity. *J Pak Med Assoc.* 2015; 65:1182-85.
23. Al-Sultan AI. Assessment of the relationship of enzymes with obesity and insulin resistance in adults in Saudi Arabia. *Sultan Qaboos Univ Med J* 2008; 8:185-92.

LETTER TO THE EDITOR

ELEVATED PLASMA IL-8 CONCENTRATION IS RELATED TO SEVERITY OF SYSTEMIC INFLAMMATION IN CHRONIC SPONTANEOUS URTICARIA

A. KASPERSKA-ZAJAC¹, A. GRZANKA¹, A. DAMASIEWICZ-BODZEK², K. BIENIEK¹,
A. SKRZYPULEC-FRANKEL¹, K. STENCEL-GABRIEL³ and A. SIKORA-ZYDEK¹

¹Department of Internal Diseases, Dermatology and Allergology in Zabrze, SMDZ in Zabrze, Medical University of Silesia in Katowice, Poland; ²Department of Chemistry in Zabrze, Medical University of Silesia in Katowice, SMDZ in Zabrze, Poland; ³Clinical Department of Pediatrics, Hospital No 2, Bytom, School of Health Sciences, Medical University of Silesia in Katowice, Poland

Received May 5, 2017 – Accepted September 7, 2017

Chronic spontaneous urticaria (CSU) is associated with activation of acute phase response. Questions arise regarding its association with other inflammatory mediators. To determine plasma IL-8 concentration in CSU patients and its association with C-reactive protein (CRP) concentration, a nonspecific inflammatory marker of the disease activity, concentrations of plasma IL-8 and serum CRP were measured in CSU patients and compared with healthy controls. IL-8 and CRP concentrations were significantly higher in CSU patients as compared with the healthy subjects. In addition, there were significant differences in IL-8 and CRP concentrations between CSU patients with moderate-severe symptoms and the healthy subjects. Plasma IL-8 and serum CRP concentrations showed a significant correlation with urticaria activity score (UAS). Additionally, a significant positive correlation was observed between IL-8 and CRP concentrations. Up-regulations of IL-8 and its association with the marker of clinical and inflammatory activity suggest a role of this cytokine in the pathogenesis of CSU.

To the Editor,

The etiopathogenesis of chronic spontaneous urticaria (CSU) may vary between patient subgroups depending on an interaction of immune, genetic, and environmental factors, including infections, emotional stress, or irritant/chemical agents. CSU has been associated with activation of immune-inflammatory and coagulation/fibrinolysis processes (1-3).

Elevated serum C-reactive protein (CRP) concentrations and high values within the normal

range are features of more active urticarial processes. CRP is associated with CSU activity, but its role in the disease is unclear. This systemic inflammatory response may contribute to urticarial activity or may reflect local inflammatory processes (4, 5). Questions arise regarding its association with other inflammatory mediators.

IL-8 is a proinflammatory cytokine that strongly mediates activity of neutrophils and it is frequently associated with inflammatory diseases (6-8). Thus, the aim of this study was to determine plasma IL-8

Key words: IL-8, chronic spontaneous urticaria, acute phase response, C reactive protein

Mailing address:

Prof. Alicja Kasperska-Zajac,
Chair and Clinical Department of Internal Diseases,
Dermatology and Allergology,
ul. M. Curie-Skłodowskiej 10, 41-800 Zabrze, Poland
Tel.: +48 323732323
e-mail: alakasperska@gmail.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

concentration in CSU patients and its association with serum CRP concentration, a nonspecific inflammatory marker of the disease activity.

MATERIALS AND METHODS

Fifty-eight active CSU patients (18 men and 40 women; median age: 38 years, range: 24–52; median disease duration of 3 years) and 22 sex-, age- and BMI (< 30)-matched with the healthy subjects were enrolled in the study. Each patient underwent tests, including the autologous serum skin test (ASST) and consultations, which were described previously (4). The study comprised 32 mild CSU patients and 26 moderate-severe CSU patients graded during four days as follows: mild: 0–8, moderate: 9–16 and severe: 17–24 urticaria activity score (UAS) according to the EAACI/GA²LEN/EDF/WAO guidelines (4). H1-antihistamines and immunosuppressants were withdrawn at least 4 days and 8 weeks, respectively before the blood sampling.

The Ethics Committee of the Medical University of Silesia (KNW-640-2-1-004/15) approved of the study and written, informed consent was obtained from all research participants.

Blood collection

Blood samples were taken using tubes with anticoagulant on fasting. Plasma were stored at -85°C until the tests were performed.

Assay of IL-8

Plasma IL-8 concentrations were measured by ELISA using (CXCL8/IL-8 R&D kit, MN, USA) according to the manufacturer's instructions. Intra- and inter-assay coefficients of variation were below 8% and 10%, respectively.

Assay of CRP

Serum CRP concentrations were measured using Roche/Hitachi cobas c system. The normal values were lower than 5.0 mg/L.

Statistical analysis

The obtained results were presented using basic parameters of descriptive statistics. Normal distribution of data was measured using Shapiro-Wilk's test. Independent data between two groups of patients with CSU (all) and the

controls were compared using non-parametric U Mann–Whitney test. Independent data between three groups of patients with mild CSU, moderate-severe CSU and the controls were compared using non-parametric ANOVA rank Kruskal-Wallis's test with post-hoc analysis. The Spearman's rank test was used for correlations. $p < 0.05$ was considered statistically significant. Calculations were performed with STATISTICA for Windows 10.0 software (StatSoft, Cracow, Poland).

RESULTS

Plasma IL-8 concentrations

Concentrations of IL-8 in plasma were significantly higher in the CSU group compared to the controls (median: 8.33 vs 2.52 pg/ml, $p < 0.001$; Fig. 1B).

IL-8 plasma concentrations were significantly higher in moderate-severe CSU group compared to the mild CSU group and controls (median: 13.63 vs 5.3 vs 2.52 pg/ml, $p < 0.01$ and $p < 0.0001$, respectively; Fig. 1B). IL-8 concentrations in mild CSU patients did not differ significantly versus the controls (5.3 vs 2.52 pg/ml, $p > 0.05$). Plasma IL-8 concentrations showed a significant positive correlation with UAS ($R = 0.43$, $p = 0.000658$; Fig. 2B).

No significant differences in IL-8 concentrations between CSU groups with positive and negative ASST response (selected according to the similar UAS) were observed.

Serum CRP concentrations

CRP concentrations were significantly higher in serum of CSU patients compared to the controls (median: 3.7 vs 0.35 mg/l; $p < 0.0001$; Fig. 1A). There were significant differences in CRP concentrations between patients with mild, moderate-severe CSU and the controls (median: 1.35 vs 9.8 vs 0.35 mg/l, respectively, $p < 0.001$; Fig. 1A). Additionally, serum CRP concentrations showed a significant positive correlation with UAS ($R = 0.89$, $p = 0.000000$; Fig. 2C).

Correlations

There was significant correlation between IL-8 and CRP ($R = 0.48$, $p = 0.000136$; Fig. 2A).

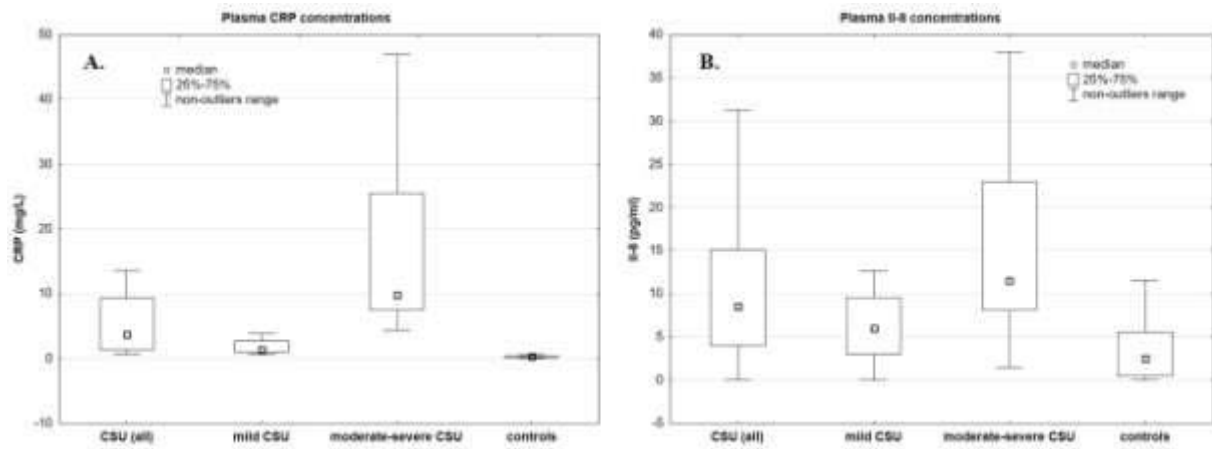


Fig. 1. *A)* Serum CRP concentration in CSU patients with different disease activity. CSU vs controls, $p < 0.0001$; mild CSU vs moderate-severe CSU vs controls, $p < 0.001$. *B)* Plasma IL-8 concentration in chronic spontaneous urticaria (CSU) patients with different disease activity. CSU vs controls, $p < 0.001$; moderate-severe CSU patients vs mild CSU vs controls, $p < 0.01$ and $p < 0.0001$, respectively.

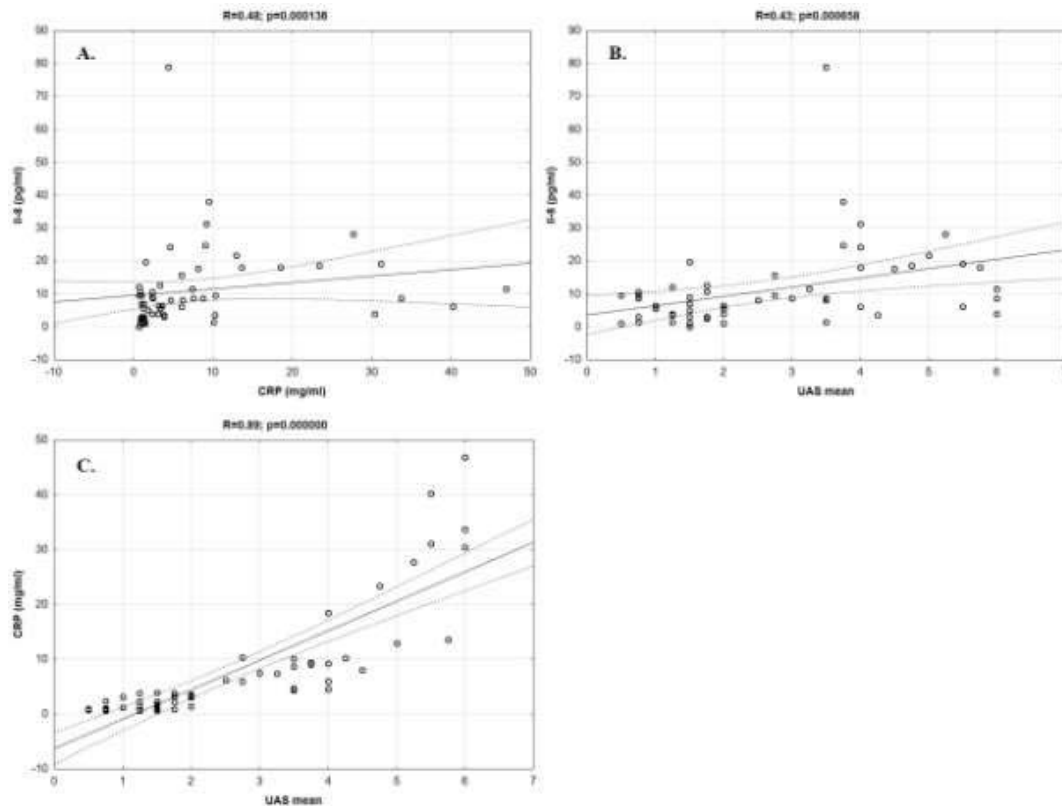


Fig. 2. *A)* Correlation between IL-8 and CRP in CSU patients. *B)* Correlation between plasma IL-8 concentration and urticaria activity score (UAS) in CSU patients. *C)* Correlation between serum CRP concentration and UAS in CSU patients.

DISCUSSION

Our findings suggest that IL-8 and CRP concentrations are associated with severity of systemic inflammation in CSU and the disease activity/severity. Plasma IL-8 and serum CRP concentrations showed a significant correlation with UAS. IL-8 and CRP concentrations were significantly higher in moderate-severe CSU versus mild CSU patients and the controls. We found raised plasma IL-8 concentrations and its correlation with increased CRP concentration, which indicate that circulating IL-8 is associated with an acute phase reaction and may be a marker of systemic inflammatory activity in CSU. It has been demonstrated that CRP stimulates IL-8 release from several cell types, including monocytes (9, 10). Therefore, it seems that a direct link may exist between CRP and IL-8 in CSU.

Similarly to our results, Santos et al. reported up-regulation of IL-8 measured by mRNA expression and the protein concentrations in the supernatant of cell cultures and serum in CSU (11). In addition, an IL-8 expression was observed in both urticarial lesions and uninvolved skin of CSU patients, but not in the healthy subjects (12). In contrast, Puxeddu et al. did not detect increased serum IL-8 concentrations in CSU (13). There were no significant differences in serum IL-8 concentration among acetylsalicylic acid (ASA)-intolerant acute urticaria, ASA-intolerant chronic urticaria, and the healthy subjects (14). Significant association was found between serum myeloperoxidase and IL-8 concentrations in ASA-intolerant acute urticaria patients, suggesting that IL-8 might be directly involved in neutrophil activation (14). Increased serum IL-8 concentration was observed in acute urticaria (15).

The clinical relevance of IL-8 in the pathogenesis of urticaria is incompletely understood. IL-8 is a strong chemoattractant and activator for neutrophils (6). Besides neutrophils, it also has diverse actions on various types of cells, including monocytes and T-lymphocytes (16, 17).

Histopathologic studies have revealed that eosinophils, neutrophils, monocytes, and T lymphocytes are the most prominent cells, although to varying degrees, depending on the stage of

the urticarial lesions; the neutrophilic infiltrate represents an early phase of wheal formation (18, 19). Therefore, it seems that IL-8 may, by its effects on some of these cells, especially neutrophils, be part of an inflammatory cascade that contributes to the development of CSU.

The present study did not explore the sources of increased plasma IL-8 concentration in CSU. It seems that many cell types associated with urticarial inflammation, mainly monocytes, macrophages and endothelial cells, may release IL-8 in response to proinflammatory stimuli. Up-regulation of IL-8 expression by monocytes has been demonstrated in CSU patients (11).

Taking into consideration that IL-8 is the main chemoattractant and activator for neutrophils, therefore, it seems that this cytokine may participate in the inflammatory processes in CSU by attracting and degranulating neutrophils. Up-regulation of IL-8 and its association with the marker of clinical and inflammatory activity suggest a role of this cytokine in the pathogenesis of CSU.

ACKNOWLEDGEMENTS

This study was supported by a research grant from the Committee for Scientific Research.

REFERENCES

1. Asero R. D-dimer: a biomarker for antihistamine-resistant chronic urticaria. *J Allergy Clin Immunol* 2013; 132:983-86.
2. Kasperska-Zajac A, Grzanka A, Kowalczyk J, et al. Refractory chronic spontaneous urticaria and permanent atrial fibrillation associated with dental infection: Mere coincidence or something more to it? *Int J Immunopathol Pharmacol* 2016; 29:112-20.
3. Kasperska-Zajac A, Jarzab J, Żerdzińska A, Bąk K, Grzanka A. Omalizumab effective treatment of different phenotypes of chronic urticaria with omalizumab: case reports and review of literature. *Int J Immunopathol Pharmacol* 2016; 29:320-28.
4. Kasperska-Zajac A, Grzanka A, Damasiewicz-Bodzek A. IL-6 signaling in chronic spontaneous urticaria.

- PLos One 2015; 10:e0145751.
5. Kasperska-Zajac A, Grzanka A, Misiolek M, Mazur B, Machura E. Pentraxin-3 as a local inflammatory marker in chronic spontaneous urticaria. *Cytokine* 2015; 76:566-68.
 6. Willems J, Joniau M, Cinque S, van Damme J. Human granulocyte chemotactic peptide (IL-8) as a specific neutrophil degranulator: comparison with other monokines. *Immunology* 1989; 67:540-42.
 7. Baggiolini M, Loetscher P, Moser B. Interleukin-8 and the chemokine family. *Int J Immunopharmacol* 1995; 17:103-8.
 8. Baggiolini M, Clark-Lewis I. Interleukin-8, a chemotactic and inflammatory cytokine. *FEBS Lett* 1992; 307:97-101.
 9. Black S, Kushner I, Samols D. C-reactive protein. *J Biol Chem* 2004; 279: 48487-90.
 10. Xie L, Chang L, Guan Y, Wang X. C-reactive protein augments interleukin-8 secretion in human peripheral blood monocytes. *J Cardiovasc Pharmacol* 2005; 46:690-96.
 11. Santos JC, de Brito CA, Futata EA, et al. Up-regulation of chemokine C-C ligand 2 (CCL2) and C-X-C chemokine 8 (CXCL8) expression by monocytes in chronic idiopathic urticaria. *Clin Exp Immunol* 2012; 167:129-36.
 12. Caproni M, Giomi B, Volpi W, et al. Chronic idiopathic urticaria: infiltrating cells and related cytokines in autologous serum-induced wheals. *Clin Immunol* 2005; 114 284-92.
 13. Puxeddu I, Panza F, Pratesi F, et al. CCL5/RANTES, sVCAM-1, and sICAM-1 in chronic spontaneous urticaria. *Int Arch Allergy Immunol* 2013; 162:330-34.
 14. Choi SJ, Ye YM, Hur GY, Shin SY, Han JH, Park HS. Neutrophil activation in patients with ASA-induced urticaria. *J Clin Immunol* 2008; 28:244-49.
 15. Papadopoulos J, Karpouzis A, Tentis J, Kouskoukis C. Assessment of Interleukins IL-4, IL-6, IL-8, IL-10 in acute urticaria. *J Clin Med Res* 2014; 6:133-37.
 16. Larsen CG, Anderson AO, Appella E, Oppenheim JJ, Matsushima K. The neutrophil-activating protein (NAP-1) is also chemotactic for T lymphocytes. *Science* 1989; 243:1464-66.
 17. Mukaida N. Interleukin-8: an expanding universe beyond neutrophil chemotaxis and activation. *Int J Hematol* 2000; 72:391-98.
 18. Ying S, Kikuchi Y, Meng Q, Kay AB, Kaplan AP. TH1/TH2 cytokines and inflammatory cells in skin biopsy specimens from patients with chronic idiopathic urticaria: comparison with the allergen-induced late-phase cutaneous reaction. *J Allergy Clin Immunol* 2002; 109:694-700.
 19. Sabroe RA, Poon E, Orchard GE, et al. Cutaneous inflammatory cell infiltrate in chronic idiopathic urticaria: comparison of patients with and without anti-FcepsilonRI or anti-IgE autoantibodies. *J Allergy Clin Immunol* 1999; 103:484-93.

LETTER TO THE EDITOR

**ANTIMICROBIAL, ANTIOXIDANT, CYTOTOXICITY AND LC-MS ANALYSES OF
AERVA JAVANICA: AN ETHNOMEDICINALLY IMPORTANT PLANT**M. KHALID¹, M. BILAL², D. HASSANI¹, H.M.N. IQBAL³ and D. HUANG¹

¹School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai, China; ²State Key Laboratory of Microbial Metabolism, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai, China; ³Tecnologico de Monterrey, School of Engineering and Sciences, Campus Monterrey, Monterrey, N.L., Mexico

Received April 5, 2017 – Accepted September 13, 2017

In this study, *Aerva javanica* was used to extract the essential oil with notable medicinal activities. The chemical composition was investigated by high-performance liquid chromatography (HPLC) and liquid chromatography/mass spectrometry (LC-MS). Ten major chemical compounds were identified as flavonoids derivatives, dihydroxylated and glycosylated metabolites. The antimicrobial, antioxidant, and cytotoxicity activities were tested using agar well-diffusion assay, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging and linoleic acid oxidation assays and hemolytic assay against human erythrocytes (RBCs), respectively. Plant extracts exhibited different extents of antimicrobial activities against selected bacterial and fungal strains; however, the essential oil displayed potent antimicrobial activity against all the tested strains. The percentage inhibition of linoleic acid oxidation and inhibitory concentration (IC₅₀) were recorded to be in the range of 42.45-96.21% and 14.21-38.18 µg/mL, respectively. Cytotoxicity profile of *A. javanica* extracts and essential oil was found in the range of 5.82 to 14.47%. In conclusion, *A. javanica* essential oil could be a potential alternative to chemical additives in the food and pharmaceutical industries.

To the Editor,

Traditional plant-derived medicines and other natural products are often available as a feasible source of primary health care needs of marginalized people in the developing countries including Pakistan (1). Medicinal plants are rich sources of naturally occurring secondary metabolites and antimicrobial compounds, which play a significant role against a myriad of ailments. Before the advent of synthetic drugs and antibiotics, phytoconstituents from crude extracts have been used in medical therapeutics (2), for example, phyto-assisted synthesis of

nanoparticles with medicinal potentialities. Plants protect themselves from microorganism outbreak by producing a wide variety of chemical constituents (2). Based on these chemical compounds, different medicines have been developed from ethnomedicinal plants which are used to treat various infectious diseases in animals and humans. More than 80% of the world's populations depend on plant-based products to encounter their health care needs; 25-45% of a modern medicinal system comprised of plant-derived molecules as basic sources (3).

A wide array of medicinal plants provides a great

Key words: essential oil, hydro distillation, chemical composition, antimicrobial, antioxidant, hemolytic activity

Mailing address:

Prof Danfeng Huang,
School of Agriculture and Biology,
Shanghai Jiao Tong University,
Shanghai-200240, China
Tel./Fax: +86 21 34206943
e-mail: hdf@sjtu.edu.cn

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
**DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF
INTEREST RELEVANT TO THIS ARTICLE.**

opportunity for the ethnobotanical investigations (2-5). Nevertheless, the majority of these plants have not yet been explored comprehensively regarding chemical constituents, pharmacological and toxicological studies. *Aerva javanica* (Desert cotton) is an ethnobotanically valuable plant belonging to the *Amaranthaceae* family and is locally recognized as Sparai. It contains an immense variety of bioactive compounds, such as alkaloids, tannins, saponins, sulfates, flavonoids and lipids, which are used as antibacterial, antifungal, anti-helminths and anti-inflammatory agents (6). The present study aimed to investigate the antimicrobial, antioxidant and cytotoxic activities of *A. javanica* plant extracts and essential oil. The identification of chemical constituents of the essential oil of the tested plant was also the focus of study.

MATERIALS AND METHODS

Plant material and extraction of essential oil

The collected plant material was washed with distilled water followed by drying at room temperature. Air-dried plant material was ground to a fine powder using a Waring blender and processed for extract separation. For essential oil extraction, the shade dried plant material was subjected to hydrodistillation with Clevenger-type apparatus, and the resulting oil was refrigerated (at 4°C) in sealed glass vials for subsequent experiments.

HPLC and LC-MS analyses

Approximately 50-100 mg of ground plant material was extracted with different solvents viz. methanol, ethanol, acetone, and water by vortex mixing (Heidolph Reax Top D-91, Schwabach) at 4°C for 24 h (7). The essential oils were extracted using hydro distillation technique, and 50 µL sample was subjected to HPLC HPLC equipped with an E2695 HPLC pump, a XBridge C18 column (5 µm, 4.6 x 250 mm), and a Dionex Photodiode Array DAD-2998 Detector (Waters, USA) at 30°C. The flow rate was 0.8 mL/min and the first detection was made in the photodiode apparatus set to 515 nm. The essential oil sample was injected into a Thermo LTQ Orbitrap XL HPLC system (Thermo Fisher Scientific, USA) equipped with an LC/MSD Trap VL electrospray ion (ESI) MS for analysis and confirmation of the identified flavonoids. Heater temperature was set at 200°C, sheath gas flow rate

at 25 AU, aux gas flow rate at 5 AU, spray voltage at 3.50 kV, capillary temperature at 350°C, capillary voltage at -35.00 v, and tube lens at -118.42 v. Scan range of m/z was set to 100-800 mass units. Data was recorded with the Chromeleon software (Dionex, USA).

Culture strains and inocula preparation

Bacterial strains, including *Staphylococcus aureus*, *Escherichia coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Marginella morgani*, and *Klebsiella pneumoniae*, were obtained from the culture collection "The Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures", whereas the fungal strains such as *Aspergillus niger*, *Rhizoctonia solani*, *Aspergillus alternate*, *Aspergillus flavus* were kindly provided by CBS Central Albureau Voor Schimmel cultures, Netherlands. Bacterial strains were pre-cultured in nutrient broth at 37°C to obtain an overnight culture. The culture was centrifuged at 10,000 rpm for 5 min, and cell density of pellet was standardized spectrophotometrically (A610 nm) using distilled water. Potato dextrose agar (PDA) medium was used to grow fungi for 3-5 days followed by spore collection using a sterile spatula after adding 10-15 mL of distilled water. The density of fungal spores was adjusted at A595 nm to the final concentration of 1×10⁵ spores/mL.

Antibacterial and antifungal activities

Agar well diffusion method was adopted to evaluate the antibacterial and antifungal activities of plant extracts and essential oil using *Ciprofloxacin* and *fungone* as reference standards for bacterial and fungal strains, respectively (8, 9). After incubating the Petri-plates for 24 h at 37°C, the antibacterial and antifungal activities were recorded by measuring the diameter inhibition zones using a zone reader (mm).

DPPH free radical scavenging activity

The DPPH free radical scavenging capacity of extracts and essential oil was determined as described (10). Briefly, 5.0 mL of a methanolic solution of DPPH (0.1 mM) was thoroughly mixed with 1.0 mL of plant extract solution, and the resulting mixture was incubated in the dark at room temperature for 30 min. The absorbance of each sample was determined on a spectrophotometer at 517 nm using methanol as a blank standard.

Inhibition percentage of linoleic acid oxidation

The antioxidant activity of plant extracts and essential oil was also investigated in terms of % inhibition of linoleic acid oxidation following the reported methodology (10). Percentage inhibition of linoleic acid oxidation was calculated using the relation given below.

Inhibition (%) = $[100 - (\text{Absorbance increase of sample at 360 h} / \text{Absorbance increase of control at 360 h}) \times 100]$

Cytotoxicity assay

The cytotoxicity profile of the *A. javanica* plant extracts and essential oil was determined using hemolytic activity assay as described (11). For each assay, Triton X-100 (0.1% v/v) and phosphate buffer saline (PBS) were taken as positive and negative controls, respectively. The optical density of each sample was recorded on a spectrophotometer at 576 nm.

Statistical analysis

Each sample was measured individually in triplicate for its antimicrobial, antioxidant and cytotoxicity activities and data were presented as mean $\pm$ S.D.

RESULTS

HPLC and LC/MS identification of essential oil

Comparing the HPLC retention time with the published data (12-14) and appropriate standards, 10 major compounds were detected in extracted essential oil, including the derivate forms of flavonoids as well as dihydroxylated and glycosylated metabolites (Fig. 1). The identities of the detected compounds were further confirmed by LC-MS analysis, which showed positively mono-charged molecular ions at m/z ratios of 100 to 300 for the peaks produced by extracts. The chemical compounds identified by LC-MS analysis of the essential oil are listed in Table I.

Antimicrobial activity

The antimicrobial results indicated that plant extracts displayed different degrees of antimicrobial activities against all tested bacterial and fungal microorganisms, and thus, different zones of inhibition (mm) (Table II). Among the plant extracts tested, methanolic extract of *A. javanica* displayed the superior antibacterial potential against *M. morganii* strain when compared with other fractions. The

inhibition zone was recorded to be 22.1 ± 0.13 mm. In case of fungal strains, the highest value of inhibition zone of 28.1 ± 0.34 mm was recorded against *R. solani*. The water as a solvent extract showed the minimum inhibitory activity against both bacterial and fungal pathogens. Notably, essential oil exhibited greater antimicrobial activity than that of the extracts tested, whereas the highest values of inhibition zones of 25.2 ± 0.80 and 29.2 ± 0.63 mm were measured against *P. aeruginosa* and *A. flavus* strains, respectively.

Antioxidant activity

The free-radical scavenging potential of different plant extracts and essential oil using the DPPH assay was evaluated, and results are portrayed as their 50% scavenging capacity (IC_{50} values). Fig. 2 shows that the radical scavenging capacity of essential oil increased in a concentration-dependent manner. The essential oil exhibited the greater scavenging potential ($IC_{50} = 14.21 \pm 0.06$ $\mu\text{g/mL}$) followed by methanol ($IC_{50} = 16.96 \pm 0.08$ $\mu\text{g/mL}$), ethanol ($IC_{50} = 17.23 \pm 0.12$ $\mu\text{g/mL}$), acetone ($IC_{50} = 27.01 \pm 0.29$ $\mu\text{g/mL}$) and water extract ($IC_{50} = 38.18 \pm 0.32$ $\mu\text{g/mL}$). The smaller values of IC_{50} represent a better antioxidant potential and vice versa. The % inhibitions of peroxidation for the methanol, ethanol, acetone and water extracts were recorded to be 75.25 ± 0.65 , 42.45 ± 0.81 , 45.86 ± 0.37 , $52.21 \pm 0.54\%$, respectively; whereas, essential oil registered the highest linoleic acid peroxidation as $96.21 \pm 0.94\%$.

Cytotoxicity assessment

The cytotoxicity profile was investigated by evaluating the hemolytic activity of *A. javanica* plant extracts and essential oil against human red blood cells (RBCs) (Fig. 3). Different plant extracts and essential oil resulted in different percentages of RBCs lysis, i.e., methanol, ethanol, acetone and aqueous extract caused 8.43 ± 0.69 , 14.47 ± 1.32 , 11.46 ± 0.84 , and 13.32 ± 1.29 , respectively, whereas essential oil showed 5.82 ± 0.73 .

DISCUSSION

Medicinal plants produce chemical compounds which possess biomedically related activities,

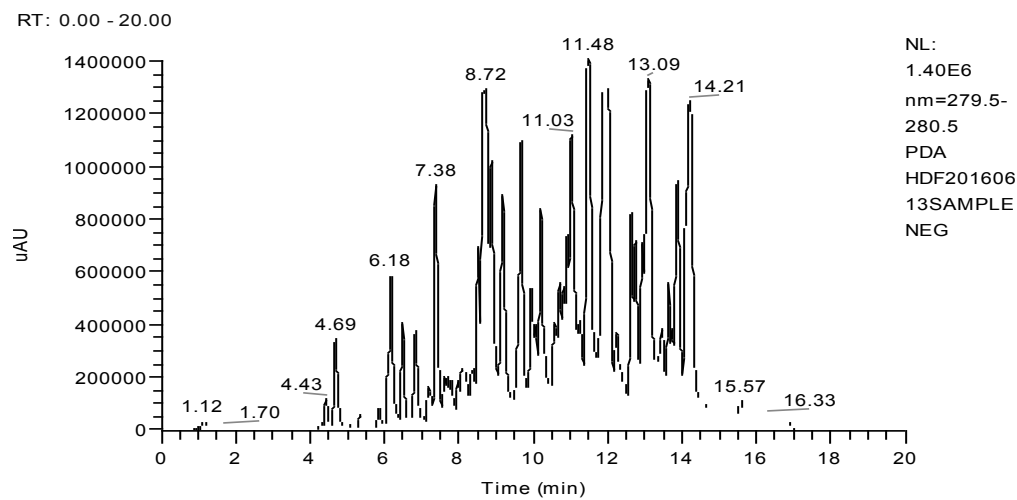


Fig. 1. The HPLC-UV chromatogram of essential oil sample obtained from *A. javanica*.

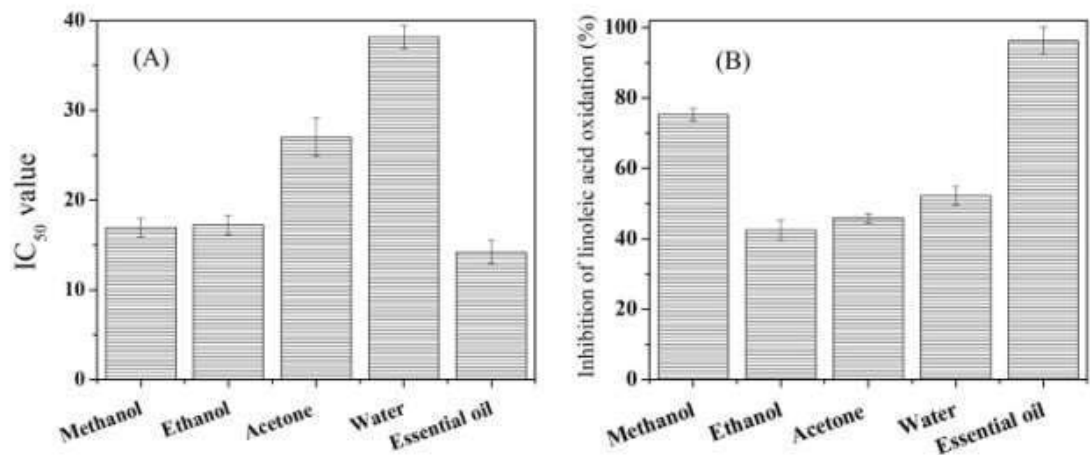


Fig. 2. Antioxidant activities of *A. javanica* extracts and essential oil. A) DPPH radical scavenging assay. B) Percent inhibition of linoleic acid peroxidation.

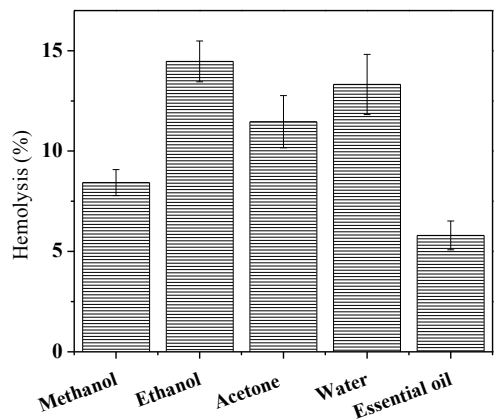
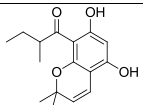
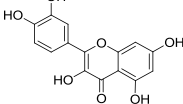
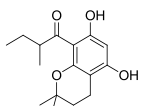
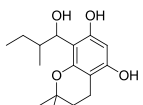
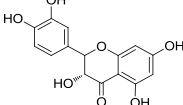
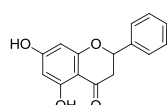
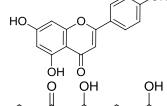
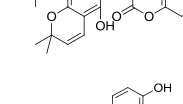
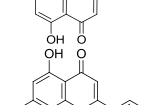


Fig. 3. Cytotoxicity assay by hemolytic activity as a percentage of hemolysis caused by *A. javanica* extracts and essential oil.

Table I. Information of compounds identified from the essential oil of *A. javanica*.

Peak No.	t _R	Negative mode (m/z)	Possible compound	Chemical Name	MS ² (-)	Possible structure
1	10.8	275	C ₁₆ H ₂₀ O ₄	1-(5,7-dihydroxy-2,2-dimethyl-2H-chromen-8-yl)-2-methylbutan-1-one	257, 231, 229, 191, 125	
2	11.7	301	C ₁₅ H ₁₀ O ₇	Quercetin	283, 271, 246, 231, 190	
3	12.6	277	C ₁₆ H ₂₂ O ₄	1-(5,7-dihydroxy-2,2-dimethylchroman-8-yl)-2-methylbutan-1-one	259, 233, 179	
4	13.0	279	C ₁₆ H ₂₄ O ₄	8-(1-hydroxy-2-methylbutyl)-2,2-dimethylchroman-5,7-diol	261, 243, 259, 207	
5	13.3	303	C ₁₅ H ₁₂ O ₇	Taxifolin	285, 257	
6	13.4	255	C ₁₅ H ₁₂ O ₄	5,7-dihydroxy-2-phenylchroman-4-one	237, 235, 217, 187	
7	13.8	269	C ₁₅ H ₁₀ O ₅	5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one	251, 179	
8	14.2	441	C ₂₅ H ₃₀ O ₇	Prenylated phloroglucinol-α-pyrone	287, 275	
9	14.4	283	C ₁₆ H ₁₂ O ₅	Genkwanin	265, 237, 167	
10	14.5	285	C ₁₅ H ₁₀ O ₆	Luteolin	271, 245	

e.g., antimicrobial and antioxidant. The superior antimicrobial activity of the essential oil in our study might be attributed to the presence of some flavonoid derivatives and other hydroxylated and glycosylated compounds identified during LC-MS analysis. Plant-extracted essential oils containing these major compounds have already been documented to present profound antimicrobial properties (15). It has been reported that biological activities of essential oils are mainly ascribed to their major phytoconstituents,

but other minor constituents also stimulate this activity. The synergistic activity of these chemical constituents may drive up the antimicrobial potential of the essential oil by disrupting bacterial or fungal membrane integrities. Furthermore, such synergistic effects may also be effective in inhibiting microorganisms from developing antimicrobial resistance (16). Previously, Legault et al. (17) reported that quercetin, taxifolin, 5, 7-dihydroxy-2-phenylchroman-4-one, and luteolin possessed

Table II. Size of inhibition zone (diameter, mm) of *A. javanica* extracts and essential oil against selected bacterial and fungal strains.

Leaf						
Tested microorganisms	Acetone	Methanol	Ethanol	Water	Essential oil	Standard
Diameter of inhibition zone (mm)						
<i>Staphylococcus aureus</i>	16.2±0.14	18.5±0.17	19.0±0.19	8.2±0.14	22.1±0.21	20.5±0.12
<i>Escherichia coli</i>	14.1±0.07	16.1±0.09	12.2±0.15	09.6±0.22	20.6±0.16	22.1±0.09
<i>Acinetobacter baumannii</i>	19.2±0.24	20.3±0.25	21.5±0.02	12.4±0.12	23.8±0.29	21.9±0.44
<i>Pseudomonas aeruginosa</i>	13.2±1.05	14.1±1.05	18.3±0.28	07.3±0.21	25.2±0.08	26.2±1.53
<i>Haemophilus influenza</i>	18.6±0.03	17.2±0.09	19.6±1.04	14.6±1.05	24.2±1.55	23.4±0.36
<i>Marginella morgani</i>	20.1±0.19	22.1±0.13	19.4±0.29	09.3±0.02	25.1±0.08	27.7±0.11
<i>Klebsiella pneumonia</i>	14.7±0.08	17.7±0.18	15.2±1.08	04.3±1.29	19.8±0.11	20.1±0.38
Fungal strains						
Leaf						
Tested microorganisms	Acetone	Methanol	Ethanol	Water	Essential oil	Standard
Diameter of inhibition zone (mm)						
<i>Aspergillus niger</i>	19.2±0.15	20.6±1.27	18.2±0.14	16.0±0.13	22.1±0.23	20.3±0.16
<i>Rhizoctonia solani</i>	22.4±8.03	24.1±0.09	28.1±0.34	14.2±0.17	25.0±0.13	23.8±4.71
<i>Aspergillus alternate</i>	25.7±0.42	26.6±0.22	26.5±0.37	15.5±0.12	28.2±0.06	27.2±0.55
<i>Aspergillus flavus</i>	27.1±7.74	23.2±1.58	26.2±1.00	14.3±0.27	29.2±0.63	13.5±1.52

potential antioxidant and anti-inflammatory activities. In agreement to this, Das et al. (18) further verified that 7-dihydroxy-2-phenylchroman-4-one 5,7-dihydroxy-2-(4-hydroxyphenyl)-4 H-chromen-4-one are flavonoid analogs having antioxidant activities. Several studies have demonstrated that the antioxidant potentiality of essential oils depends on the presence of phenolic compounds (10). The essential oil derived from *A. javanica* was recorded to be rich in flavonoids or phenolic constituents. Therefore it presented good antioxidant activity. Moreover, insignificant RBCs lysis ranging from 5.82-14.47 suggests that the plant extract and essential oil from *A. javanica* may be used as herbal medicine.

In conclusion, the current report provides comprehensive data on the antibacterial, antifungal, and antioxidant activities of the extracts and essential oil obtained from *A. javanica*. The essential oil displayed comparatively potent antimicrobial and antioxidant activities than the solvent extracts due to the presence of flavonoid derivatives and other phytoconstituents. The cytotoxicity analysis of plant extracts, and essential oil through examining

hemolytic activity revealed minor cytotoxicity concerning the positive control.

ACKNOWLEDGEMENTS

The authors are grateful to the School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai-200240, China for providing experimental facilities and financial support. The technical and analytical help extended by the Instrumental Analysis Center of SJTU is also thankfully acknowledged.

REFERENCES

1. Ammer MR, Zaman S, Khalid M, Bilal M, Erum S, Huang D, Shengquan C. Optimization of antibacterial activity of Eucalyptus tereticornis leaf extracts against *Escherichia coli* through response surface methodology. J Radiat Res Appl Sci 2016; 9(4):376-85.
2. Bilal M, Rasheed T, Iqbal HMN, Hu H, Zhang X. Silver nanoparticles: biosynthesis and antimicrobial potentialities. Int J Pharmacol 2017; 13(7):832-45.
3. Ahmad I, Beg AZ. Antimicrobial and phytochemical

- studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. *J Ethnopharmacol* 2001; 74(2):113-23.
4. Bilal M, Rasheed T, Iqbal HMN, Li C, Hu H, Zhang X. Development of silver nanoparticles loaded chitosan-alginate constructs with biomedical potentialities. *Int J Biol Macromol* 2017; In-Press, DOI: 10.1016/j.ijbiomac.2017.07.047.
 5. Rasheed T, Bilal M, Iqbal HMN, Li C. Green biosynthesis of silver nanoparticles using leaves extract of *Artemisia vulgaris* and their potential biomedical applications. *Colloids Surf B Biointerfaces* 2017; 158:408-15.
 6. Vetrichelvan T, Jegadeesan M, Palaniappan MS, Murali N, Sasikumar K. Diuretic and antiinflammatory activities of *aerva lanata* in rats. *Indian J Pharm Sci* 2000; 62(4):300-302.
 7. Hassani D, Liu H, Chen Y, Wan Z, Zhuge Q, Li S. Analysis of biochemical compounds and differentially expressed genes of the anthocyanin biosynthetic pathway in variegated peach flowers. *Genet Mol Res* 2015; 14(4):13425-36.
 8. Mishra MP, Padhy RN. In vitro antibacterial efficacy of 21 Indian timber-yielding plants against multidrug-resistant bacteria causing urinary tract infection. *Osong Public Health Research Perspect* 2013; 4(6):347-57.
 9. Rath S, Padhy RN. Monitoring in vitro antibacterial efficacy of *Terminalia alata Heyne ex. Roth*, against MDR enteropathogenic bacteria isolated from clinical samples. *J Acute Med* 2013; 3(3):93-102.
 10. Iqbal S, Bhanger M, Anwar F. Antioxidant properties and components of some commercially available varieties of rice bran in Pakistan. *Food Chem* 2005; 93(2):265-72.
 11. Powell W, Catranis C, Maynard C. Design of self-processing antimicrobial peptides for plant protection. *Lett Appl Microbiol* 2000; 31(2):163-68.
 12. Shad AA, Asmat S, Bakht J, Jan S, Khan MA. Antimicrobial potentials and phytochemical analysis of desert cotton (*A. Javanica*) and flax (*L. Ustitatissimum*). *Pak J Pharm Sci* 2016; 29(3):861-68.
 13. Casero C, Machín FI, Méndez-Álvarez S, et al. Structure and antimicrobial activity of phloroglucinol derivatives from achyrocline satureioides. *J Nat Prod* 2014; 78(1):93-102.
 14. Arredondo M, Blasina F, Echeverry C, et al. Cytoprotection by *Achyrocline satureioides* (Lam) DC and some of its main flavonoids against oxidative stress. *J Ethnopharmacol* 2004; 91(1):13-20.
 15. Hanif MA, Al-Maskari MY, Al-Maskari A, Al-Shukaili A, Al-Maskari AY, Al-Sabahi JN. Essential oil composition, antimicrobial and antioxidant activities of unexplored Omani basil. *J Med Plants Res* 2011; 5(5):751-57.
 16. Ud-Daula AS, Demirci F, Salim KA, Demirci B, Lim LBL, Baser KHC, Ahmad N. Chemical composition, antioxidant and antimicrobial activities of essential oils from leaves, aerial stems, basal stems, and rhizomes of *Etlingera fimbriobracteata* (K. Schum.) RM Sm. *Ind Crops Prod* 2016; 84:189-98.
 17. Legault J, Perron T, Mshvildadze V, et al. Antioxidant and anti-inflammatory activities of quercetin 7-O- β -D-glucopyranoside from the leaves of *Brasenia schreberi*. *J Med Food* 2011; 14(10):1127-34.
 18. Das S, Mitra I, Batuta S, Alam MN, Roy K, Begum NA. Design, synthesis and exploring the quantitative structure-activity relationship of some antioxidant flavonoid analogues. *Bioorg Med Chem Lett* 2014; 24(21):5050-54.

LETTER TO THE EDITOR

ANALYSIS OF SPECIALIZED NURSING ON RESPIRATORY FUNCTIONS
IN THORACOTOMY PATIENTSM. SHU¹, BY. WANG², J. ZHANG³, CY. GUO⁴ and XH. WANG⁵

¹Orthopedic Surgery Section 4, the 2nd Affiliated of Harbin Medical University, Harbin, China;
²The Operation Room, the 2nd Affiliated of Harbin Medical University, Harbin, China; ³General
 Surgery Section 7, the 2nd Affiliated of Harbin Medical University, Harbin, China; ⁴Chest Surgery
 Section, the 2nd Affiliated of Harbin Medical University, Harbin, China; ⁵Intensive Care Unit, the
 2nd Affiliated Hospital of Harbin Medical University, Harbin, China

Received June 1, 2017 - Accepted August 25, 2017

To analyze the nursing effect on the respiratory function of thoracotomy patients, sixty thoracotomy hospitalized patients were studied. The subjects were divided into a normal group (A) and an observation group (B). The patients in group A received routine nursing only, while those in group B received chest physiotherapy as well as routine nursing. Afterwards, the respiratory function indicators of the two groups were compared and a data analysis was performed. The results showed that the partial pressure of oxygen (PO₂) value of the patients in group B was greater than that of the patients in group A while the partial pressure of carbon dioxide (PCO₂) value in group B was smaller than that in group A, and there was a significant difference between the two groups ($p < 0.05$). The vital capacity under normal circumstances and forced breathing of group B were greater than that of group A and the difference was statistically significant ($p < 0.05$). The incidence of complications (atelectasis, respiratory infections, pleural effusion) was statistically significant between the two groups ($p < 0.05$). The degree of autonomic respiratory dysfunction in group B was lower than that in group A, and there was a significant difference ($p < 0.05$), suggesting that the respiratory function in patients receiving chest physiotherapy improved significantly.

To the Editor,

Major thoracic surgery can cause oppression on the patient's lungs, which may greatly influence breathing (1). Moreover, thoracic surgery is very likely to cause great harm to the chest of patients (2). Qiao W et al. (3) applied the three-dimensional thoracoscopic system to thoracic surgery to provide a better visual effect and increase. During the operation, the body's water balance and lymphatic balance were destroyed and increased lung injury (4). Due to the pain caused by surgical incision

after thoracic surgery, most patients chose thoracic breathing, which caused the accumulation of residual air deep in the lungs, leading to adhesions, aging of the lung and atelectasis (5). Hence, proper care is particularly important. Fagundes et al. (6) used the MD Anderson symptom measurement table and Kaplan-Meier analysis to test the effect of care on symptoms of fatigue, pain and shortness of breath of thoracic surgery patients and found that the postoperative recovery phase was an effective strategy for assessing perioperative care. Park et al.

Key words: major thoracic surgery, respiratory function, recovery, nursing, efficacy

Mailing address:

Dr Xiaohui Wang
 No. 246, Xuefu Road,
 Nangang District,
 Harbin City, 150086,
 Heilongjiang Province, China
 e-mail: wangxiao_hui321@sina.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
**DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF
 INTEREST RELEVANT TO THIS ARTICLE.**

(7) performed video-assisted thoracic surgery and thoracic incision surgery on early stage non-small cell lung cancer patients, compared the patient indexes of lung capacity, loss of lung function and pain, and found that there was no statistical difference in these indexes between patients ($P>0.05$). In this paper, different clinical care was performed on critically ill patients after thoracic surgery, and PO_2 and PCO_2 , vital capacity, complications and subjective respiratory dysfunction were analyzed. The results showed that respiratory function recovery care was very necessary for the patients and should be strengthened.

MATERIALS AND METHODS

Experimental subjects

Sixty thoracic surgical patients were selected from those treated in our hospital from December, 2016 to May, 2017 as the research subjects, which were then randomly divided into group A and group B, with 30

patients in each group. As shown in Table I, there was no statistical difference in the basic information between the two groups ($p>0.05$). All the patients and their families were informed of the experimental procedures and signed informed consents.

Experimental method

After surgery, the nursing started in both groups, as follows:

Group A: basic routine care, including monitoring of vital signs, nursing care and routine attention to drainage tubes (8).

Group B: in addition to the basic routine care, chest physiotherapy was carried out.

Respiratory tract nursing

After surgery, the patients lay flat, with their heads turned to one side to prevent the secretion of liquid into the respiratory system. After awaking from anesthesia, the patients took a half sitting position to relieve abdominal muscle tension and pain.

Table I. Basic information of patients.

Group	Age	Gender (male: female)	Height	Weight	Diagnostic results (lung cancer: esophageal cancer: chest trauma: other)	Pain level
Group A	42.5±18.5	16:14	1.68±2.12	64.17±16.24	13:8:5:4	Level 4
Group B	44.3±17.7	17:13	1.71±2.14	65.22±15.31	15:10:4:1	Level 4

Table II. Oxygen saturation.

Group		Day 1	Day 3	Day 5	Day 7
Arterial PO_2 (mmHg)	Group A	78.29±5.14	79.12±6.06	79.21±6.16	78.42±4.75
	Group B	85.79±5.89*	85.36±6.41*	85.11±6.98*	85.06±7.04*
Arterial PCO_2 (mmHg)	Group A	42.69±4.18	42.31±4.54	42.16±4.85	41.12±5.16
	Group B	39.95±4.99*	39.87±4.96*	39.53±4.28*	39.46±3.91*

* $p < 0.05$ compared to group A

Oxygen and expectoration care

In order to enable the patients to restore breathing after surgery, proper amount of oxygen was given; when the patients had foreign bodies in the mouth, they were cleaned timely to avoid respiratory obstruction; for the effective expectoration of the patients, their respiratory mucosa was wetted, accompanied with deep breath guidance. If it failed, then atomization inhalation was adopted (9).

Closed thoracic drainage care

This care required proficiency. Firstly, the thoracic drainage tube was fixed with adhesive tape. Then, whether the drainage was smooth at each interface was checked. Afterwards, the color of the drainage implant was recorded. The liquid in the drainage bottle was replaced daily.

Respiratory function training

First is the care of the respiratory system. Respiratory examination should be performed on patients with poor breathing caused by chest trauma. Then, drug treatment is required to reduce the permeability of capillaries. Abdominal breathing was guided for the patients, as shown below.

By relaxing the abdominal muscles, the patients were asked to inhale through the nose, hold their breath and then exhale three seconds later. This process was performed two times a day, 10 min each time, which is beneficial to improve lung ventilation and oxygen saturation. Inspiratory resistance devices can also be used to train the patients' respiratory functions and promote their respiratory muscle strength and endurance recovery (10).

Secondly whole body exercise was carried out. By

doing rehabilitation gymnastics, the patients' physical strength and pulmonary function levels can be improved effectively (11).

Pain care

Psychological counseling was carried out to encourage the patients to maintain a positive and optimistic mood; patients' attention was transferred by talking and listening to music.

The patients' condition was monitored, and when the VAS pain score was above 7 points, aspirin was given. One week later, the PO_2 and PCO_2 values, vital capacity and complications (atelectasis, respiratory infections, pleural effusion), and subjective respiratory dysfunction of the two groups were compared. Venous blood was taken and a blood gas analyzer was applied to detect PO_2 and PCO_2 values. The results showed that the smaller the PO_2 value, the more serious the respiratory failure; the greater the PO_2 value, the more difficult it was for the patient to breathe (12). Evaluation of subjective dysfunctional breathing disorders was realized through judging whether shortness of breath happened based on VAS pain score, with 5 levels of 0-5, as follows: 0: with different degrees of emphysema, patients could still live normally; 1: shortness of breath during intense exercise or high-intensity labor; 2: shortness of breath quickly going up stairs; 3: shortness of breath when walking slowly; 4: shortness of breath when talking and dressing; 5: unable to lie down and shortness of breath all the time even when not moving (13).

Data statistics

The statistical method used in this experiment is

Table III. Comparison of lung capacity.

Group		Day 1	Day 3	Day 5	Day 7
Vital capacity L	Group A	2.48±0.25	2.54±0.14	2.61±0.37	2.68±0.54
	Group B	2.96±0.78*	3.08±0.96*	3.14±0.65*	3.19±0.38*
Vital capacity of forced breathing (L)	Group A	2.55±0.28	2.63±0.27	2.75±0.24	2.88±0.21
	Group B	3.09±0.93*	3.17±0.87*	3.19±0.52*	3.23±0.41*

* $p < 0.05$ compared to group A.

SPSS17.0, with measurement data expressed by $\bar{x} \pm a$ and data comparison by χ^2 . $P < 0.05$ was used for statistical differences between the two groups.

RESULTS

Oxygen partial pressure and carbon dioxide partial pressure

As shown in Table II, there was a significant difference in arterial oxygen pressure between group A and group B in the same time period ($p < 0.05$). However, there was no significant difference in arterial carbon dioxide partial pressure between the two groups at any time point ($p > 0.05$), suggesting that the effect was superior in arterial oxygen partial pressure than in arterial blood carbon dioxide partial pressure.

Lung function index

As shown in Table III, with the increase of nursing time, there was no significant difference in the vital capacity of group A. At the same time point, the lung capacity of group B was significantly different from that of group A ($p < 0.05$). There was a significant difference in vital capacity during forced breathing between the two groups ($p < 0.05$), indicating that the vital capacity

would increase with the extension of treatment time under normal circumstances, and respiratory care contributed to a further increase in vital capacity.

Incidence of complications

The main complications were atelectasis, respiratory infection and pleural effusion. In group A, there were 2 cases of atelectasis, 2 cases of respiratory infection and 3 cases of pleural effusion, with an incidence of complications of about 23.3%. In group B, there were 2 cases of atelectasis and 1 case of respiratory infection, with an incidence of complications of about 10%. Hence, the incidence of complications was statistically significant between the two groups ($p < 0.05$).

Subjective respiratory dysfunction

As shown in Fig. 1, the overall degree of autonomic respiratory dysfunction in group B was lower than that in group A and was significantly different between the two groups on the third, fifth and seventh day ($p < 0.05$). However, no difference was found on the first day between the two groups ($p > 0.05$), suggesting that the autonomous breathing ability of patients in group B who received respiratory function recovery care was higher than that of patients in group A.

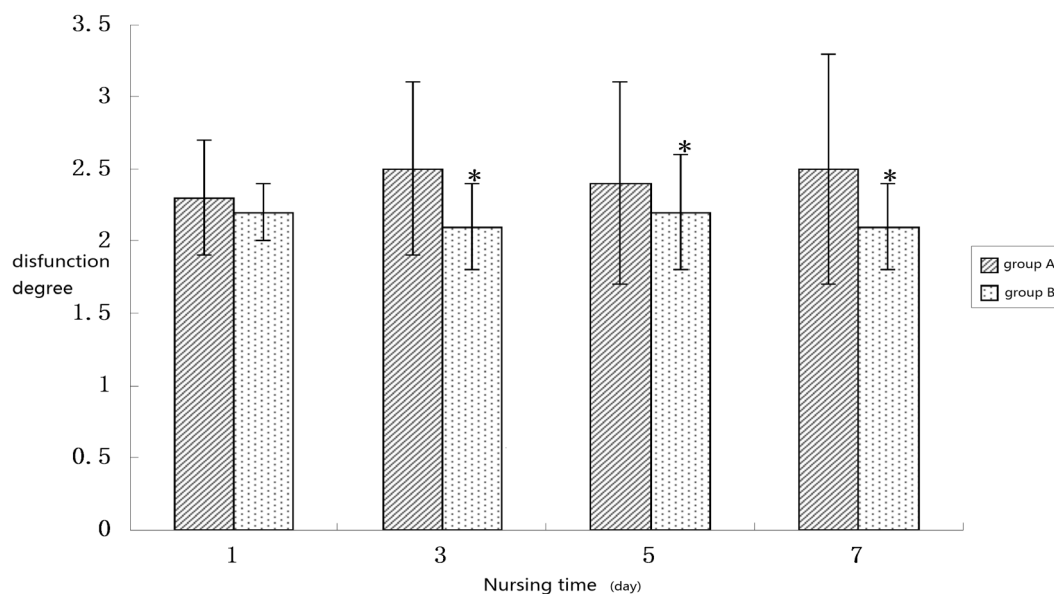


Fig. 1. Comparison of the degree of respiratory dysfunction. * $p < 0.05$ compared to group A.

DISCUSSION

In recent years, although thoracic surgery success rates have increased with the progress of the level of science and technology, the injury to patients undergoing surgery is unavoidable. Moreover, tracheal intubation can cause complex secretions to remain in the respiratory system which obstruct breathing and can result in complications if not cleaned timely.

Kao et al. (14) studied anesthesia and analgesia in thoracic surgery, suggesting that anesthesia also causes atelectasis as well as progressive hypoxia, hypercapnia, and hypotension. Sasaki N et al. (15) found that the correct use of respiratory inhibitors and neuromuscular blockers can have a role in the recovery of respiratory function. In the present paper, the values of the oxygen saturation, pulmonary function, complications, and subjective respiratory dysfunction of the patients receiving regular care and the patients receiving chest physiotherapy were used to study the effect of recovery of respiratory function. The results showed that the value of oxygen partial pressure in patients receiving chest physiotherapy was significantly higher than that in the routine nursing group, and its partial pressure of carbon dioxide was significantly lower than that of the routine nursing group ($p < 0.05$). Furthermore, the vital capacity of the chest physiotherapy group was also higher than that of the conventional nursing group, suggesting that the breathing of the patients receiving chest physiotherapy was better than that of the patients receiving regular care. Vital capacity under normal breathing and vital capacity under forced breathing are important manifestation of the therapeutic effects on patients after major thoracic surgery and the results obtained in this study were consistent with those of Ercegovic et al. (16). The number of complications and the number of patients with complications were higher in the routine care group than in the chest physiotherapy group. The degree of autonomic respiratory dysfunction in patients receiving chest physiotherapy was lower than that in the routine care group, indicating that chest physiotherapy has a considerable role in promoting respiratory function recovery in patients.

In summary, the patients receiving chest physiotherapy had better respiratory function recovery and the restoration nursing method is worthy of being used in the recovery of respiratory function and has good development prospects.

REFERENCES

1. Shibasaki T, Asano H, Maruzima H, Yamashita M, Odaka M, Morikawa T. Development of video-assisted thoracic surgery by the Department of Thoracic Surgery, The Jikei University School of Medicine, and treatment outcomes. *J Tokyo Felix Med Univ* 2014; 129:220-20.
2. Foroulis C, Kleontas A, Tossios P, Anastasiadis K. Early reoperation for the management of complications in patients undergoing general thoracic surgical procedures in an academic cardiothoracic department. *J Thoracic Dis* 201; 6(suppl 1):S21-S31.
3. Qiao W, Zhou J, Liu F, Al E. Application of three-dimensional thoracoscope system in minimally invasive thoracic surgery. *Chin J Cancer* 2015; (04):305-10.
4. Şen S, Şen S, Şentürk E, Kuman NK. Postresectional lung injury in thoracic surgery pre and intraoperative risk factors: a retrospective clinical study of a hundred forty-three cases. *J Cardiothorac Surg* 2010; 5(1):62.
5. He YD, Li MJ, Wang GX. Respiratory management and lung physical therapy in patients undergoing thoracic surgery. *J Zunyi Med Coll* 2008; 31(04):423-25.
6. Fagundes CP, Shi QL, Vaporciyan AA, Rice DC, Popat KU, Cleeland CS, Wang XS. Symptom recovery after thoracic surgery: measuring patient-reported outcomes with the MD Anderson Symptom Inventory. *J Thorac Cardiovasc Sur* 2015; 150(3):613-19.
7. Park TY, Park YS. Long-term respiratory function recovery in patients with stage I lung cancer receiving video-assisted thoracic surgery versus thoracotomy. *J Thorac Dis* 2016; 8(1):161-68.
8. Khatiban M, Shirani F, Oshvandi K, Soltanian A, Ebrahimiyan R. Effect of supportive-educative nursing system on self-care skills in trauma patients with chest drainage system. *Hayat* 2014; 20(1):48-58.
9. Cai XL, Dong MN, Lv HX, Tang XY, Wu

- WN. Research on contamination situation of oxygen humidification bottles with and without humidification fluid and oxygen inhalation effect for patients. *Nurs Rehab J* 2013; 12(12):1122-23.
10. Eikermann M, Kurth T. Apply protective mechanical ventilation in the operating room in an individualized approach to perioperative respiratory care. *Anesthesiology* 2015; 123(1):12-14.
 11. Cho SH, Lee JH, Jang SH. Efficacy of pulmonary rehabilitation using cervical range of motion exercise in stroke patients with tracheostomy tubes. *J Phys Ther Sci* 2015; 27(5):1329-31.
 12. Jensen D, Webb KA, O'Donnell DE. The increased ventilatory response to exercise in pregnancy reflects alterations in the respiratory control systems ventilatory recruitment threshold for CO₂. *Resp Physiol Neurobi* 2010; 171(2):75-82.
 13. Sheean PM, Peterson SJ, Gomez PS, et al. The prevalence of sarcopenia in patients with respiratory failure classified as normally nourished using computed tomography and subjective global assessment. *Jpen-Parenter Enter* 2014; 38(7):873-79.
 14. Kao MC, Lan CH, Huang CJ. Anesthesia for awake video-assisted thoracic surgery. *Acta Anaesthesiol Taiwan* 2012; 50(3):126-30.
 15. Sasaki N, Meyer MJ, Eikermann M. Postoperative respiratory muscle dysfunction: pathophysiology and preventive strategies. *Anesthesiology* 2013; 118(4):961-78.
 16. Ercegovac M, Subotic D, Zugic V, Jakovic R, Moskovljevic D, Bascarevic S, Mujovic N. Posttct complications do not affect the pattern of early lung function recovery after lung resection for lung cancer in patients at risk. *J Cardiothorac Surg* 2014; 9:92.

LETTER TO THE EDITOR

INHIBITION OF PAR2 AND TRPA1 SIGNALS ALLEVIATES NEUROPATHIC PAIN
EVOKED BY CHEMOTHERAPEUTIC BORTEZOMIBQ. WANG¹, J. WANG¹, D. GAO² and J. LI³

¹Tumor Center, The First Hospital of Jilin University, Changchun, Jilin, China; ²Department of Otorhinolaryngology, The First Hospital of Jilin University, Changchun, Jilin, China; ³Department of Radiology, The First Hospital of Jilin University, Changchun, Jilin, China

Received august 14, 2017 – Accepted September 18, 2017

The first two authors contributed equally to this work

Bortezomib (BTZ) is generally used as a chemotherapeutic agent for the treatment of multiple myeloma; however, one of the significant limiting complications of BTZ is painful peripheral neuropathy observed during BTZ therapy. There is a lack of drugs which can prevent and/or treat the painful symptoms induced by BTZ, as the underlying molecular mechanism leading to neuropathic pain remains largely unclear. In the present study, we examined engagement of proteinase-activated receptor 2 (PAR2) and transient receptor potential ankyrin 1 (TRPA1) in neuropathic pain induced by BTZ in rats. Our results demonstrated that systemic injection of BTZ increased mechanical pain and cold sensitivity as compared with control animals ($P < 0.05$ vs control rats). Our data further showed that blocking respective PAR2 and TRPA1 attenuated mechanical pain and cold sensitivity observed in control rats and BTZ rats ($P < 0.05$ vs vehicle control). Notably, the attenuating effect of blocking PAR2 and TRPA1 on mechanical pain and cold sensitivity was significantly less in BTZ rats than that in control rats. In addition, protein expression of PAR2 and TRPA1 was upregulated in the lumbar dorsal root ganglion of BTZ rats, and inhibition of PAR2 decreased the levels of TRPA1 and attenuated its downstream pathways (namely, PKC ϵ and PKA). Overall, we revealed specific signaling pathways leading to neuropathic pain induced by chemotherapeutic BTZ and that blocking PAR2 and TRPA1 in sensory nerves is beneficial to improve neuropathic pain during BTZ intervention.

To the Editor,

Bortezomib (BTZ), a reversible inhibitor of the proteasome complex, is used primarily as a chemotherapeutic agent for the treatment of multiple myeloma (1). However, clinical and animal studies have shown that dose-limiting painful peripheral neuropathy is one of the significant adverse events which are observed during BTZ therapy (2). Treatment

options for these abnormal sensations have been restricted, partly due to a poor understanding of the underlying mechanisms responsible for neuropathic pain induced by chemotherapeutic agents such as BTZ. As yet no drug can better prevent and/or treat the painful symptoms evoked by BTZ therapy.

Proteinase-activated receptors (PARs) are a family member of G-protein-coupled receptors

Key words: bortezomib, neuropathic pain, PAR2, TRPA1

Mailing address:

Dr Dongmei Gao,
Department of Otorhinolaryngology,
Department of Radiology,
The First Hospital of Jilin University,
71 Xinmin Street, Changchun, Jilin 130021, China
e-mail: gaodongmei64@aol.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
**DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF
INTEREST RELEVANT TO THIS ARTICLE.**

and are activated by a proteolytic mechanism (3). Among the four members of PARs, subtype PAR2 is largely distributed in various tissues including neural systems (4) and dorsal root ganglion (DRG) neurons at the L4-6 levels contain PAR2 (4). Stimulation of PAR2 by peripheral or central administration of non-inflammatory doses of PAR2 agonists evokes mechanical and thermal hyperalgesia in rodents (5) and contribute to neuropathic pain (6).

Transient receptor potential ankyrin 1 (TRPA1) plays a functional role in regulating pain and neurogenic inflammation resulting from channel activation to a variety of compounds including pungent agents, irritant chemicals, reactive oxygen, and products of oxidative stress-induced lipid peroxidation (7). TRPA1 appears in DRG neurons and is engaged in development of mechanical hypersensitivity and painfully cold temperature (8). TRPA1 also mediates mechanical hyperalgesia and cold hypersensitivity induced by chemotherapeutic agents (9). In addition, PAR2 plays an important role in regulating TRPA1 functions in evoking neuropathic pain because blocking PAR2 can attenuate the protein expression of TRPA1 in the DRG in engagement of mechanical and thermal hyperalgesia (10).

Thus, we postulated that PAR2 and TRPA1 signals in sensory nerves play a role in regulating neuropathic pain in rats during BTZ intervention. We hypothesized that BTZ amplifies expression of PAR2 and TRPA1 in the DRG, thereby resulting in mechanical hyperalgesia and cold hypersensitivity. Blocking these receptors attenuates neuropathic pain in BTZ rats. We further hypothesized that BTZ increases PAR2 in the DRG, which subsequently activate TRPA1 via protein kinases A and C (PKC ϵ and PKA), resulting in neuropathic pain. Blocking PAR2 by systemic injection of FSLLRY-NH₂, an antagonist to PAR2, would attenuate expression of PKC ϵ /PKA and TRPA1 and thereby alleviate BTZ-induced hyperalgesia.

MATERIALS AND METHODS

Animals

A total of 148 Wistar rats were used in this study. The

rats were divided into a control group of 72 animals and a BTZ group of 76 animals. All animal protocols were in accordance with the guidelines of the International Association for the Study of Pain and approved by the Institutional Animal Care and Use Committee of Jilin University. Both genders of rats (200-250 g) were housed in individual cages with free access to food and water and were kept in a temperature-controlled room (25°C) on a 12/12 h light/dark cycle.

A model of neuropathic pain and administration of drugs

BTZ (0.4 mg/kg body weight; Haoran BioTech Co., Shanghai, China) was administrated intraperitoneally (i.p.) once daily for 5 consecutive days. Control animals received an equivalent volume of saline. After BTZ intervention, PAR2 antagonist FSLLRY-NH₂ (0.01, 0.1, 1 mg/kg; Tocris Biosci) was administrated i.p. each day for the following 3 consecutive days. In the same way, TRPA1 antagonist HC030031 (1, 3, 10 mg/kg body weight; Sigma) was also given i.p. each day for 3 consecutive days.

Behavioral test

To quantify the mechanical sensitivity of the hindpaw, rats were placed in individual plastic boxes and allowed to acclimatise for > 30 min. Mechanical paw withdrawal threshold (PWT) of rat hindpaw in response to the stimulation of von Frey filaments was determined. A series of calibrated von Frey filaments (ranging from 0.5 to 18.0 g) were applied perpendicularly to the plantar surface of the hindpaw with a sufficient force to bend the filaments for 60s or until the paw withdrew. In the presence of a response, the filament of the next lower force was applied. In the absence of a response, the filament of the next greater force was applied. To avoid injury during tests, the cutoff strength of the von Frey filament was 18 g. The tactile stimulus producing a 50% likelihood of withdrawal was determined using the "up-down" method (11). Each trial was repeated 2 times at approximately 2 min intervals. The mean value was used as the force produced a withdrawal response.

To examine cold sensitivity, Thermal Place Preference System (Coulburn Instruments) was used to perform the thermal place preference test in order to assess a cold avoidance behavior. Two connecting metal plates were surrounded by a plastic enclosure. The first plate was kept at neutral temperature (25°C) and the second plate was

kept at cold temperature (12°C). The test was performed in darkness and each session lasted 3 min. During the session, the rats were left free to explore both plates. The time spent on the cold plate during the entire session was recorded using an infrared camera connected to a computer to determine cold avoidance behavior. To better control behavior test, the rats were repeatedly placed on the apparatus with both plates held at room temperature (25°C) for 3 min 2 days before the beginning of the experiment. The rats spent an equal amount of time on each plate under these conditions, suggesting that the animals showed no place preference. Also, to avoid learning or any place preference unrelated to cold, the temperature of the plates were inverted between two consecutive sessions. Two trials were performed for each dosage and data were averaged. All behavioral tests were performed in a blind fashion.

Western blot analysis

All the tissues from individual rats were sampled for the analysis. In brief, DRG tissues (L4-L6) of the rats were removed. Total protein was then extracted by homogenizing the sample in ice-cold immunoprecipitation assay buffer with protease inhibitor cocktail kit. The lysates were centrifuged and the supernatants were collected for measurements of protein concentrations using a bicinchoninic acid assay reagent kit.

After being denatured, the supernatant samples containing 20 µg of protein were loaded onto gels and electrically transferred to a polyvinylidene fluoride membrane. The membrane was incubated with primary antibodies (at 1:500, Abcam): rabbit anti-PAR2, anti-TRPA1, anti-PKCε and anti-PKA. The membranes were washed and incubated with an alkaline phosphatase conjugated anti-rabbit secondary antibody (1:1000). The immunoreactive proteins were detected by enhanced chemiluminescence. The bands recognized by the primary antibody were visualized by exposure of the membrane onto an X-ray film. The membrane was stripped and incubated with anti-β-actin to show equal loading of the protein. The film was then scanned and the optical density of PAR2, TRPA1, PKCε, PKA and β-actin bands was analyzed using Scion Image software (National Institute of Health, USA).

Statistical analysis

All data were analyzed using a two-way repeated-

measures analysis of variance. Values were presented as means ± standard deviation of mean. For all analyses, differences were considered significant at $P < 0.05$. All statistical analyses were performed by using SPSS for Windows version 13.0 (SPSS Inc.).

RESULTS

BTZ injection significantly decreased PWT as compared with saline control. PWT was 8.5 ± 0.9 g in control rats and 4.5 ± 0.8 g in BTZ rats ($P < 0.05$ vs control rats). Fig. 1 shows that PWT was increased in a dose-dependent way after injection of FSLLRY-NH2 (0.01, 0.1, 1 mg/kg) in control rats and BTZ rats. As 1 mg/kg of FSLLRY-NH2 was given, the effects were observed 1 day after its administration, and peaked 3 days after injection. The effects began to reduce after injection was discontinued. In addition, the percentage increase of PWT evoked by FSLLRY-NH2 was smaller in BTZ rats than that in control rats. i.e. as 1 mg/kg of FSLLRY-NH2 was given, PWT was increased by 48% in BTZ rats ($P < 0.05$, control rats/ $n=10$ vs BTZ rats/ $n=12$) and 76% in control rats 3 days after injection of FSLLRY-NH2.

Fig. 1 also shows that BTZ injection significantly diminished % time spent on the cold plate as compared with saline injection. The percentage time spend was $47 \pm 9\%$ in control rats and $33 \pm 7\%$ in BTZ rats ($P < 0.05$ vs controls). The injection of FSLLRY-NH2 (1 mg/kg) significantly attenuated cold sensitivity to a greater degree in control rats (% time increased by 86%, $n=10$) than in BTZ rats (% time increased by 51%, $n=12$, $P < 0.05$ vs control rats) (Fig. 1).

We further examined the role played by inhibition of TRPA1 in mediating mechanical and cold sensitivity. Injection of HC030031 (1, 3, 10 mg/kg) attenuated mechanical sensitivity in control rats and BTZ rats in a dose-dependent way (Fig. 2). As 10 mg/kg of HC030031 was given, the effects were observed 1 day after its administration, and peaked at 3 days after injection. The effects began to reduce after injection was discontinued. In addition, the percentage increase of PWT evoked by HC030031 was smaller in BTZ rats than in control rats. i.e. as 10 mg/kg of HC030031 was given, PWT was

increased by 52% in BTZ rats ($P < 0.05$, control rats/ $n=12$ vs BTZ rats/ $n=12$) and 75% in control rats 3 days after injection of HC030031. Likewise, the inhibitory effects of HC030031 on cold sensitivity were smaller in BTZ rats than in control rats (Fig. 2).

In additional groups, the regulatory effects of PAR2 on TRPA1 signal pathway were examined. As can be seen in Fig. 3, BTZ administration amplified the levels of PAR2 and TRPA1 in DRG as compared with control rats ($P < 0.05$, BTZ rats vs control rats, $n=6$ in control; and $n=8$ in BTZ group). BTZ also upregulated protein expression of intracellular signal PKC ϵ , PKA as compared with control rats ($P < 0.05$, BTZ rats vs control rats, $n=6-8$ in each group). Furthermore, PAR2 inhibition using FSLLRY-NH2 decreased upregulation of PKC ϵ , PKA and TRPA1

induced by BTZ ($P < 0.05$ vs BTZ without FSLLRY-NH2) (Fig. 3).

DISCUSSION

One of the most common and distressing symptoms suffered by patients with progression of cancer is pain (12). Cancer pain mainly arises from a tumor compressing or infiltrating tissue; from nerve and other changes caused by a hormone imbalance or immune response; and/or from treatments and diagnostic procedures. Chemotherapy may produce painful conditions persisting long after treatment has ended (13).

BTZ is a compound commonly used to treat multiple myeloma (1). It has a significant activity

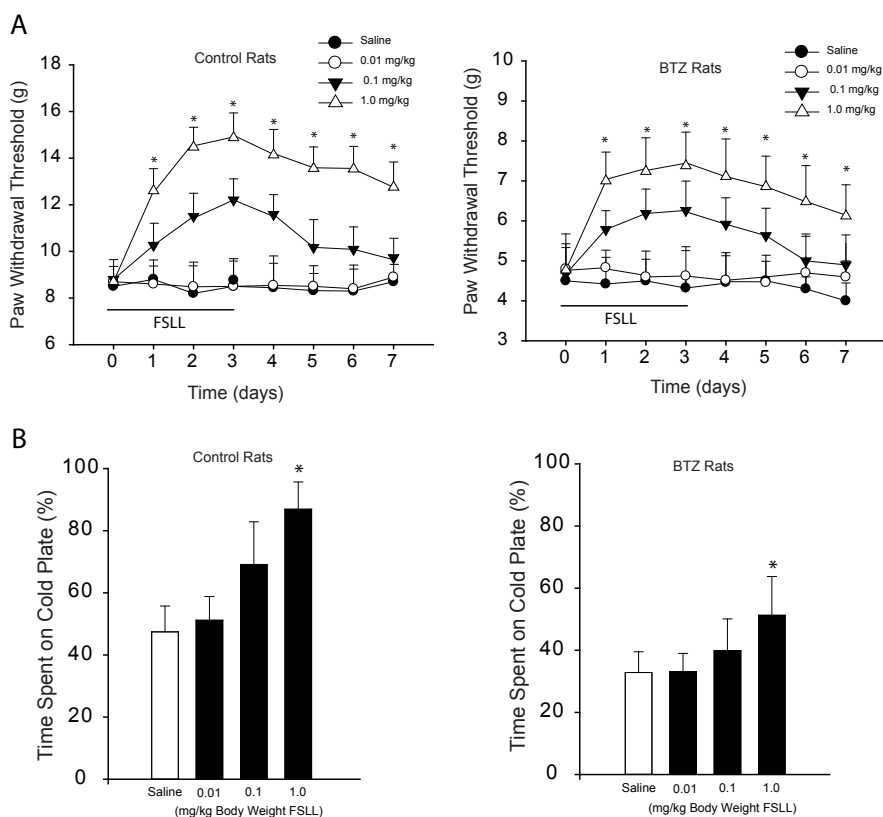


Fig. 1. Effects of blocking PAR2 by FSLLRY-NH2 (FSL) on paw withdrawal threshold (PWT) and cold sensitivity expressed as time spent on the cold plate (%) in control rats and BTZ rats. FSL increased PWT in control rats and BTZ rats as compared with saline control, but the amplitude of PWT increases evoked by FSL was smaller in BTZ rats than that in control rats (A). FSL elevated % time spent on the cold plates in control rats and BTZ rats 3 days after its injection (B). * $P < 0.05$ vs saline and other dosages. The number of rats in each group = 8-12.

against advanced and/or metastatic tumors, but painful neuropathy is a main limiting complications (2). A heightened cold sensitivity is another complication observed in cancer patients undergoing BTZ treatment (2). Prior studies have shown that BTZ can induce neuropathic pain, including mechanical hyperalgesia and cold hypersensitivity in rats after initiation of the chemotherapy regimen (14). These abnormalities were ablated several days after discontinuation of BTZ. Thus, we employed the same rat model to study the mechanisms of neuropathic pain induced by BTZ. Consistent with previous findings (14), we observed that BTZ decreased PWT and evoked less time (%) spent on the cold plate in rats after its injection. We further demonstrated that PAR2 and TRPA1 antagonists

significantly increased PWT and % time spent on the cold plate in both groups, and that the effects of blocking PAR2 and TRPA1 were smaller in BTZ rats. We also showed that BTZ amplified PAR2 and TRPA1 in the DRG. Interestingly, blocking PAR2 attenuated upregulation of TRPA1 and neuropathic pain in BTZ rats. PKC ϵ and PKA signals were engaged in the effects of PAR2. Accordingly, our data indicate that injection of BTZ activates PAR2, which next amplifies TRPA1 in DRG, likely via PKC ϵ and PKA, thereby resulting in mechanical hyperalgesia and cold hypersensitivity.

TRPA1 has a role in regulating pain and neurogenic inflammation resulting from channel activation to a variety of compounds (7, 8). Our current study suggests that TRPA1 contributes to

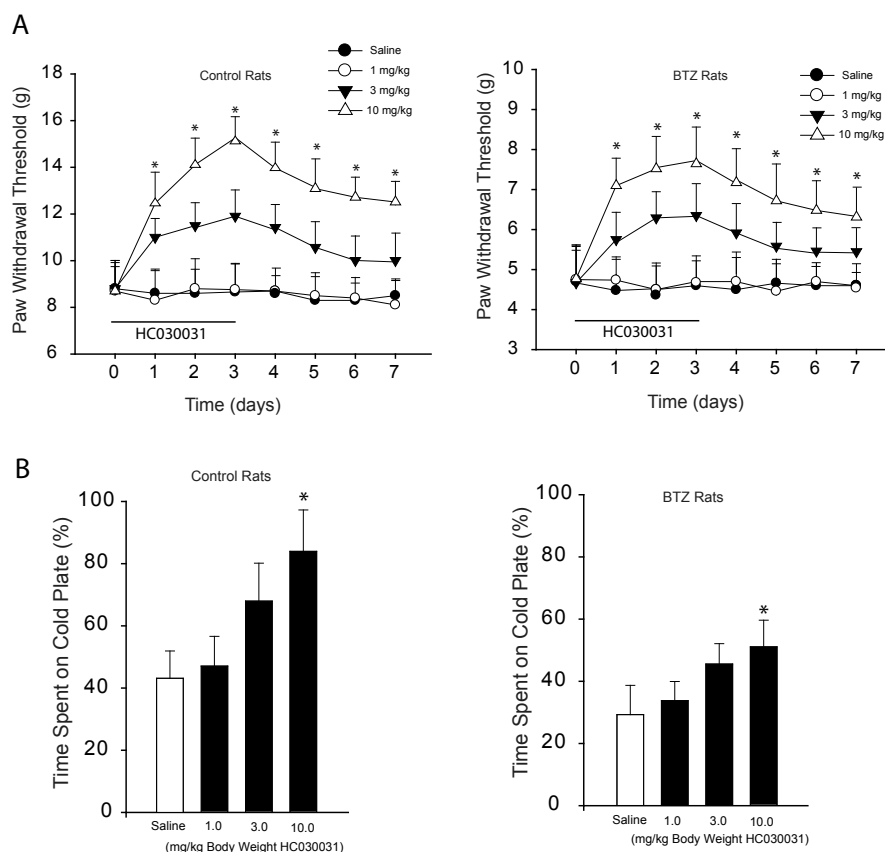


Fig. 2. Effects of blocking TRPA1 by administration of HC030031 on paw withdrawal threshold (PWT) and cold sensitivity expressed as time spent on the cold plate (%) in control rats and BTZ rats. As compared with saline injection, HC030031 increased PWT in control rats and BTZ rats (A). Note that increases of PWT evoked by attenuation of TRPA1 were smaller in BTZ rats than those in control rats (A). HC030031 elevated % time spent on the cold plates in control rats and BTZ rats 3 days after its injection (B). * $P < 0.05$ vs saline control and other dosages. The number of rats in each group = 8-12.

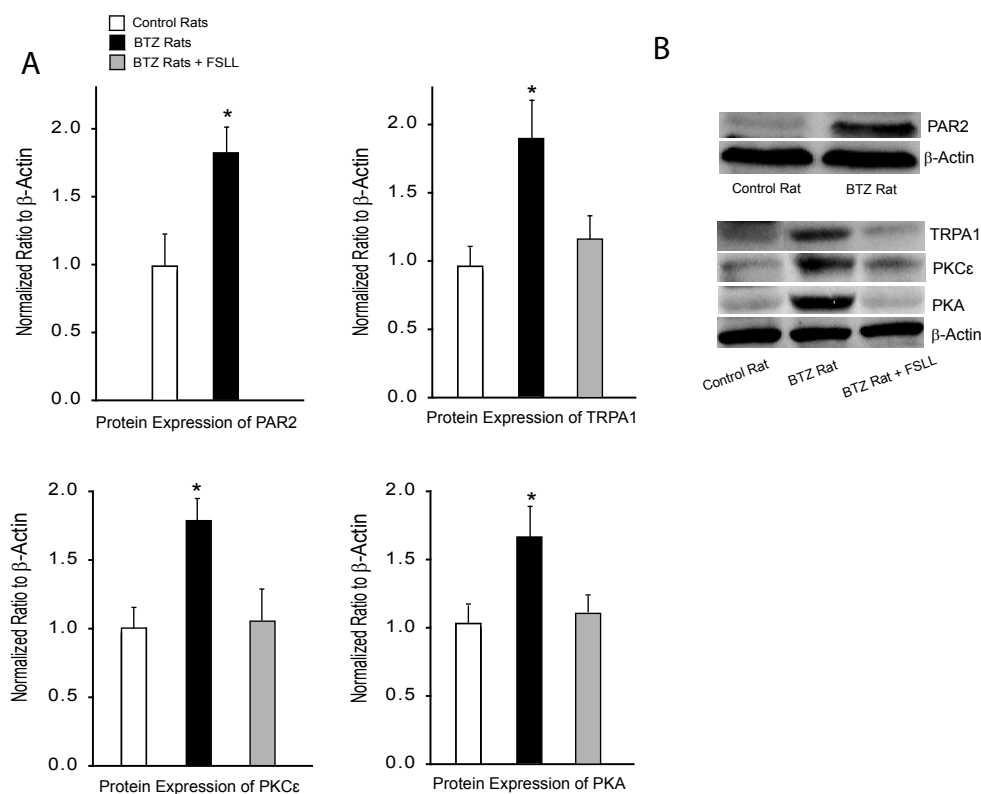


Fig. 3. *A)* The protein levels of PAR2 were amplified in the DRG tissues of BTZ rats and attenuation of PAR2 by systemic administration of FSLNRY-NH2 (FSLN) decreased expression of TRPA1, intracellular signals PKC ϵ and PKA. * $P < 0.05$, BTZ rats vs control rats and BTZ rats treated with FSLN. The number of rats in each group = 6–8. *B)* Typical bands show that BTZ increased the protein levels of PAR2 (top panel), TRPA1 and PKC ϵ /PKA (bottom panel) and FSLN inhibited amplification of TRPA1, PKC ϵ and PKA evoked by BTZ (bottom panel).

BTZ-evoked neuropathic pain and PAR2 is a signal to activate TRPA1 because inhibition of PAR2 decreases BTZ-evoked upregulation of TRPA1 and neuropathic pain. We provide the first evidence that PAR2 has effects on TRPA1 by which it mediates mechanical hyperalgesia and cold hypersensitivity induced by BTZ. TRPA1 can regulate substance P and calcitonin gene-related peptide (CGRP) CGRP in sensory nerves and/or at the spinal levels for neuropathic pain (15). We speculated that BTZ would increase substance P and CGRP in the DRG, and blocking PAR2 and TRPA1 receptors would attenuate the amplified substance P and CGRP.

In conclusion, protein expression of PAR2 and TRPA1 in sensory nerves is upregulated by injection of BTZ; inhibition of PAR2 and TRPA1 signals antagonizes mechanical hyperalgesia and cold

hypersensitivity during BTZ intervention; and PAR2 plays a role in TRPA1 engagement of BTZ-induced neuropathic pain via intracellular PKC ϵ and PKA signals.

REFERENCES

1. Adams J, Kauffman M. Development of the proteasome inhibitor Velcade (Bortezomib). *Cancer Invest* 2004; 22:304-11.
2. Curran MP, McKeage K. Bortezomib: a review of its use in patients with multiple myeloma. *Drugs* 2009; 69:859-88.
3. Cottrell GS, Amadesi S, Schmidlin F, Bunnett N. Protease-activated receptor 2: activation, signalling and function. *Biochem Soc Trans* 2003; 31:1191-97.
4. Steinhoff M, Vergnolle N, Young SH, et al. Agonists

- of proteinase-activated receptor 2 induce inflammation by a neurogenic mechanism. *Nat Med* 2000; 6:151-58.
5. Alier KA, Endicott JA, Stemkowski PL, et al. Intrathecal administration of proteinase-activated receptor-2 agonists produces hyperalgesia by exciting the cell bodies of primary sensory neurons. *J Pharmacol Exp Ther* 2008; 324:224-33.
 6. Bao Y, Hou W, Liu R, et al. PAR2-mediated upregulation of BDNF contributes to central sensitization in bone cancer pain. *Mol Pain* 2014; 10:28.
 7. Andersson DA, Gentry C, Moss S, Bevan S. Transient receptor potential A1 is a sensory receptor for multiple products of oxidative stress. *J Neurosci* 2008; 28:2485-94.
 8. Kwan KY, Allchorne AJ, Vollrath MA, Christensen AP, Zhang DS, Woolf CJ, Corey DP. TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. *Neuron* 2006; 50:277-89.
 9. Zhao M, Isami K, Nakamura S, Shirakawa H, Nakagawa T, Kaneko S. Acute cold hypersensitivity characteristically induced by oxaliplatin is caused by the enhanced responsiveness of TRPA1 in mice. *Mol Pain* 2012; 8:55.
 10. Wei H, Wei Y, Tian F, Niu T, Yi G. Blocking proteinase-activated receptor 2 alleviated neuropathic pain evoked by spinal cord injury. *Physiol Res* 2016; 65:145-53.
 11. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 1994; 53:55-63.
 12. Hanna, Magdi, Zylicz, (Ben) Z. *Cancer Pain*, Springer 2013.
 13. Portenoy RK. Treatment of cancer pain. *Lancet* 2011; 377:2236-47.
 14. Zhang J, Su YM, Li D, et al. TNF-alpha-mediated JNK activation in the dorsal root ganglion neurons contributes to Bortezomib-induced peripheral neuropathy. *Brain Behav Immun* 2014; 38:185-191.
 15. Nassini R, Materazzi S, Benemei S, Geppetti P. The TRPA1 channel in inflammatory and neuropathic pain and migraine. *Rev Physiol Biochem Pharmacol* 2014; 167:1-43.

LETTER TO THE EDITOR

DOSE-DEPENDENT ACUTE PHASE RESPONSE OF AQUEOUS LEAF DECOCTION OF *NERIUM OLEANDER* IN WISTAR RATS

M.H. ABBASI^{1,2}, S. FATIMA³, M.B. KHAWAR², N. NAZ⁴, K.A. MUJEEB⁵,
T. AKHTAR² and N. SHEIKH^{2,6}

¹Department of Zoology, Government College of Science, Lahore, Pakistan; ²Cell and Molecular Biology Laboratory, Department of Zoology, University of the Punjab, Lahore, Pakistan;
³University of Health Sciences, Lahore, Pakistan; ⁴Faculty of Life Sciences, University of Manchester, UK; ⁵Centennial College, Scarborough, Toronto, ON, Canada; ⁶Centre of Applied Molecular Biology (CAMB), University of the Punjab, Lahore, Pakistan

Received April 20, 2017 - Accepted May 22, 2017

Many studies have been carried out in order to determine the toxicity of medicinal plants. The objective of this study was to compare and analyze the hepatic response against two doses of *Nerium oleander*, (*N. oleander*) “kaner” leaf decoction. Aqueous leaf decoction was injected intramuscularly into both hind limbs of male rats (200±10g), assigned into three categories (n=4): control group with no treatment; group I, injected with 5 ml/ kg; and group II injected with 10 ml/ kg of leaf decoction, respectively. Animals were sacrificed 6 h after administration and hepato-histological changes were then observed. The decoction induced an acute phase reaction reflected by a more significant recruitment of inflammatory cells in group II than in group I and controls, as observed by histological studies. These results indicated that both doses can induce an acute-phase condition. Hence, traditional practice of medicinal plants without preliminary dose assessment must not be administered.

To the Editor,

Acute phase reaction (APR) is a wound-healing response accompanied with the release of soluble mediators, recruitment of inflammatory cell populations, changes in hemogram and level of liver derived acute-phase proteins (APPs). Body organs respond differently against APR. The changes occurring during APR are believed to guard the host from further detrimental effects during the acute stage of infection (1).

The liver has a central role in maintaining body energy homeostasis. Its damage causes activation and proliferation of a variety of inflammatory cell populations (2). Concentrations of these cells along

with changes in APPs are commonly used parameters to evaluate liver functions. Significant reduction in the level of the proteins, such as albumin, is an indication of severe liver damage, a principal organ responsible for the synthesis of various metabolites (3).

Plants have always been reputed as therapeutic remedies. *Nerium oleander* (*N. oleander*), (Apocynaceae) is one of such plants with multiple therapeutic activities (4, 5). Two toxic cardiac glycosides (cardenolides) oleanderin and neriine, have been isolated from all parts of the plant and reported as positive inotropic, negative chronotropic, cross reactive and very similar to the toxin in foxglove (*Digitalis*). Patel et al. (2010) isolated various alkaloids,

Key words: acute phase response, DAPI, ED-1, inflammatory cells, liver, *Nerium oleander* (*N. oleander*), immunofluorescence

Mailing address:

Dr Nadeem Sheikh,
Cell and Molecular Biology Lab,
Department of Zoology, University of the Punjab, Lahore, Pakistan
Tel.: +92 3224222036
e-mail: s_nadeem77@yahoo.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties

DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

glycosides, tannins and phenolic compounds for methanolic extracts of *Nerium* sp. (leaves and roots) (6, 7). However, significant time-dependent cytotoxic effects of this plant have been reported in various experimental animal models (8-10).

To the best of our knowledge, little is reported about the concentration-dependent cytotoxic effects of this plant or its products through muscle abscess in Wistar rats to date. Thus, it is imperative to study the concentration of aqueous leaf decoction of this plant to induce and assess an acute phase response by histological changes in the liver sections.

MATERIALS AND METHODS

Animals

Male Wistar rats (about 200±10 g bodyweight) were used from the Department of Zoology, Govt. College of Science (GCS), Lahore, Pakistan, and were maintained and acclimatized before the experiment as described by Abbasi et al. (2013) (8).

Experimental design

Fresh leaves of *N. oleander* were collected from GCS and further processed for decoction preparation as described elsewhere (11). Briefly, the leaves were boiled and steam distilled in 0.9% NaCl solution (1:1, w/v) for 3 h. The decoction was filtered and used for the experimental animals. Wistar rats were categorized into three groups (n=4): groups I and II and a control group. Groups I and II were intramuscularly administered 5 and 10 ml/kg, respectively, of *N. oleander* leaf decoction in both hind limbs, while the control group received no treatment. All the animals were anesthetized and sacrificed as described previously 6 h after decoction administration (12).

Tissue sampling

Livers of animals from each group were excised after dissection and were preserved in 10% formalin separately and further cut into thin sections (4-5 µm) and stained for H&E and Masson's trichrome as per the manufacturer's instructions.

Immunohistology and immunofluorescence

Immunohistochemical evaluation was performed with the first antibody directed against ED-1 (1:100) according

to the method previously described (8).

For immunofluorescence, acetone fixed cryostat sections were incubated in mouse monoclonal anti-ED-1 primary antibodies (1:50) (Serotec, Germany) overnight at 4°C, rinsed in phosphate buffered saline (PBS). The sections were then incubated in Alexa Fluor and rhodamine-conjugated anti-mouse secondary antibody (1:200) (Molecular Probes, Germany) at room temperature for 1 h, and washed three times for 5 min in PBS. Finally, the nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI), the sections were then washed, mounted and photographed.

RESULTS

Liver sections showed enlarged sinusoidal spaces with infiltration of some leukocytes in group I while massive extravasations of similar type of cells were observed in group II compared to control in H & E staining (Fig. 1A-C). Sections of group II showed more negative changes characterized by congestion in sinusoids, loss of usual concentric arrangement of hepatocytes with hypergranulation in nuclei indicating severity of toxins in decoction following Masson's trichrome staining compared to group I and controls (Fig. 1D-F).

Recruitment of maximum ED-1⁺ cells was noted in group II compared to I with less ED-1⁺ cells while no such cells were observed in the control group (Fig. 2 A-C). Immunofluorescence double staining with the same antibodies also demonstrated a strong expression of ED-1⁺ (green) in hepatocytes and in sinusoidal spaces in group II compared to Group I and controls (blue DAPI nuclear staining) (Fig. 3 A-C).

DISCUSSION

In the current study two doses of leaf decoction of *N. oleander*, 5ml/kg and 10ml/kg bodyweight, were injected into rats to induce acute phase response and analyze the hepatic response. Infiltration reflected in histological sections in both of the experimental groups might be an indication of severe systemic damage as evident by raised levels of transaminases (13). In a similar experimental model, toxic effects on the liver tissue were reported previously (8).

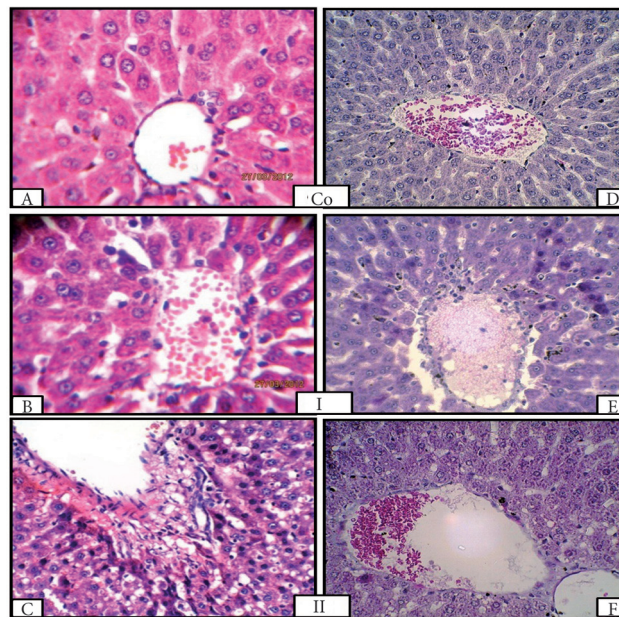


Fig. 1. Hematoxylin–eosin (A–C) and Masson's trichrome (D–F) stainings of liver sections of *N. oleander* leaf decoction induced APR in rats after 6h compared with control. Co (A, D) showed no histological alteration however, sinusoidal widening was noted for group I (B, E). Group II (10ml/kg) dose resulted in congestion and moderate aggregation of inflammatory cells in hepatic sinusoid lumen (C) while vacuolization of cytoplasm and amorphous hepatocytes was observed in section (F) (Magnification = 400×).

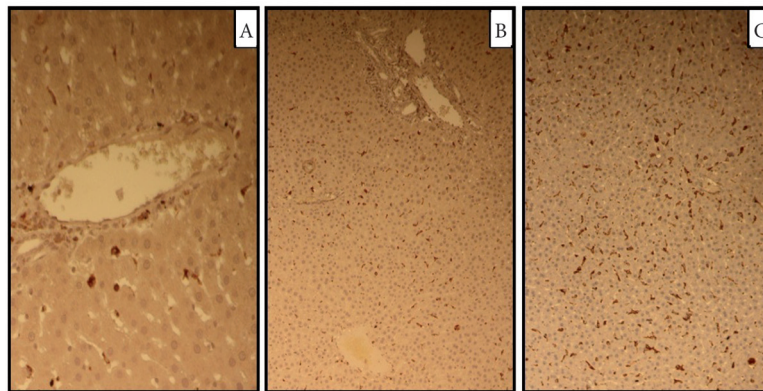


Fig. 2. Immunohistology: Sections of liver stained with an antibody directed against ED-1 followed by peroxidase staining of *N. oleander* leaf decoction induced APR in rats after 6 h compared with controls (A). Massive infiltration of polymorphic nuclear cells (as indicated by increase in ED-1⁺) was observed in the liver section of group II (C). (Magnification: A=400×, B and C=200×)

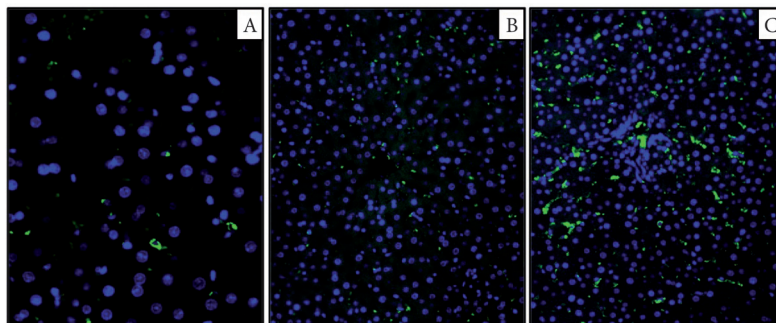


Fig. 3. Immunofluorescence staining of liver sections 6 h after leaf decoction with monoclonal antibodies directed against ED-1.

Single dose (110 mg/ kg bodyweight) of dried oleander leaves led to infiltration of mononuclear inflammatory cells into the portal space, with scattered necrosis of hepatocytes (14).

Immunohistochemistry and Immunofluorescence staining revealed recruitment of ED-1⁺ cells in both experimental groups though less in group I, compared to controls. This might be due to pronounced inflammatory damage to the liver even if it was a muscular abscess. Hepatic damage causes activation of neutrophil granulocytes (NGs) evident by strong ED-1⁺ cells, which play an important role in the inflammatory reaction that occurs during the multistep process of liver injury and repair. Activation of NGs in the inflamed tissue generates reactive oxygen radicals and releases proteases (15), destroying microorganisms and foreign antigens, and ultimately undergoing apoptotic death and phagocytosis by macrophages (16).

This study underlines the fact that excessive use of phyto-medicines can cause undesired and serious adverse ailments. A dose of 5ml/kg bodyweight induced APR and caused changes in hepatic response, although these changes were less vivid than those caused by 10ml/kg dose. Therefore, evaluation of plant decoctions is indispensable in order to consider them safe for treatment.

ACKNOWLEDGEMENTS

The authors are thankful to the Vice Chancellor, University of the Punjab, Lahore, Pakistan for providing financial support to publish the article.

REFERENCES

1. Malik I, Naz N, Sheikh N, Khan S, Moriconi F, Blaschke M, Ramadori G. Comparison of changes in gene expression of transferrin receptor-1 and other iron-regulatory proteins in rat liver and brain during acute-phase response. *Cell Tissue Res* 2011; 344:299-312.
2. Saile B, Ramadori G. Inflammation, damage repair and liver fibrosis-role of cytokines and different cell types. *Z Gastroenterol* 2007; 45:77-86.
3. Tian YW, Smith PGJ, Yeoh GCT. The oval-shaped cell as a candidate for a liver stem cell in embryonic, neonatal and precancerous liver: identification based on morphology and immunohistochemical staining for albumin and pyruvate kinase isoenzyme expression. *Histochemistry* 1997; 107:243-250.
4. Derwich E, Benziane Z, Boukir A. Antibacterial activity and chemical composition of the essential oil from flowers of *Nerium oleander*. *Electron J Environ Agric Food Chem* 2010; 9:1074-84.
5. Wang X, Plomley JB, Newman RA, Cisneros A. LC/MS/MS analyses of an oleander extract for cancer treatment. *Anal Chem* 2000; 72:3547-3552.
6. Al-Farwachi MI, Rhaymah MS, Al-Badrani BA. Acute toxicity of *Nerium oleander* aqueous leaf extract in rabbits. *Iraqi J Vet Sci* 2008; 22:1-4.
7. Patel G, Nayak S, Shrivastava S. Physical evaluation and qualitative chemical examination of methanolic extract of *Nerium indicum*. *Inter Jours Trends Sci Tech* 2010; 1:32-36.
8. Abbasi MH, Fatima S, Naz N, Malik IA, Sheikh N. Effect of *Nerium oleander* (NO) leaves extract on serum hepcidin, total iron, and infiltration of ED1 positive cells in albino rat. *BioMed Res Int* 2013; 2013:125671.
9. Barbosa RR, Fontenele-Neto JD, Soto-Blanco B. Toxicity in goats caused by oleander (*Nerium oleander*). *Res Vet Sci* 2008; 85:279-81.
10. Smith PA, Aldridge BM, Kittleson MD. Oleander toxicosis in a donkey. *Journal of veterinary internal medicine* 2003; 17:111-14.
11. Abbasi MH, Fatima S, Sheikh N. Histological comparison of natural lung injury in *Rattus norvegicus* induced by a natural herb (*Nerium oleander*) and a known carcinogen (thioacetamide). *Biologia (Pakistan)* 2014; 60:135-38.
12. Abbasi MH, Akhtar T, Malik IA, Fatima S, Khawar B, Mujeeb KA, Mustafa G, Hussain S, Iqbal J, Sheikh N. Acute and Chronic Toxicity of Thioacetamide and Alterations in Blood Cell Indices in Rats. *J Cancer Ther* 2013; 4: 251-59.
13. Abbasi MH, Khawar B, Mehmood R, Akhtar T, Sheikh N. Changes in proteins, transaminases activity and leucocyte count during *Nerium oleander* induced toxicosis in wistar rats. *Biologia (Pakistan)* 2015; 61:47-53.
14. Aslani MR, Movassaghi AR, Janati-Pirouz H, Karazma M. Experimental oleander (*Nerium*

- oleander) poisoning in goats: a clinical and pathological study. Iran J Vet Res 2007; 8:58-63.
15. Jaeschke H, Farhood A, Smith CW. Neutrophils contribute to ischemia/reperfusion injury in rat liver in vivo. FASEB J 1990; 4:3355-59.
 16. Haslett C. Resolution of acute inflammation and the role of apoptosis in the tissue fate of granulocytes. Clin Sci (Lond). 1992; 83(6):639-48.

LETTER TO THE EDITOR

COAGULATION IN HINDBRAIN MEMBRANE MENINGIOMA PATIENTS TREATED WITH DIFFERENT INJECTIONS USING ACUTE HYPERVOLEMIC HEMODILUTION

JK. LI, C. WANG, HD. GONG and HZ. LI

*Department of Neurosurgery, Affiliated HongQi Hospital of Mu Dan Jiang Medical University, Mudanjiang City, China**Received August 3, 2017 – Accepted September 18, 2017*

The aim of this study was to analyze the changes in coagulation in meningioma patients treated with different injections using the method of acute hypervolemic hemodilution (AHH). One hundred fifty hindbrain membrane meningioma patients were randomly divided into 5 groups, 30 per group. The first group were injected 40ml/time with Danhong after anesthesia induction; the second group were injected with 40ml~60ml/time Kangai and combined with interventional chemotherapy and embolization procedure; the third group of AHH were injected with polygeline 15ml/kg; the fourth group were injected with hydroxyethyl starch (130/0.4) sodium chloride in doses of 15ml/kg; the control group underwent basic treatment for lowering blood pressure and lowering blood fat. The changes of coagulation index were recorded before and after surgery and before and after the injection of different medications. Compared to the control group, for the first group of AHH, after being treated for 10 days and 30 days, the concentrations of bone specific alkaline phosphatase (BALP), bone Gla protein (BGP) and pro-collagen carboxy-terminal propeptide (PICP) were higher than that of the control group, the levels of endotoxin (ET) and C-reactive protein (CRP) were decreased compared to the control group ($p<0.05$); for the second group of AHH, after being treated for 10 days, the index of prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), fibrinogen (Fg) were not significantly changed, but the related level of vascular endothelial growth factor (VEGF) significantly decreased ($p<0.05$). Comparing the coagulation function index after surgery in the third and fourth groups, there were no significant changes in mean arterial pressure (MAP) level, heart rate (HR) value presented a low decrease, central venous pressure (CVP) level increased and the level of interleukin IL-6 showed a steady state after increasing. Analyzing the levels of interleukin IL-8 and tumor necrosis factor- α (TNF- α) after surgery, it was seen that in the third group they increased and in the fourth group they decreased ($p<0.05$). Danhong injection improved the coagulation function and microcirculation of patients, Kangai injection and interventional chemotherapy and embolization restrained the appearance of tumor angiogenesis, AHH operation with polygeline injection and hydroxyethyl starch (130/0.4) sodium chloride kept blood flow in normal parameters.

To the Editor,

Patients suffering from a severe disease such as cirrhosis with hepatitis C virus (1), non-alcoholic

fatty liver (2), post transplant complications (3) or critically ill patients with severe parapneumonic pleuresia (4) generally have coagulopathy function

*Key words: AHH, coagulation function, Danhong injection, Kangai injection, hydroxyethyl starch**Mailing address:*

Dr Hongzhe Li,
TongXiang Road No 5,
Affiliated HongQi Hospital
Mu Dan Jiang Medical University,
Mu Dan Jing City, Hei Long Jiang Province, China
e-mail: dr_lihongzhe@163.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

disorders determined by vitamin K-dependent coagulation factors (5). Once the blood coagulation function deteriorates, thrombus formation can seriously threaten the patient's life.

Brain tumors are generally removed by surgery. During this process, when the amount of bleeding is high, allogeneic transfusion needs to be carried out.

Hemodilution is used widely as a method of blood conservation, including acute normovolemic hemodilution (ANH), acute hypervolemic hemodilution (AHH) (6) and acute non-isovolemic hemodilution (ANIH). The onset of a brain tumor can cause the obstacle of coagulation function and lead to increased bleeding, therefore the application of blood substitute for alleviating the illness become very important. Danhong injection (7) is a traditional Chinese medicine which stimulates blood circulation. Kangai injection (8) contains astragalus mongholicus, oxymatrine and ginseng which act as nourishing agents for the blood, stimulating the immunity and tumor resistance. Hydroxyethyl starch (130/0.4) sodium chloride injection (9) and polygeline injection (10) can be used as blood supplement.

The aim of this study was to analyze the influence of different treatments applicable by the AHH method on coagulation function of patients with brain tumors.

MATERIALS AND METHODS

We included in the study 150 patients with hindbrain membrane meningioma who were treated in our hospital from September 2015 to February 2017. The patients were randomly divided into 5 groups, 30 patients per group. Each group was composed of 17 men and 13 women patients, aged between 23 and 54 years (average 40 ± 7.6 years) with no infection before surgery and who were under treatment with immunosuppressants, chemotherapy and radiotherapy. All the patients from the 5 groups had coagulopathy function disorders, affected blood supply and had indication for surgery.

No differences were observed between the groups regarding gender and age. The study was approved by the medical expert group and the Ethics Committee of the Affiliated Hong Qi Hospital of Mu Dan Jiang Medical

University, and all the patients signed informed consent to participate in the study.

Experimental methods

The patients in group A had osteoporosis or bone injuries and they were treated using the method of internal fixation. Twenty ml blood was collected on anticoagulant at 10 and 25 days after the surgery; 10 ml serum was separated and saved at the temperature of -15°C ; bone-specific alkaline phosphatase (BALP), alkaline phosphate (ALP), C Reactive Protein (CRP) and coagulation indexes as prothrombin time (PT) and activated partial thromboplastin time (APTT) were determined.

Group B was formed by patients with abnormal levels of vascular endothelial growth factor (VEGF) and they were treated with brain protective drugs and received anti-infection treatment. In this group T cell and Natural Killer Cell (NK) cell activity, VEGF and coagulation parameters were determined.

Groups C and D were comprised of patients with inflammation. Hemodynamic data such as heart rate (HR), mean arterial pressure (MAP) and central venous pressure CVP were detected at different intervals before and after AHH. The changes in IL-6, IL-8 and TNF- α value were determined by ELISA at D0 (before surgery), D1 (3 days after surgery), D2 (7 days after surgery) and D3 (10 days after surgery).

Group E was the control group that received standard treatment for normalizing blood pressure, reducing blood liquid and alleviating meningioma progression.

Patients from group A were treated with the AHH method and received intravenously Danhong injection (Heze Step Pharmaceutical Co., Ltd, State Medical Permit No. Z20026866) diluted in 5% glucose, 40 ml once a day for three weeks.

Patients from group B were also treated with AHH, and received interventional chemotherapy and embolization. As chemotherapy, platinum coordination compounds or Mitomycin were used. Lipiodol was injected and gelatin sponge was attached for the embolism and was combined with 40-60 ml of Kangai injection diluted in 5% glucose (Changbai Mountain Pharmaceutical Limited by Share Ltd, State Medical Permitment No. Z20026868). The treatment was administered for four weeks once a year, for a total of two courses of treatment.

Patients in groups C and D were treated using

anesthesia and AHH. The patients had been strictly fasting for 8 h and had received no liquids 6 h prior to surgery. 0.5 mg anisodamine was injected 30 min before conventional oxygen inhalation. Before anaesthesia, patients from group C received a polygeline injection (Wuhan DaAn Pharmaceutical Co. Ltd, Specification: 250ml:1.6g) and those from group D received a hydroxyethyl starch (130/0.4) sodium chloride injection (Faison Jukka Be Limited by Share Ltd, Specification: 250ml:15g).

Statistical analysis

SPSS17.0 software was used for statistical analysis and measurement data were expressed as the mean $\pm$ standard deviation ($\bar{x} \pm s$). Basic data of patients, coagulation index and other measurements were compared using Student's *t* test. The pathogeny and the damaged part were checked by χ^2 . $p < 0.05$ indicated that the difference was statistically significant.

RESULTS

There were three kinds of patients in the study, focusing on the index performance related with coagulation function. Hindbrain membrane meningioma patients with osteoporosis and bone injuries group A; patients with abnormal VEGF index group B; patients with inflammation groups C and D.

We can see from Table I that no significant differences were detected for the type A patients suffering from osteoporosis and bone injuries in terms of morphological integration, flow integration, ambient value, summary score, vessel velocity and leukocyte growth rate. Ten and 25 days after the treatment, the relevant indexes in the experimental group were all decreased compared to the control group; the vessel velocity significantly increased compared to the control group and leukocyte growth rate decreased ($p < 0.05$).

For type B patients, the effect of the treatment was judged by the following standards: CR (Complete Relief), PR (Part Relief), NE (No Effect) and PP (Progress Proportion). Total effective rate was the sum of CR and PR, expressed as TP.

We can see from Fig. 1a that the NE in the experimental group was significantly lower than that of the control group ($p < 0.05$).

For type B patients, the indexes of coagulation function were compared, and were expressed as prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), and fibrinogen concentration (Fg). We can see from Fig. 1b that there were no significant change in coagulation function between the control group and experimental group with regard to PT, APTT, TT and Fg ($p > 0.05$).

The adverse reactions of type B patients before and after the treatment were also analyzed by determining the decrease of white blood cells (WBCL), the decrease of hemoglobin (HGBL), the increase of bilirubin (BH), damage of the liver by liver fibrosis (LF), and nausea and vomiting (N&V) (Table II).

The incidence of adverse reactions was correlated with coagulation function. The levels of WBCL and HGBL indexes in the experimental group were better than the control group ($p < 0.05$).

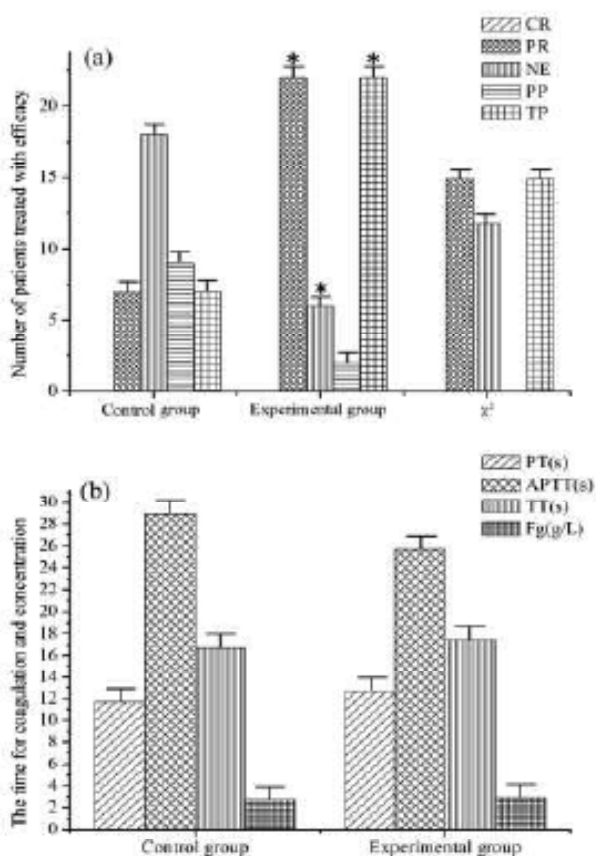


Fig. 1. The comparison of overall treatment effect and coagulation function of type B patients in both groups. Compared with control group, * $p < 0.05$

Table I. Relevant indexes of blood microcirculation of type A patients.

		Morpho- logical integration	Flow integration	Ambient value	Summary score	Vessel velocity (mm/s)	Leukocyte growth rate
Control group	Before treatment	1.75±0.64	2.78±0.37	2.12±0. 42	6.65±1.01	702.95±47. 12	1.88±0.38
	10 days after treatment	1.65±0.57	2.27±0.41	1.75±0. 38	5.65±1.36	622.34±43. 65	1.61±0.52
	25 days after treatment	1.45±0.57	2.05±0.28	1.62±0. 40	5.12±1.25	785.64±50. 54	1.34±0.57
Experimental group	Before treatment	1.72±0.60	2.67±0.42	1.97±0. 36	6.35±0.98	698.55±42. 87	2.01±0.44
	10 days after treatment	1.21±0.62 *	1.98±0.31 *	1.37±0. 38*	4.56±1.00 *	732.34±47. 31*	1.35±0.32*
	25 days after treatment	0.83±0.60 *	1.83±0.30 *	1.32±0. 24*	2.98±0.75 *	813.45±41. 62*	1.12±0.39*

Compared with control group, * $p < 0.05$

Table II. Comparison of the adverse reactions of type B patients in both groups.

Group	Amount	WBCL	HGBL	BH	LF	N&V
Control group	30	22	15	12	21	12
Experimental group	30	10*	7*	6	10	11

Compared with control group, * $p < 0.05$

For patients with inflammation, the effect of polygeline injection (group C) and hydroxyethyl starch (130/0.4) sodium chloride (group D) was analyzed.

MAP index showed no obvious changes before, after AHH and 30 minutes after AHH. HR indexes decreased after AHH, the effect being more pronounced in group D. The CVP indexes increased significantly 30 min after AHH compared with the levels immediately after AHH in both groups ($p < 0.05$) (Fig. 2a).

Fig. 2b shows that IL-6 changed more than the other parameters, and in group D the levels at different times were lower than that in group C after the surgery, ($p < 0.05$). In terms of IL-8 and TNF- α , in group C, the

levels were relatively stable. In D₃ the level became the same as before the surgery. In D group injected with hydroxyethyl starch (130/0.4) sodium chloride, IL-8 and TNF- α decreased compared with group C ($p < 0.05$).

DISCUSSION

The coagulation process is a complex process in which thrombin can catalyze fibrinogen materials soluble in plasma into the formation of protein monomers and activate XIII factor into XIIIa factor. This will bond the protein monomers and generate protein polymers insoluble in water, which interact with each other (5). Bleeding and coagulation problems usually appear during neurosurgery in

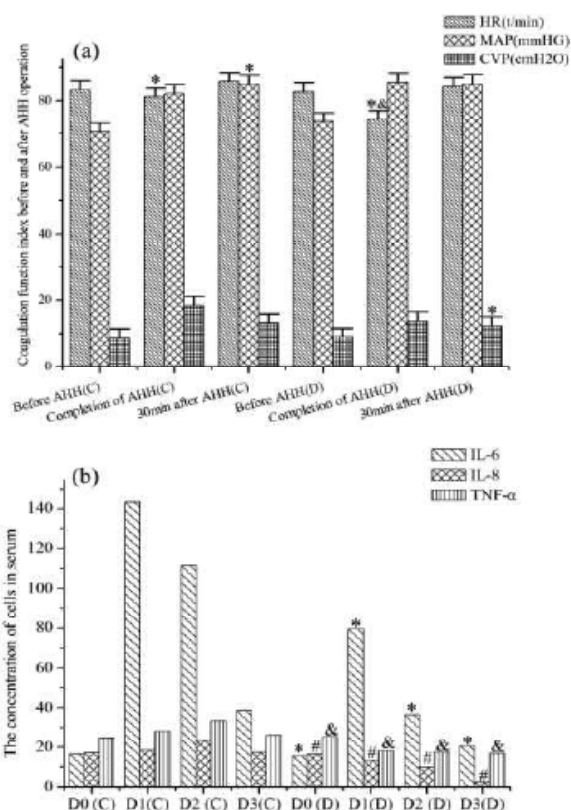


Fig. 2. Relevant indexes of coagulation function and IL-6/8 and TNF- α of type C patients in C and D
a) Compared with before AHH, * $P < 0.05$; Compared with completion of AHH in the group C, $\Delta P < 0.05$
b) Compared with IL-6 in group C, * $P < 0.05$; Compared with IL-8 in group C, $\#P < 0.05$; Compared with TNF- α in the group C, $\Delta P < 0.05$
D0 (before surgery), D1 (3 days after surgery), D2 (7 days after surgery) and D3 (10 days after surgery)

patients with hindbrain membrane meningioma. AHH method can solve this problem by replacing the blood function, but the method is still in the developing stage.

Hindbrain membrane meningioma could lead to intracranial hemorrhage if improper treatment is applied. Danhong injection can eliminate oxygen anion and inhibit blood coagulation due to lipid oxidation (11).

VEGF (12) promotes tumor blood supply leading to the tumor stimulation. When Kangai injection is used, an improvement could be seen in WBCL (13)

and HGBL after surgery (14) in the experimental group compared with the control group, and 10 days after the treatment, the level of VEGF in the experimental group was lower compared with the control group.

Analysis of the inflammatory markers at different times before and after surgery when polygeline and hydroxyethyl starch (130/0.4) sodium chloride are used showed that IL-8 and TNF- α increased after surgery and after that decreased to the level before the surgery in D3.

In summary, the above injections were used with the method of AHH for patients suffering hindbrain membrane meningioma. As a result, the coagulation function became relatively stable or improved.

REFERENCES

1. Docea AO, Gofiță E, Călina D, Zaharie SI, Vâlcea DI, Mitruț P. Autoimmune disorders due to double antiviral therapy with Peginterferon and Ribavirin in patients with hepatitis C virus infection. *Farmacia* 2016; 64(4):605-11.
2. Cioboată R, Găman A, Trașcă D, et al. Pharmacological management of non-alcoholic fatty liver disease: Atorvastatin versus pentoxifylline. *Exp Ther Med* 2017; 13(5):2375-81.
3. Tănase A, Coliță A, Ianoși G, et al. Rare case of disseminated fusariosis in a young patient with graft vs. host disease following an allogeneic transplant. *Exp Ther Med* 2016; 12(4):2078-82.
4. Călina D, Roșu L, Roșu AF, et al. Etiological diagnosis and pharmacotherapeutic management of parapneumonic pleurisy. *Farmacia* 2016; 64 (6): 946-52.
5. Wojciechowski VV, Călina D, Tsarouhas K, et al. A guide to acquired vitamin K coagulopathy diagnosis and treatment: the Russian perspective. *Daru* 2017; 25(1):10.
6. Qiao H, Zhang J, Liang WM. Feasibility and safety of acute hypervolemic hemodilution in neurosurgical patients. *Zhonghua Yi Xue Za Zhi* 2011; 91(37):2630-33.
7. Wang H, Ren S, Liu C, Zhang X. An overview of systematic reviews of danhong injection for ischemic stroke. *Evid Based Complement Alternat Med* 2016;

- 2016;8949835.
8. Fan HT, Lin HS. Review on clinical application and basic research of kang'ai injection on cancer. *Cancer Res Prev Treat* 2014; 41(9):1045-48.
 9. Ma PL, Peng XX, Du B, Hu XL, Gong YC, Wang Y, Xi XM. Sources of heterogeneity in trials reporting hydroxyethyl starch 130/0.4 or 0.42 associated excess mortality in septic patients: a systematic review and meta-regression. *Chin Med J (Engl)* 2015; 128(17):2374-82.
 10. Milutinovic D, Simin D, Zec D. Risk factor for phlebitis: a questionnaire study of nurses' perception. *Rev Lat Am Enfermagem* 2015; 23(4):677-84.
 11. Mohanty JG, Nagababu E, Rifkind JM. Red blood cell oxidative stress impairs oxygen delivery and induces red blood cell aging. *Front Physiol* 2014; 5(5):84.
 12. Tabrizi MA, Shamsipur M, Farzin L. A high sensitive electrochemical aptasensor for the determination of VEGF(165) in serum of lung cancer patient. *Biosens Bioelectron* 2015; 74(12):764-69.
 13. Nguyen AQ, Cherry BH, Scott GF, Ryou MG, Mallet RT. Erythropoietin: powerful protection of ischemic and post-ischemic brain. *Exp Biol Med (Maywood)* 2014; 239(11):1461-75.
 14. Pappo AS, Vassal G, Crowley JJ, et al. A phase 2 trial of R1507, a monoclonal antibody to the insulin-like growth factor-1 receptor (IGF-1R), in patients with recurrent or refractory rhabdomyosarcoma, osteosarcoma, synovial sarcoma, and other soft tissue sarcomas: results of a Sarcoma Alliance for Research Through Collaboration study. *Cancer* 2014; 120(16):2448-56.

LETTER TO THE EDITOR

AGE-RELATED CHANGES OF BONE MINERAL DENSITY IN MANDIBLE BY
QUANTITATIVE COMPUTED TOMOGRAPHY

X. DU¹, J. JIAO¹, X. CHENG², L. WANG², K. LI², H. LIU¹, C. WANG¹, C. ARENA³,
K. ZHURAKIVSKA³, G. GUGLIELMI⁴ and L. LO MUZIO³

¹Department of Radiology, Affiliated Hospital of Guizhou Medical University, Guiyang, Guizhou, China; ²Department of Radiology, Beijing Jishuitan Hospital, Xicheng District, Beijing, China;

³Department of Clinical and Experimental Medicine, University of Foggia, Foggia, Italy;

⁴Department of Radiology, University of Foggia, Italy

Received May 22, 2017 – Accepted October 5, 2017

Quantitative Computed Tomography (QCT) was used to investigate age-related changes in trabecular bones of the mandible. The volumetric Bone Mineral Density (vBMD) of mandibles was found to be increasing with aging in the 20-29, 30-39, 40-49 age groups, while resulted decreasing in the 50~ years age group in a Chinese population. The aim of this study was to validate QCT as a reliable method to measure the vBMD of the mandible and to assess vBMD of the mandible in a Chinese population. A total of seven hundred and eighteen volunteers (299 males, 419 females), ranging from 20 to 63 years of age, previously enrolled in another study, were included for vBMD measurements by QCT. A total of 50 subjects were enrolled for the validation of QCT measurement. Basic information (age, height and weight), and vBMD of the mandible (middle, body and angle) were recorded. Comparisons among age and sex groups were analyzed using the Mann-Whitney test. Linear correlation analysis between vBMD and age was performed. The mandibular vBMD was twice measured with QCT by one operator and resulted highly repeatable (ICC 0.97, P 0.05). The vBMD in the middle of the mandible was the highest, followed by that in the body and at the angle of mandible. In each age group, the mean vBMD increased with aging except for the 50~ years age group (P<0.05) in which it decreased. The mean mandibular vBMD was higher in females than males in each age group (P<0.05). Correlation between vBMD and age was statistically significant (r=0.15, P<0.01). QCT shows high precision for the measurement of BMD in the mandible. The present study provides normative data of the mandible in an age- and sex-stratified manner, which can be helpful in clinical practice.

To the Editor,

The mandible is part of the maxillofacial complex, the evaluation of bone mineralization of which has important clinical implications. The mandibular BMD reflects the strength of bones as represented by calcium content and it is widely used in clinical practice. The BMD of mandible is

closely related to bone metabolic diseases and it is useful for evaluation of periodontal procedures and dental treatment planning, especially bone grafting and dental implants (1-4). The ability to move teeth orthodontically depends on the level of bone mineralization within the alveolar bone. The chance to place successfully a dental implant for a

Key words: quantitative computed tomography, bone density, mandible, age

Mailing address:

Prof. Lorenzo Lo Muzio,
Department of Clinical and Experimental Medicine,
University of Foggia,
Via Rovelli 50,
71122 Foggia, Italy
e-mail: lorenzo.lomuzio@unifg.it

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties

**DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF
INTEREST RELEVANT TO THIS ARTICLE.**

prosthodontic device and to predict its acceptance by the mandibular bone also depends on the level of bone mineralization (5). The level of mandibular mineralization changes according to age and sex, therefore needs to be evaluated in humans during growth (6).

At present, the dual-energy X-ray absorptiometry (DEXA) and quantitative computed tomography (QCT) are commonly used in clinical and laboratory-based measurements of bone mineralization, but the measurement principle and osteoporosis diagnostic criteria are different. The DEXA measures the area of BMD, whereas the QCT measures the volume of BMD. The DEXA has been used to measure BMD of the mandible and to correlate it with that of other parts of the skeleton (7). This method measures an integrated sum of cortical and trabecular bone density.

Computed tomography (CT) is the only non-invasive pre-operative method that allows to obtain information on the degree of mineralization of trabecular bone as distinct from the cortex (8). The QCT has the major advantage of enabling trabecular and cortical bone density to be evaluated precisely and separately.

MATERIALS AND METHODS

Study subjects

The subjects included in this study were participants of an ongoing study on degeneration of the spine and knee since June 2014. The present study analyzed existing data in the cervical degeneration study, which was approved by the Ethics Committee of Beijing Jishuitan Hospital, Peking University Fourth School of Clinical Medicine, China (9).

Seven hundred and twenty-seven volunteers were enrolled. Some vBMD values of mandible were missing due to the following reasons: periodontitis, mandibular dental implant, artifacts caused by dentures and motion. Consequently, a total of 718 individuals were included in the final sample size, including 299 males (20 to 61 years old) and 419 females (20 to 63 years old). The criteria for inclusion were healthy adult, apart from the pathology that was the subject of previous study (i.e. degeneration of the spine and knee), aged 20–65 years. Participants affected by any disease that could influence bone metabolism, including trauma and tumor, and those who were taking bone metabolism regulating drugs were excluded. The exclusion criteria also included diabetes,

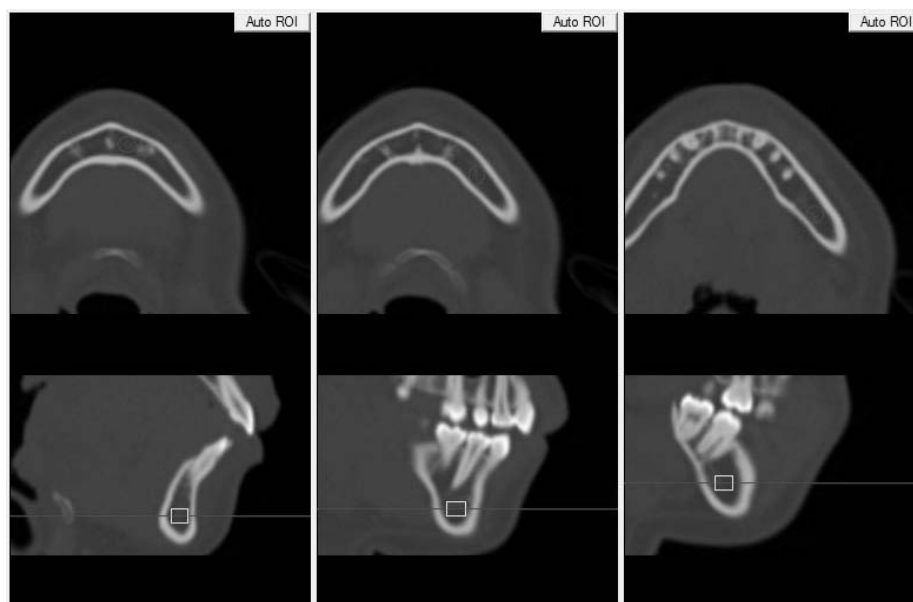


Fig. 1. QCT bone mineral density was analyzed using spine model, and the ROI was placed manually at the middle, body and angle of mandible, including only trabecular bone.

thyroid and parathyroid disease, or other chronic illnesses affecting the bone. All the subjects signed informed consent. Basic information, including age (years), height (cm), weight (cm), body mass index (BMI, kg/cm²), was recorded before scanning.

Mandibular scanning by QCT

QCT scans were obtained using Toshiba CT scanner (Aquilion PRIME ESX-302A, Toshiba Medical Systems Corporation, Otawara, Japan), as in a previous study (9). A QCT calibration phantom (Mindways Inc., Austin, TX, USA) was placed beneath the neck and scanned simultaneously according to the standard scanning protocol by Lang et al. (10). The scanning parameters were as follows: 120 kV, 187 mAs, field-of-view 40 cm, 1 mm slice thickness, and reconstruction matrix: 512 x 512.

Volumetric BMD (vBMD) measurement

After scanning Volumetric BMD (vBMD) measurement, the CT datasets were translated to the QCT workstation for further analysis with the spine model of QCT Pro 5.0.3 (Mindways Inc.). The regions of interest (ROI) were defined as the round and oval-shaped in mandibular middle (beneath the incisors), body (beneath the fangs) and angle (beneath the first and second molar) (Fig. 1). The areas of ROI contained 20

to 40 mm² of trabecular bone, not including the cortical bone. The vBMD values of three ROI were then recorded and analyzed, respectively. The reproducibility of QCT measurement was evaluated with a subset of 50 subjects analyzed by one observer in duplicate, two weeks apart. Fifty subjects were randomly selected to do bilateral mandibular measurements, and the other measurements were completed on the left side of the mandible.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows version 20.0 (IBM SPSS Inc., Chicago, IL, USA). Differences were considered significant at $p < 0.05$. A descriptive study was made for each variable. The baseline characteristics of the subjects were calculated as mean values. The intraclass correlation coefficient (ICC) was used to evaluate the reproducibility of the same measurement twice. The closer the result is to 1, the better the consistency. Wilcoxon test was performed to compare the differences between left and right mandibular vBMD. Data were grouped based on sex and age (10 year intervals) and expressed as the median and percentile. Kruskal-Wallis test was used to determine the statistically significant differences between all 4 age groups. Mann-Witney test was used to determine the statistically significant differences between 2 different

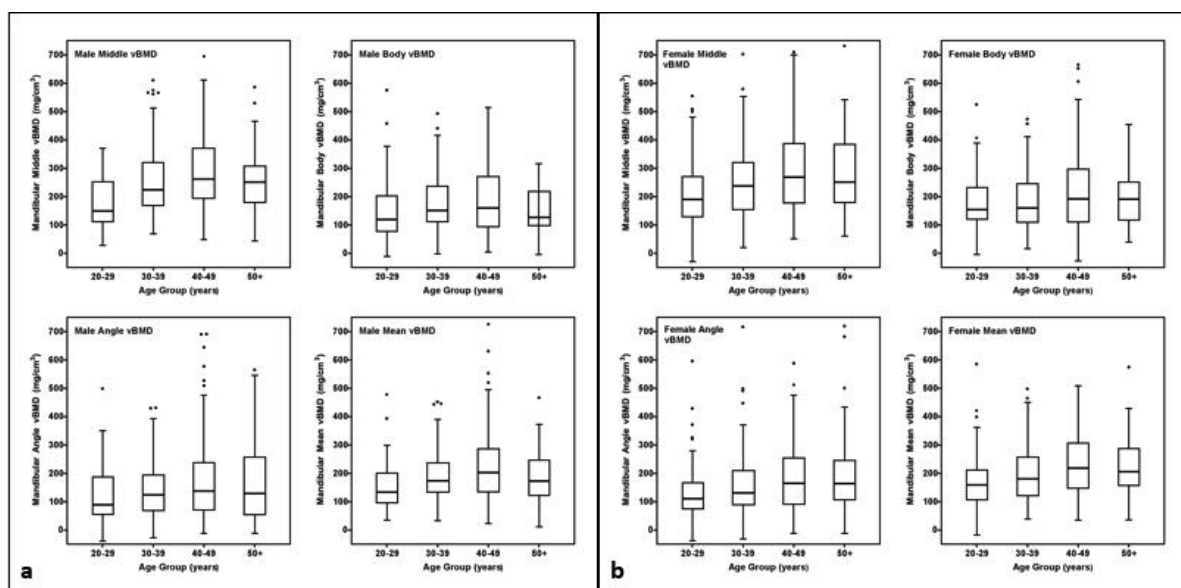


Fig. 2. Middle, body, angle and mean of mandible vBMD with age in male (a) and female (b) Chinese population.

age groups. To compare age differences in mandibular vBMD, we defined four age groups: 20-29, 30-39, 40-49, ≥ 50 years, and performed linear regression analysis on the measurements of the different age groups. Mann-Witney test was performed to compare differences between sexes in age, weight, height and vBMD.

RESULTS

Study population characteristics

Baseline characteristics of the subjects are shown in Table I. Seven hundred and eighteen volunteers (299 males, mean age 39.6 ± 9.2 years; 419 females, mean age 40.3 ± 9.5 years) were enrolled in this study. There were 114 participants in the 20-29-year-old age group (45 men, 69 women), 230 in the 30-39-year-old age group (106 men, 124 women), 248 in the 40-49-year-old age group (100 men, 148 women) and 126 in the ≥ 50 -year-old age group (48 men, 78 women). The 25th, 50th and 75th percentiles of mandible vBMD are shown in Table II. No age difference between the two sex groups was found ($p = 0.296$). Certainly, males were taller and heavier than females in average, and vBMI difference between the sexes was statistically significant.

Precision of QCT measurement

The middle, body and angle of the mandible vBMD of Intra-observer measurement were similar, and showed no statistically significant differences ($P > 0.05$), with a high consistency between measured values (ICC 0.90).

The difference between the left and right mandible

The left and right measurement of middle, body and angle mandible vBMD were similar, the difference between left and right measurement showed no statistically significant ($P > 0.05$).

Age-related differences in variables of mandible vBMD of the subjects

Based on multiple linear regression analysis between height, weight, BMI, age and mandible vBMD, the age was correlated with vBMD ($r = 0.15$, $P < 0.01$), while the rest of the correlations did not prove to be significant. Fig. 2 shows the middle,

body, angle and mean of mandible vBMD changes with age in males and females. Table III illustrates the differences between age group of the middle, body, angle and mean of mandible vBMD in both sexes. There was significant difference in the mean and middle vBMD between all 4 age groups in males and females ($P < 0.05$). The angle vBMD was significantly different among the age groups in females ($P < 0.05$). The angle vBMD in males and the body vBMD in males and females showed no significant difference ($P > 0.05$). In each age group, the middle, body and angle vBMD increased with aging but decreased in the 50~ years age group.

Sex-related differences in mandible vBMD of the subjects

The Mann-Witney test was used in order to compare differences in sex in mandibular vBMD. No age differences between the two sex groups were found. The middle vBMD showed no significant difference in variables of age groups ($P > 0.05$). There was a significant difference in the body, angle and mean vBMD between males and females ($P < 0.05$).

DISCUSSION

This is the first study on the trabecular bone of the mandible measured in a large Chinese population by QCT. The mandible is the only active bone of the head that articulates on two bilaterally situated joints and contains a dental arch that interdigitates with the maxillary one (5). The mandible is divided into a horizontal part, called mandibular body and a vertical part, the mandibular branch. The mandibular angle is the connecting part between body and branch. Evaluating trabecular bone mineral density of the mandible has important clinical implications, especially in the treatment of periodontal disease and for the placement of dental implants.

Previous studies have investigated mandibular growth and its change in BMD using different techniques. Many studies have investigated the mandibular BMD using conventional panoramic radiographs, which are semi-quantitative research and allow to perform only a morphological analysis of the

Table I. *Characteristics of the subjects.*

Parameters	Male	Female	P value
Sample size	299	419	
Age(years)	39.6±9.2	40.3±9.5	0.296
Height(cm)	172.4±5.9	160.4±7.3	<0.001
Weight(kg)	78.4±11.8	62.2±10.1	<0.001
BMI(kg/cm ²)	26.3±3.5	24.5±8.3	<0.001

BMI: body mass index

Table II. *The percentiles of mandible vBMD.*

Parameters	Male			Female		
	25th	Median	75th	25th	Median	75th
Middle vBMD	173.21	235.69	321.86	158.62	240.44	335.11
Body vBMD	93.28	145.39	239.91	113.98	177.41	255.95
Angle vBMD	65.39	124.96	223.22	88.11	144.60	226.50

Table III. *Age-related differences in variables of mandible vBMD of the subjects.*

Group	Male					Female				
	20-29 years	30-39 years	40-49 years	≥50 years	P value	20-29 years	30-39 years	40-49 years	≥50 years	P value
Sample size	45	106	100	48	#	69	124	148	78	#
Middle vBMD	152.80	226.38	262.50	253.45	<0.001	191.75	239.89	266.77	249.10	<0.001
Body vBMD	125.69	153.46	158.86	125.88	0.219	155.39	162.51	193.38	184.25	0.454
Angle vBMD	90.76	125.93	141.14	129.47	0.451	110.84	131.26	165.98	156.35	0.007
Mean vBMD	131.60	174.67	200.93	174.68	0.010	160.91	182.41	214.89	200.86	<0.001

p values were for the differences among sex-specific age groups analyzed using Kruskal-Wallis test

mandible. Mental foramen index (MFI), mandibular cortical index (MCI) and panoramic mandibular index (PMI) are used to evaluate the bone mineral density of the mandible on panoramic radiographs (11-13). DEXA and QCT have been well accepted and widely used in the diagnosis of osteoporosis, which needs quantitative measurement. Differences between DEXA and QCT in BMD values are widely recognized (14-16). DEXA is not able to selectively measure a specific area, thus will inevitably include the cortical bone. However, QCT is more advanced in selectively measuring the trabecular bone without any superimposition of surrounding tissues (17). QCT measures the vBMD of the mandible in different parts selectively and accurately. It is able to evaluate the trabecular BMD of mandible with high accuracy and with good reproducibility. Our study showed that the precision of duplicate QCT scans of the mandible was excellent.

Mandibular body is more important for dental clinical treatment and fracture risk prediction. In our study, we choose the trabecular BMD in the middle, body and angle of mandible for measurement using QCT, which is expected to represent the BMD of the mandibular horizontal area. Since the mandible BMD values on both sides resulted very similar, showing the differences were not statistically significant, we only measured the left mandible BMD. In both males and females, the vBMD values of the mandible gradually reduced from the middle to the angle. As demonstrated in other studies, the bone densities may vary when different areas of mandible are compared (18). The vBMD in middle, body and angle of mandible resulted to be different, which may be due to the different distribution of mandibular muscle and tooth movement. Mandibular body vBMD and the mean vBMD were closest, so we thought that mandibular body vBMD can better represent the mandible vBMD.

Mandibular alveolar bone undergoes aging processes that are similar to those of other bones in the body (19). Pham et al. showed that starting from puberty to middle age and adulthood, the mandible trabecular bone becomes more dense (20). The vBMD data from our study are in general agreement with previous studies. In our study, the trabecular

vBMD of the mandible was associated with age only, and there were no significant correlations with height, weight and body mass index. In the 20~ years age group, the vBMD in the middle, body and angle of the mandible gradually increased with age, and in the 40~ years age group resulted the highest. Subsequently, the mandible BMD began to decrease in the 50~ years age group. Such findings are not consistent with previous studies on lumbar vertebrae BMD. Zhang Y et al. showed that the lumbar vertebrae BMD was the highest in the 20~ years age group and decreased with age in adults. This may be due to the unique characteristics of mandible anatomy, phylogenetic factors and complex motions from the surrounding muscles.

The present study shows that the mean vBMD of mandibular trabecular bone, as detected on QCT, was determined to be higher in females than males. Noticeably, our findings that mandibular body, angle and mean vBMD showed statistically significant differences in males and females is not consistent with previous studies. Pham et al. showed that sex differences were not found to be significant according to their research using conventional panoramic radiographs (20).

To note, our study has several limitations. We performed a cross-sectional study and also evaluated age-related differences in the mandible vBMD. The sample size of advanced elderly people is relatively small, particularly for those over 60 years old, thereby limiting the ability to explore age differences and trends in more detail.

To our knowledge, this is the first clinical study to investigate and establish the normative data on mandible in an age-related manner. QCT can provide a precise measurement in trabecular vBMD of mandible. Additionally, these normative data of the mandible from the QCT could be helpful to comprehensively evaluate the mandibular trabecular status and design a better pre-operative surgical plan.

REFERENCES

1. Jemt T, Lekholm U. Implant treatment in edentulous maxillae: a 5-year follow-up report on patients with different degrees of jaw resorption. *Int J Oral Maxil-*

- lofac Implants 1995; 10(3):303-11.
2. Jaffin RA, Berman CL. The excessive loss of Branemark fixtures in type IV bone: a 5-year analysis. *J Periodontol* 1991; 62(1):2-4. doi:10.1902/jop.1991.62.1.2
3. Klemetti E, Collin HL, Forss H, Markkanen H, Lassila V. Mineral status of skeleton and advanced periodontal disease. *J Clin Periodontol* 1994; 21(3):184-88.
4. Tezal M, Wactawski-Wende J, Grossi SG, Ho AW, Dunford R, Genco RJ. The relationship between bone mineral density and periodontitis in postmenopausal women. *J Periodontol* 2000; 71(9):1492-98. doi:10.1902/jop.2000.71.9.1492
5. Maki K, Miller A, Okano T, Shibasaki Y. Changes in cortical bone mineralization in the developing mandible: a three-dimensional quantitative computed tomography study. *J Bone Miner Res* 2000; 15(4):700-709. doi:10.1359/jbmr.2000.15.4.700
6. Maki K, Miller AJ, Okano T, Shibasaki Y. A three-dimensional, quantitative computed tomographic study of changes in distribution of bone mineralization in the developing human mandible. *Arch Oral Biol* 2001; 46(7):667-78.
7. Corten FG, van 't Hof MA, Buijs WC, Hoppenbrouwers P, Kalk W, Corstens FH. Measurement of mandibular bone density ex vivo and in vivo by dual-energy X-ray absorptiometry. *Arch Oral Biol* 1993; 38(3):215-19.
8. Lindh C, Nilsson M, Klinge B, Petersson A. Quantitative computed tomography of trabecular bone in the mandible. *Dentomaxillofac Radiol* 1996; 25(3):146-50. doi:10.1259/dmfr.25.3.9084263
9. Zhang Y, Zhou Z, Wu C, et al. Population-stratified analysis of bone mineral density distribution in cervical and lumbar vertebrae of Chinese from quantitative computed tomography. *Korean J Radiol* 2016; 17(5):581-89. doi:10.3348/kjr.2016.17.5.581
10. Lang TF, Li J, Harris ST, Genant HK. Assessment of vertebral bone mineral density using volumetric quantitative CT. *J Comput Assist Tomogr* 1999; 23(1):130-37.
11. Horner K, Devlin H. The relationship between mandibular bone mineral density and panoramic radiographic measurements. *J Dent* 1998; 26(4):337-43.
12. Klemetti E, Kolmakov S, Kroger H. Pantomography in assessment of the osteoporosis risk group. *Scand J Dent Res* 1994; 102(1):68-72.
13. Hastar E, Yilmaz HH, Orhan H. Evaluation of mental index, mandibular cortical index and panoramic mandibular index on dental panoramic radiographs in the elderly. *Eur J Dent* 2011; 5(1):60-67.
14. Yu W, Qin M, Xu L, et al. Normal changes in spinal bone mineral density in a Chinese population: assessment by quantitative computed tomography and dual-energy X-ray absorptiometry. *Osteoporos Int* 1999; 9(2):179-87. doi:10.1007/s001980050133
15. Pickhardt PJ, Bodeen G, Brett A, Brown JK, Binkley N. Comparison of femoral neck BMD evaluation obtained using Lunar DXA and QCT with asynchronous calibration from CT colonography. *J Clin Densitom* 2015; 18(1):5-12. doi:10.1016/j.jocd.2014.03.002
16. Setiawati R, Di Chio F, Rahardjo P, Nasuto M, Dimpudus FJ, Guglielmi G. Quantitative assessment of abdominal aortic calcifications using lateral lumbar radiograph, dual-energy X-ray absorptiometry, and quantitative computed tomography of the spine. *J Clin Densitom* 2016; 19(2):242-49. doi:10.1016/j.jocd.2015.01.007
17. Grampp S, Jergas M, Gluer CC, Lang P, Brastow P, Genant HK. Radiologic diagnosis of osteoporosis. Current methods and perspectives. *Radiol Clin North Am* 1993; 31(5):1133-45.
18. de Oliveira RC, Leles CR, Normanha LM, Lindh C, Ribeiro-Rotta RF. Assessments of trabecular bone density at implant sites on CT images. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2008; 105(2):231-38. doi:10.1016/j.tripleo.2007.08.007.
19. Jonasson G, Billhult A. Mandibular bone structure, bone mineral density, and clinical variables as fracture predictors: a 15-year follow-up of female patients in a dental clinic. *Oral Surg Oral Med Oral Pathol Oral Radiol* 2013; 116(3):362-68. doi:10.1016/j.oooo.2013.06.009.
20. Pham D, Kiliaridis S. Evaluation of changes in trabecular alveolar bone during growth using conventional panoramic radiographs. *Acta Odontol Scand* 2012; 70(2):127-32. doi:10.3109/00016357.2011.600706.

LETTER TO THE EDITOR

PREOPERATIVE EVALUATION AND SURGICAL TECHNIQUE OF FUNCTIONAL AND COSMETIC ASPECTS IN ZYGOMATIC COMPLEX FRACTURE PATIENTSQ. LIN¹, X-Y. HONG², D. ZHANG¹ and H-J. JIN¹¹Department of Plastic and Reconstructive Surgery, the First Hospital of Jilin University, Changchun, China; ²Department of Vascular Surgery, China-Japan Hospital of Jilin university, Changchun, China*Received August 28, 2017 – Accepted October 5, 2017*

The zygomatico-maxillary complex functions as the principle buttress of the face and is the cornerstone to an individual's aesthetic appearance. Its fracture not only creates cosmetic deformities owing to its position and facial contour, but can also cause disruption of ocular and mandibular functions. The aim of this study was to evaluate the quality, efficacy and impact of internal fixation of zygomatic complex fractures on functional and cosmetic outcomes. A prospective study was carried out on 100 patients who were divided according to the classification and the severity of injury. Subjective evaluation was submitted based on the patient's perception of signs and symptoms in the preoperative and postoperative periods. Intraoperative and postoperative assessment of bone reduction quality was made according to the type of the fracture and related difficulties; also, the difference between these groups was observed as functional and esthetic outcome. To optimize the treatment of zygomatic bone fractures, a pre-designed questionnaire was used for subjective evaluation of symptoms and treatment outcome. In 70% of cases, ophthalmologic consultation was taken and was most common in type VII fractures (100% cases). Neurosensory disturbance was the most common finding (60%), followed by diplopia (56%), pain upon mouth opening (54%) and malar depression (50%). Out of all possible 400 fracture sites in 100 patients of zygomatic complex fractures, 266 (66.5%) fractures were detected by clinical examination, in contrast to 330 (82.5%) on radiological examination, which were highest at zygomatic-maxillary buttress (93%) followed by infraorbital rim (91%) and almost equal among fronto-zygomatic site (72%) and zygomatic arch (74%). The scores from the questionnaire for annoyance were significantly higher for paraesthesia (23%) than for trismus (10%), pain (8.5%), or deformity (8.25%). Residual deformity and pain significantly influenced the total satisfaction. Conclusively, there are many treatment modalities available for zygomatic complex fractures, and the preferred methods should be selected on the basis of fracture type, fracture severity, pre-operative signs and symptoms. Regarding the requirements of fracture site exposure and actual fixation, one priority should be to minimize postoperative complications, morbidity and residual deformities.

To the Editor,

Zygoma fracture management has very controversial information regarding its name related to anatomy, incidence, classification, associated

fractures/ injuries, signs and symptoms particular to one classification group with potential early and late complications. Above all, there is little evidence which can exactly correlate the severity of injury and

Key words: zygomatico-maxillary complex fracture, treatment, internal fixation, residual deformities, patient perception; esthetic outcome

Mailing address:

Dr. Hongjuan Jin,
Department of Plastic and Reconstructive Surgery,
the first hospital of Jilin university,
71 Xinmin Street,
Changchun, China
e-mail: hongjuan.jin@yahoo.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

degree of post-operative complications (1). Although these complications are relatively heterogeneous and can be prevented with correct diagnosis, fracture reduction and fixation, for a surgeon ocular problems involving antero-posterior and vertical globe projections, lateral canthal positions, palpebral fold changes and extraocular movements can be the most difficult and most disfiguring to correct (1).

Surgical technique is usually selected to achieve qualitative reformation of facial bones to minimize the outcome, such as exophthalmos, enophthalmos, malar malposition and neurosensory changes (2). Although to achieve precise reduction and rigid fixation, direct exposure and visualisation of all buttresses are required, however resulting morbidity to soft tissues and blood supply cannot be avoided (3). It has been demonstrated that all the requirements cannot be satisfied with a single procedure simultaneously. One has to prioritize among the residual symptoms for treatment optimization but the patient's annoyance towards residual symptoms or deformities must be taken into consideration. The aim of the present study is to preoperatively evaluate the quality, efficacy and impact of treatment of different combinations of internal fixation of sites in zygomatic complex fracture on functional and cosmetic aspects along with the comparison of using different scales and methods.

The objectives of this study were to:

- i) identify signs and symptoms preoperatively and postoperatively at different follow-up times;
- ii) to assess fracture sites by clinical examination and radiological examination;
- iii) assess requirement of fixation for stability;
- iv) identify associated complications and residual deformities;
- v) evaluate the treatment outcome.

MATERIALS AND METHODS

A prospective study was conducted on patients with isolated zygomatic complex fractures reported in the emergency department and outpatient department of our hospital. All the patients were informed about the study and gave written informed consent.

One hundred patients aged between 20-55 years of

either gender with isolated ZMC fractures who were fairly fit for surgery and anaesthesia (ASA grade I and II) were included. Patients were diagnosed and classified with the help of detailed history, clinical examination and radiological examination (plain radiographs and, if necessary, CT scans). The groups were divided according to classification used for diagnosis, and subjective evaluation was made based on the patient's perception of deformity, pain and paraesthesia as well as the measurement of range of mouth opening, pain upon mouth opening and annoyance with incisions in the pre- and post-operative periods. Previously, special attention had been given to signs and symptoms presented at the time of first consultation or examination and noted carefully. Standard scales, rates, conventional examination methods or specialist references (e.g., ophthalmologist) were used to assess all the signs and symptoms associated with different types of fracture.

All the patients were considered for open reduction and internal fixation using bone plates and screws with standard surgical protocols. Various incisions were selected for the repairs. Based on the location, displacement of fracture segments, and comminution of fracture segments and severity of fractures. A lower eyelid incision was used to expose the inferior orbital rim, intraoral upper vestibular incision for zygomaticomaxillary buttress and lateral brow incision for zygomaticofrontal suture. The rationale behind choosing different surgical approaches was to achieve proper alignment and fixation. During reduction of fracture segments, quality of bone was assessed for the evaluation of their intra- and post-operative complications, esthetic and functional outcome. Occipitomental radiograph was obtained on the first post-operative day and patients were followed-up at regular intervals as per study design until radiological confirmation of healed fractured bones. Control examinations were performed by the outpatient clinic team. At last follow-up after 3 months, assessment of fractured bone stability was made by applying lateral manual pressure over the zygoma, as the patients' subjective evaluations of symptoms were necessary to optimize treatment of zygomatic bone fractures.

A pre-designed questionnaire was used for the quantitative assessment of evaluation of symptoms and treatments using the degree of satisfaction and annoyance resulting from residual symptoms (deformity, pain,

paraesthesia, and trismus). Incisions used for exposure of the fracture site were also investigated. Patients were asked to answer a self-designed questionnaire to evaluate the patient's perception of total satisfaction for the treatment, deformity, pain, paraesthesia, trismus and annoyance associated with incisions, and subjective assessment for answers to the above parameters was evaluated as 0%, 25%, 50% and 100%.

RESULTS

The modified Rowe and Killey's (1971) classification was used to divide all the fractures into seven types as demonstrated by occipito-mental radiographs which are non-displaced fractures, fractures of zygomatic arch, rotation around vertical axis (medial and lateral), rotation around the longitudinal axis (medial and lateral); displacement without rotation (medial, inferior, lateral and posterior); orbital rim fractures and complex fractures (4).

Distribution of pre-operative signs and symptoms

In 70% cases, ophthalmologic consultation was

taken and was most common in type VII fractures (100% cases). In 60% cases, neurosensory disturbance was the most common finding, followed by diplopia (56%), pain upon mouth opening (54%), malar depression (50%), and trismus. Subconjunctival ecchymosis and cheek ecchymosis were present equally in 44% cases. Restricted eye mobility was presented in 32% cases followed by exophthalmos (23%), globe injury (22%), enophthalmos (21%); ectropion (20%); pupil level difference (18%); retro-bulbar haemorrhage (18%) and lacrimal injury (16%). Lastly, epistaxis was present in 20% cases and malocclusion in 11% cases. There was no case in which there was complete absence of the above examined signs and symptoms (Fig. 1).

When these signs and symptoms were distributed according to the classification system used, the group type VII fractures have the maximum number of signs and symptoms present followed by VC (118); VD (87); VA (75); VI (60); IVB (46); IIIB (41); II (39); IIIA (21); VB (20); IVA (13); and I (4).

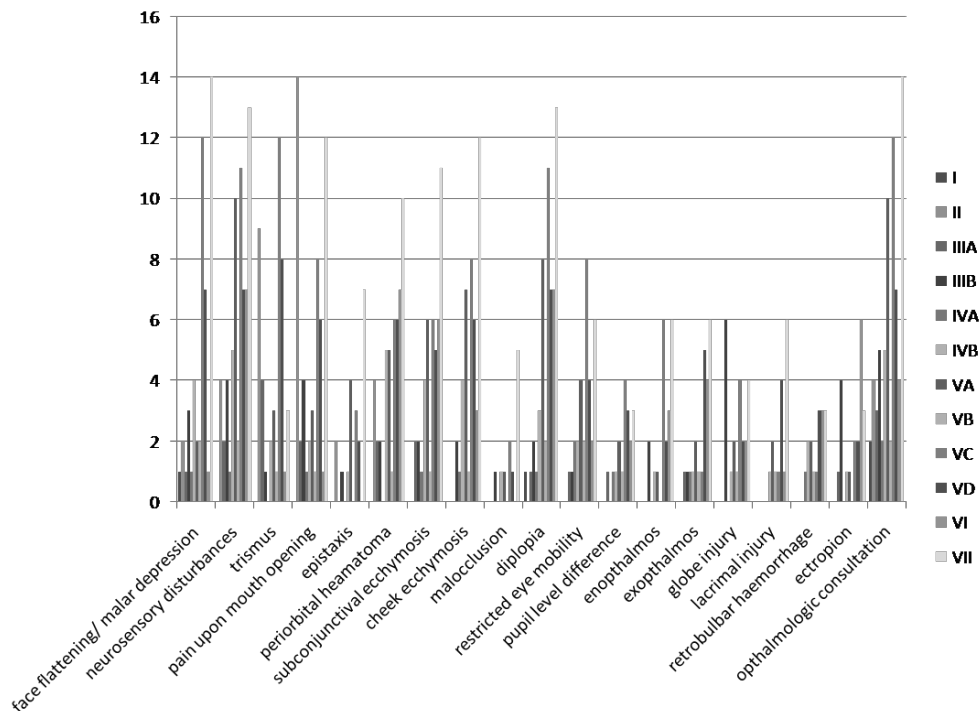


Fig. 1. Distribution of signs and symptoms: noted at first consultation or examination.

type and VA (displacement without rotation-medial) following complex fracture group type. Trismus was found in the highest number in VC followed by II. Pain upon mouth opening was significantly found highest in II, similar to findings of trismus. Epistaxis, apart from complex fractures, was found in VA. Periorbital haematoma, subconjunctival ecchymosis, cheek ecchymosis and malocclusion were highest in VII but also found in other fractures types in almost equal proportions. Diplopia again showed interesting variation of its presentation as apart from VII type fractures, it is present in VA and VC. Retrobulbar haemorrhage, pupil level difference and enophthalmos symptoms were highest in VA. Globe injury was highest in type IIIB. And ectropion was highest in VI.

Distribution of identified fracture sites

When analysis was carried out for the total number of fracture sites identified on clinical examination, radiological examination, surgically explored sites, of possible 400 fracture sites in 100 patients of zygomatic complex fractures, 266 (66.5%) fracture sites were found by clinical examination. However, on radiological examination 330 (82.5%) fractures sites were identified for these patients, which were highest at zygomatic-maxillary buttress (93%) followed by infraorbital rim (91%) and almost equal

among fronto-zygomatic site (72%) and zygomatic arch (74%) (Table I).

On comparing fracture sites identified on clinical examination with radiological examination, significant increases in percentages were seen at infraorbital rim, fronto-zygomatic site, and zygomatic arch. Depending on many factors, such as age and sex, the situation and extent of severity and displacement of fractured segments, fracture site type and decision of operating surgeon, out of total number of fracture sites identified clinically and radiologically, 204 (51%) sites were surgically explored. After reduction of fractured zygomatic complex fractures, it was found that not all fracture sites were amenable for fixation and as per stability needs of reduced fractured segments, 163 (40.75%) fracture sites were actually fixed. On comparison of four fracture sites identified, zygomatic maxillary buttress was found in highest number (95.25%) among clinical examination, radiological examination, surgical exploration and sites actually fixed. Other sites identified were 59.25%, 47.75%, 38.5% for infraorbital rim, fronto-zygomatic site and zygomatic arch, respectively.

Distribution of post-operative findings on follow-up assessments

The assessments at follow-ups or facial photographs

Table I. Total number of fracture sites found.

	Infraorbital Rim	Fronto-Zygomatic Site	Zygomatico-Maxillary Buttress	Zygomatic Arch	Total
Clinical	76	52	92	46	266
Radiological	91	72	93	74	330
Surgically Explored	44	38	98	24	204
Actually Fixed	26	29	98	10	163
Total	54	58	111	23	246

(photos collected from patients preoperatively) showed significant variations among various signs and symptoms noted. All the previously noted signs and symptoms were assessed at diverse post-operative follow-ups: after 1 day, 2 weeks, 1 months, 2 months and 3 months, respectively (Fig. 2).

Among 50 patients who had facial flattening or malar depression, two patients had observable asymmetry at follow-up visits. Face flattening or malar depression was not observed immediately post-operatively to reduce bias or error due to postoperative swelling at the operated site. These were assessed by two different observers. These patients were among those previously noted to have an inadequate reduction at all fracture sites due to early malunion.

Although retrobulbar haemorrhage, pupil level difference, enophthalmos and exophthalmos diminished significantly at post-operative day one follow-up assessment, other symptoms or signs showed a graph of significant correction or reduction in the problem at subsequent follow-ups.

Neurosensory disturbance which was noted in 66% of cases was present in 40 cases at immediate

post-operative day one assessment and subsequently reduced to 32 cases at 1 week, 16 cases at 2 weeks and 6 cases after 3 months. The data for neurosensory disturbances showed 2 peaks of recovery in neurosensory disturbance, one at the 2-week follow-up and the other at the 3-month follow-up. Trismus was present in only 25% of cases at immediate post-operative day, which reduced to 4 cases after 2 months and only one case after 3 months. Pain upon mouth opening, showed an interesting finding: it reduced to 50% at immediate post-operative day but almost diminished after 2 weeks. Periorbital haematoma and subconjunctival ecchymosis were not decreased at first post-operative day assessment, 1 week and 2 week assessment. But at 1 month follow up, these were almost diminished.

One unexpected finding was subconjunctival ecchymosis which was present in 46 cases at one day post-operative assessment (preoperatively it was in 44 cases). Both of these cases had enophthalmos and underwent orbital reconstruction.

Diplopia has a delayed healing and subsequently decreased in every follow-up assessment but the optimum recovery peak was noted at immediate

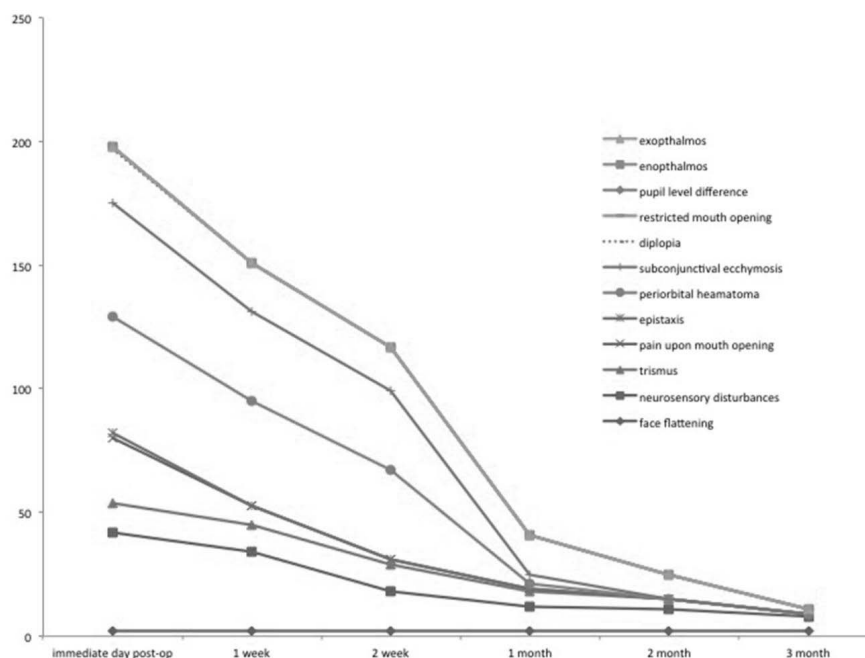


Fig. 2. Distribution of post-operative signs and symptoms at different follow-up assessments.

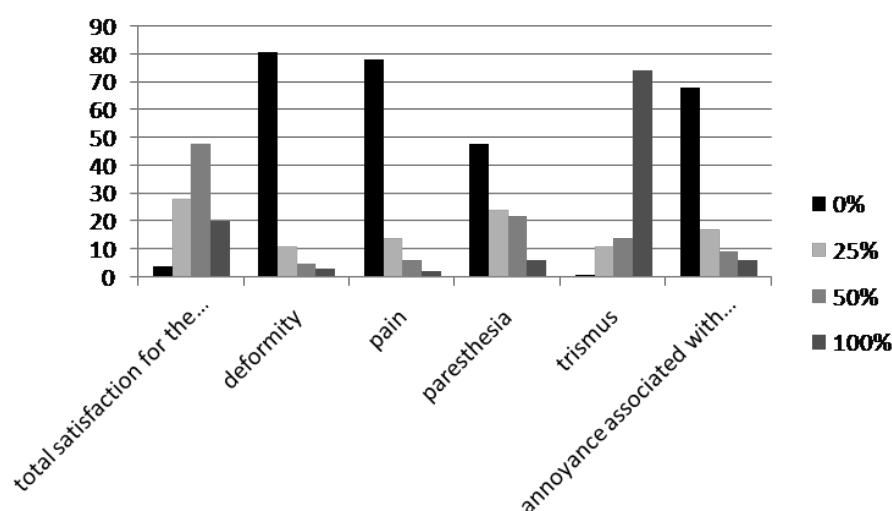


Fig. 3. Distribution of subjective evaluations, quantitative assessments and qualitative assessments of the treatment by patients.

post-operative day assessment.

Uneventful healing was noted in all the fractures. There were no cases of osteomyelitis or infection. There was no requirement for corrective operations and no pseudo arthrosis was seen. No reaction to an alloplastic material or any other material was noted.

Quantitative assessments and qualitative evaluations for residual symptoms

Total satisfaction of the treatment

Total treatment satisfaction was noted as 100% in 20 patients, 50% in 48 cases, 25% in 28 cases and 0% in 4 cases. The mean total satisfaction in 100 patients was 51%. The four patients who had 0% satisfaction were aged between 22-25 years and had complex zygomatic-complex fractures (Fig. 3).

Deformity

Amongst 100 patients who provided explanations of their residual deformity, either noted by them or by friends/parents, 81 had no perception of residual deformity on their face, which was statistically significant, and 11 had a perception of 25% deformity at the end of three months. These 11 patients were mostly females and had received 2 point exposure or 2 point fixation. Moreover, 50% deformity was perceived in 5 cases among which 3 patients underwent lower lid incisions which they referred as being eyelid

deformities. The remaining 3 patients described 100% deformity at indefinite locations. Conclusively, according to the above data, the mean perception of residual deformity was 8.25% in 100 cases.

Pain

Among the 100 cases, only 2 described 100% pain at fracture fixations and exposure sites, but they were not able to describe the frequency, onset, or associated relieving and aggravating factors. Six patients who described their pain as 50% relieved were those who had pain once in a week early in the morning which was relieved by analgesics. Eleven cases who had described their pain perception as 25% at the end of follow-up were those who felt pain only while yawning and wider mouth opening. Of 100 cases, 78 had a perception of 0% pain at every site, therefore the mean percentage of pain existence after three months of follow-up was 8.5%.

Paraesthesia

Six patients who reported residual paraesthesia as 100% (0% relieved) at the end of follow-up described the degree of paraesthesia as stronger than preoperatively and affecting their daily life. Moreover, 22 patients thought the degree of paraesthesia was 50% and was improving with time, but very slowly, and 24 patients reported 25%

paraesthesia from the upper lip to lateral orbital region. Forty-eight cases had complete relief of any neurosensory disturbance. The mean percentage of paraesthesia perception was 23%.

Trismus

Of 32 patients who complained about residual trismus, one patient considered that trismus was affecting his daily life and considered it as 100%. Nine patients had 50% trismus as compared to pre-injury perception. The rest (17 patients) had 25% trismus and described it as being more psychological as there was nothing significant when examined clinically. Sixty-eight patients had total relief from trismus. The mean trismus percentage resulted 10% in 100 cases.

Annoyance associated with incisions

Sixty-eight patients reported no discomfort regarding any incision. However, 17 patients graded it as 25% but mostly described it as due to anxiety and psychologically felt an inferiority complex in public. Nine patients had feeling of contracture and discomfort of sensation at incision sites and graded it as 50% annoyance. Only six patients graded 100% annoyance associated with incisions and provided the reason as visible scarring. Overall, the mean annoyance was calculated as 14.75%.

DISCUSSION

Our results were consistent with the study by Hwang and Kim (5) regarding the initial physical examination, in which hypaesthesia was the most common associated symptom, followed by limitation of mouth opening or closure, diplopia, and malocclusion and it was contrary to the study by Kovacs and Ghahremani (6) in which the most common symptom of ZMC fractures was pain (91.4%) followed by oedema and ecchymosis of the cheek or eyelids (90.6%); enophthalmos (65.8%); anesthesia or paraesthesia of the cheek, nose, upper lip, lower eyelid (58.5%); and diplopia (49.1%).

When we analysed the total number of fracture sites identified, our results were similar to the findings by Hwang and Kim (5) in which 78.9 %

of zygomatic maxillary buttress site was found and used in treatment which is contrary to the findings of Calderoni et al. (7) who found Inferior orbital rim as the most common fracture site (73.5%) followed by lateral wall (49.2%). Postoperatively, the most common complications reported in literature are hypoesthesia, followed by diplopia, limitation of mouth opening or closure, infection, and hematoma (5). But in this study, it was neurosensory disturbance, face flattening, trismus and diplopia.

Significant facial asymmetry requiring surgical revision occurs in 3-4% of patients (8, 9). In our study, the major asymmetry was described in 2% of the patients operated. The most frequent complications described in the literature are infections and opening soft tissue flaps, mild residual deformity, and complications associated with internal fixation techniques (9). In this study, complications after three months were limited to face flattening, neurosensory disturbances, trismus and diplopia. In our study the overall total satisfaction towards treatment was 51% in comparison to 72.5% in a study by Kurita, et al. (10). Similar results were also evident in the literature (10), paraesthesia and annoyance with the incisions caused the strongest annoyance on subjective evaluation and assessment.

In conclusion, there are many treatment possibilities available but the preferred methods should be selected on the basis of fracture type, fracture severity, severity of pre-operative signs and symptoms, and requirement of fracture site exposure; and actual fixation, attempts to minimize postoperative complications, morbidity and residual deformities should be a priority.

REFERENCES

1. Stanley RB, Mathog RH. Evaluation and correction of combined orbital trauma syndrome. *Laryngoscope* 1983; 93(7):856-65.
2. Forrest CR. Application of endoscope-assisted minimal-access techniques in orbitozygomatic complex, orbital floor, and frontal sinus fractures. *J Craniomaxillofac Trauma* 1999; 5(4):7-12.
3. Yonehara Y, Hirabayashi S, Tachi M, Ishii H. Treatment of zygomatic fractures without inferior

- orbital rim fixation. *J Craniofac Surg* 2005; 16(3):481-85.
4. Yanagisawa E. Symposium on maxillo-facial trauma. III. Pitfalls in the management of zygomatic fractures. *Laryngoscope* 1973; 83(4):527-46.
 5. Hwang K, Kim DH. Analysis of zygomatic fractures. *J Craniofac Surg* 2011; 22(4):1416-21.
 6. Kovacs AF, Ghahremani M. Minimization of zygomatic complex fracture management. *Int J Oral Maxillofac Surg* 2001; 30(5):380-83.
 7. Calderoni DR, Guidi Mde C, Kharmandayan P, Nunes PH. Seven-year institutional experience in the surgical treatment of orbito-zygomatic fractures. *J Craniomaxillofac Surg* 2011; 39(8):593-9.
 8. Peretti N, MacLeod S. Zygomaticomaxillary complex fractures: diagnosis and treatment. *Curr Opin Otolaryngol Head Neck Surg* 2017; 25(4):314-19.
 9. Balakrishnan K, Ebenezer V, Dakir A, Kumar S, Prakash D. Management of tripod fractures (zygomaticomaxillary complex) 1 point and 2 point fixations: A 5-year review. *J Pharm Bioallied Sci* 2015; 7(Suppl 1):S242-47.
 10. Kurita M, Okazaki M, Ozaki M, Tanaka Y, Tsuji N, Takushima A, Harii K Patient satisfaction after open reduction and internal fixation of zygomatic bone fractures. *J Craniofac Surg* 2010; 21(1):45-49.

LETTER TO THE EDITOR

SOCIAL SELECTION FAVOURS OFFSPRING PRONE TO THE DEVELOPMENT OF ANDROGENETIC ALOPECIA

A. GOREN^{1,2}, J. SHAPIRO³, T. NACCARATO¹, M. SITUM⁴, M. KOVACEVIC²,
N. LONKY⁵, T. LOTTI² and J. McCOY¹

¹*Applied Biology, Inc., Irvine, CA, USA;* ²*Department of Dermatology and Venereology, University of Rome "G.Marconi", Rome, Italy;* ³*Department of Dermatology, New York University Langone Medical Center, New York City, New York, USA;* ⁴*Department of Dermatology and Venereology, University Hospital Center "Sestre milosrdnice", Zagreb, Croatia;* ⁵*University of California, School of Medicine, Irvine, California, USA*

Received August 14, 2017 – Accepted October 20, 2017

In recent years, dermatologists have observed an increase in the incidence of male androgenetic alopecia (AGA). In a survey of 41 dermatologists, 88% reported an increase in incidence of AGA in men younger than 30 years. This phenomenon has no apparent explanation. However, due to the strong genetic inheritance component of AGA, a social or environmental factor which favours the inheritance of genes that increase the risk of developing AGA is suspected. To date, the strongest predictor of AGA in men has been the length of the CAG repeat located in the androgen receptor gene (AR gene) on the X chromosome. The same genetic variant in women is associated with ovulation at a later age, higher antral follicle count, and lower risk for premature ovarian failure. This led us to theorize that, due to social pressure to conceive later in life, women carriers of the short CAG repeat in the AR gene would have a selective advantage to conceive later in life and would thus favour male offspring exhibiting AGA.

To the Editor,

Androgenetic alopecia, also known as male pattern baldness, is the most common form of hair loss in men. In 1975, Norwood established a classification scale of male pattern baldness based on a survey of 1,000 Caucasian males. In his survey, he observed an incidence of Norwood III and above pattern baldness in 12% of males ages 18-29. The percentage of males exhibiting Norwood III and above pattern baldness increased to 38% of men by the age of 30 and plateaued at 70% by the age of 80 (1). Since Norwood's seminal work, it has been generally accepted that the majority of men initially

present with symptoms of AGA in their third or fourth decade, however, a survey conducted by Gan and Sinclair (2005) support the notion of an increase in incidence of AGA. In their survey of 655 men (primarily Caucasian), Gan and Sinclair observed an incidence of 17.14% of alopecia grade III and above in males ages 20-29 plateauing at 73.5% in males by the age of 80 (2). Interestingly, in a survey conducted in the intervening years between the Norwood and Gan surveys, Rhodes et al. (1998) observed that 16% of men aged 18-29 suffer from moderate to extensive hair loss (grade III or above) (3). In Asian populations the incidence of AGA is lower than in

Key words: androgenetic alopecia, androgen receptor, fertility

Mailing address:

Andy Goren, MD

Applied Biology, 17780 Fitch, Suite 192,
Irvine, CA 92614, USA.

Tel.: +1 (949) 387-4526

e-mail: andyg@appliedbiology.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties

DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

Caucasians, however, a similar increase in AGA has been reported. In a 5-year observational study at a dermatological clinic, Jang et al. (2013) noted that the average age of AGA onset decreased from 34.1 ± 10.1 years to 31.6 ± 10.9 (4). To extend this knowledge base, our group conducted a survey of 41 dermatologists.

MATERIALS AND METHODS

Forty-one dermatologists in four countries were surveyed. The dermatologists varied from private clinic dermatologists to hospital based dermatologists. The dermatologists were selected based on their expertise in alopecia. Each dermatologist was in the physician network of at least one of the co-authors. Phone calls were placed and the survey was conducted and scored by one of the co-authors. 98% of the patients attended by these dermatologists were Caucasian men. The survey was conducted between 2016-2017.

RESULTS

In a survey of 41 dermatologists in the US and Europe, we found that the majority (88%) of dermatologists believe that the incidence of AGA is increasing as is evident in younger patients (<29 years old) presenting with AGA.

DISCUSSION

No apparent explanation for the increased incidence of AGA at younger age groups exists. However, due to the strong genetic inheritance component of AGA, we theorized the underlying cause was genetic. The risk for developing AGA is multi-factorial, however, a genetic disposition is necessary for AGA to develop. In the presence of elevated dihydrotestosterone (DHT), hair follicles highly sensitive to androgens miniaturize, eventually leading to the development of AGA (5). Androgen sensitivity is primarily mediated by the androgen receptor (AR). The length of the polyglutamine (PolyQ) repeat in the AR N-terminal domain affects DHT binding affinity to the AR and hence mediates AR sensitivity to androgens (6). The length of the PolyQ repeat is determined by the CAG tri-nucleotide repeat encoded in the AR gene. Several genetic association studies have established that a lower number of CAG repeats in the androgen receptor gene of males is associated with the AGA phenotype. Sawaya et al. (1998), demonstrated that males with AGA had 19 ± 3 CAG repeats in the AR gene compared to normal controls who had 22 ± 4 CAG repeats in the AR gene (7). In another study, Ellis et al. (2001), demonstrated that short (<21) CAG and GGC repeats were present in 50% of young

Table I. *Dermatologist survey responses.*

Survey Question: In the past five years have you witnessed an increase in incidence of AGA in males <29 years old?			
Country	Dermatologists Surveyed	Yes	No
US	23	20	3
Italy	9	7	2
Spain	6	6	0
Croatia	3	3	0
Total	41	36 (88%)	5 (12%)

men (18-39 years) presenting with AGA compared to 29.5% in controls (8).

As the androgen receptor gene is located on the X chromosome, the inheritance is maternal. As such, we looked for social, behavioral, or environmental factors that may increase the frequency of alleles with shorter version of the CAG repeat in the AR gene. Chatterjee et al. (2009) demonstrated in 168 patients (78 patients with POF and 90 control) that longer CAG repeat length was associated with higher risk of premature ovarian failure (POF) (9). Shiina et al. (2006) studied mice that lack AR activity, a condition similar to androgen insensitivity syndrome most often associated with a longer CAG repeat length. In their study, they demonstrated that mice lacking AR activity suffered from POF and lower antral follicle numbers (10). This suggests, a short CAG repeat is associated with a phenotype exhibiting a higher antral follicle count and lower risk for POF.

Furthermore, a shorter CAG repeat length and increased AR activity is associated with the development of polycystic ovary syndrome (PCOS), a common endocrine disorder in pre-menopausal women that is characterized by hyperandrogenism, ovulatory dysfunction, and polycystic ovaries (11). However, despite increased difficulty in conception and childbirth, results of a recently published study by Carmine et al. (2015), demonstrate that approximately 50% of anovulatory patients with PCOS are able to ovulate in their late reproductive age (40-45 years), with approximately 10% of patients exhibiting a complete remission of all PCOS-related symptoms (12). This supports the data from Elting et al. (2000) who demonstrated a strong trend between age and regular menstrual cycle length in women with PCOS, with the percentage of women with regular menstrual cycles increasing from approximately 41% of women aged 30-35 to 100% in women aged 51-55 (13). In addition, Tehrani et al. (2010) observed in a study on 174 women (85 with PCOS) that estimated age of menopause in PCOS cases was 2 years later (age 51) compared to controls (age 49) (14).

The short CAG allele in women is associated with ovulation at a later age, higher antral follicle count, and lower risk for premature ovarian failure. Taken together, the evidence suggests that women with

shorter CAG repeat (< 22 repeats) in the AR gene are likely to be able to conceive later in life compared to women with a longer CAG repeat. Given the social selective pressure to conceive later in life, these women would have a selective advantage to conceive later in life and would thus favor offspring exhibiting AGA.

In conclusion, in the past three decades, women in first world societies are delaying parenthood to a later age. Attempting to conceive at a later age is associated with childlessness due to declined fertility and increased risk of fetal death (15). Thus, social selection to reproduce later in life favors females that have a higher chance of late-age conception, i.e., carriers of a short CAG repeat allele in the AR gene. Consequently, we put forth the theory that social selection for later conception favors offspring prone to the development of AGA.

REFERENCES

1. Norwood OT. Male pattern baldness: classification and incidence. *South Med J* 1975; 68(11):1359-65.
2. Gan DC, Sinclair RD. Prevalence of male and female pattern hair loss in Maryborough. *J Investig Dermatol Symp Proc* 2005; 10(3):184-9.
3. Rhodes T, Girman CJ, Savin RC, et al. Prevalence of male pattern hair loss in 18-49 year old men. *Dermatol Surg* 1998; 24(12):1330-2.
4. Jang WS, Son IP, Yeo IK, et al. The annual changes of clinical manifestation of androgenetic alopecia clinic in Korean males and females: an outpatient-based study. *Ann Dermatol* 2013; 25(2):181-8.
5. Trüeb RM. Pharmacologic interventions in aging hair. *Clin Interv Aging* 2006; 1(2):121-29.
6. Chamberlain NL, Driver ED, Miesfeld RL. The length and location of CAG trinucleotide repeats in the androgen receptor N-terminal domain affect transactivation function. *Nucleic Acids Res* 1994; 22(15):3181-86.
7. Sawaya ME, Shalita AR. Androgen receptor polymorphisms (CAG repeat lengths) in androgenetic alopecia, hirsutism, and acne. *J Cutan Med Surg* 1998; 3(1):9-15.
8. Ellis JA, Stebbing M, Harrap SB. Polymorphism of the androgen receptor gene is associated with

- male pattern baldness. *J Invest Dermatol* 2001; 116(3):452-55.
9. Chatterjee S, Singh R, Kadam S, Maitra A, Thangaraj K, Meherji P, Modi D. Longer CAG repeat length in the androgen receptor gene is associated with premature ovarian failure. *Hum Reprod* 2009; 24(12):3230-35.
 10. Shiina H, Matsumoto T, Sato T, et al. Premature ovarian failure in androgen receptor-deficient mice. *Proc Natl Acad Sci USA* 2006; 103(1):224-29.
 11. Peng CY, Xie HJ, Guo ZF, Nie YL, Chen J, Zhou JM, Yin J. The association between androgen receptor gene CAG polymorphism and polycystic ovary syndrome: a case-control study and meta-analysis. *J Assist Reprod Genet* 2014; 31(9):1211-19.
 12. Carmina E. Reproductive system outcome among patients with polycystic ovarian syndrome. *Endocrinol Metab Clin North Am* 2015; 44(4):787-97.
 13. Elting MW, Korsen TJ, Rekers-mombarg LT, Schoemaker J. Women with polycystic ovary syndrome gain regular menstrual cycles when ageing. *Hum Reprod* 2000; 15(1):24-28.
 14. Tehrani FR, Solaymani-Dodaran M, Hedayati M, Azizi F. Is polycystic ovary syndrome an exception for reproductive aging? *Hum Reprod* 2010; 25:1775-81.
 15. Schmidt L, Sobotka T, Bentzen JG, Nyboe Andersen A. Demographic and medical consequences of the postponement of parenthood. *Hum Reprod Update* 2012; 18(1):29-43.

LETTER TO THE EDITOR

EFFECTIVENESS OF ACUPUNCTURE IN THE MANAGEMENT OF CERVICAL SPONDYLOSIS: A META ANALYSIS

Y-Z. DENG¹, L-G. XU¹, L. CHEN², D. ZHOU³ and Y. LIU¹

¹Department of Acupuncture, The affiliated Hospital to Changchun University of Chinese Medicine, Changchun, China; ²Department of Hematology and Oncology, The affiliated Hospital to Changchun University of Chinese Medicine, Changchun, China; ³Department of Ophthalmology, The affiliated Hospital to Changchun University of Chinese Medicine, Changchun, China

Received April 11, 2017 – Accepted October 25, 2017

Cervical spondylosis is the most common type of spinal pathology which is more common in middle-aged or senile populations with a high potential to affect physical and mental health. This study evaluates the effectiveness of acupuncture in the management of cervical spondylosis. After a detailed literature search in electronic databases, the required data were acquired from selected research articles and meta-analyses were performed to obtain the percent cure, failure and total effectiveness rates under random effects model. Meta-regression was performed to identify the factors affecting the efficacy. Twenty-seven studies were selected for data acquisition (2,853 patients; average age 46.2 ± 9.5 ; $51.7 \pm 12.5\%$ males). Acupuncture alone had 33.41% (25.50, 41.24) cure rate but in combination with other therapies it rose to 53.36% (41.9, 64.8). Similarly, total effectiveness rate was 87.01% (83.40, 90.62) with acupuncture alone and 93.62% (89.85, 97.38) with acupuncture in combination with other therapies. Age was inversely associated with the percent cure rate and the number of combination therapies with acupuncture was positively associated with the cure rate. In conclusion, acupuncture therapy alone can provide cure and total effectiveness rates of 33% and 87%, respectively, but acupuncture with additional therapies can improve the cure and total effectiveness rates to 53% and 94%, respectively. Age inversely affects efficacy and the number of additional therapies improves the efficacy.

To the Editor,

Cervical spondylosis (CS) is a chronic spinal disc degenerative pathology which manifests as cervical radiculopathy, cervical myelopathy, or cervical radiculomyelopathy. The degenerative changes may affect the nerve root, spinal cord, vertebral artery, or sympathetic nerve to induce clinical manifestations (1). It can be asymptomatic or may present with neck/shoulder pain or stiffness and numbness or lack of strength of the upper limbs and fingers (2). Poor posture, anxiety, depression, neck strain, and sporting or occupational activities are considered as

the contributing factors for its etiology (3).

CS is the most common type of spinal pathology and is more common in middle-aged or senile populations with potential to affect physical and mental health to a greater extent (4). The prevalence of cervical spondylosis in over-40-year-old individuals is approximately 50%, and in individuals over 60 years of age, the prevalence is 85% upon radiological diagnosis (5). In normal Chinese adults, CS incidence is estimated at 3.8-17.6% (6).

Surgery is the most effective treatment for patients with intractable pain but non-surgical treatments are

Key words: cervical spondylosis, acupuncture, moxibustion, cure, efficacy

Mailing address:

Prof. Yang Liu,
Department of Acupuncture,
The affiliated Hospital to Changchun University of Chinese
Medicine, No. 1478 Gongnong Road,
Changchun, China.
e-mail: drliuyang@outlook.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
**DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF
INTEREST RELEVANT TO THIS ARTICLE.**

usually considered for the first line management. Stress management and/or postural exercises, physiotherapy, and the use of non-steroidal anti-inflammatory drugs and muscle relaxants have been found to be effective (7). Another strategy utilizes various forms of traditional Chinese medicine of which acupuncture and related therapies such as traction (8), massage (9), Chinese herbal decoction (10) and acupoint injections (11) are popular.

A number of studies have evaluated the effectiveness of acupuncture in CS patients, either as sole treatment or along with other related therapies, but the outcomes vary across the studies. The aim of the present study was to evaluate the efficacy of acupuncture alone or in combination with other related therapies for CS patients in a weighted pooled analysis.

MATERIALS AND METHODS

This meta-analysis was performed using Cochrane Collaboration guidelines and is reported in accordance with PRISMA statement.

Literature search

A literature search was carried out in several electronic databases (Embase, Google Scholar, PubMed, Ovid SP, EBSCO) by using relevant keywords including cervical spondylosis, radiculopathy, myelopathy, vertigo, acupuncture, electro-acupuncture, hydro-acupuncture, tuina, massage, chiropractice, neck pain, moxibustion, warm needle, stone needle, plum-blossom needle, cupping, and points which were used in logical combinations. The search encompassed original research papers published in English before August 2016.

Inclusion and exclusion criteria

Studies which evaluated acupuncture therapy in cervical spondylosis patients, either as sole therapy or along with other related therapies in a single arm or multi-arm design, were included in the meta-analysis. The participants in these studies were CS (radiculopathy, myelopathy, vertigo) patients. Studies which evaluated the efficacy of acupuncture therapy with or without a related non-medical therapy were included in the meta-analysis, whereas studies which involved acupuncture or related medical therapy/surgery were excluded.

Data analysis

Data were extracted from respective research articles independently by 2 reviewers. Inter-rater reliability was good (Cohen kappa = 0.94). Outcomes of interest for this meta-analysis were the percent cure, improvement, failure, and total effectiveness rates. Random effect meta-analyses were performed to achieve overall effects of the percent cure, improvement, failure and total effectiveness rates in which the overall effect of a treatment was a weighted average of the inverse variance adjusted individual effects. Significance of difference between groups was tested by two-tailed z-test. Between-study inconsistency was assessed with I^2 index. Meta-analyses were performed with Stata software (version 12; Stata Corporation; Texas, USA).

For sub-group analyses, the following subgroups were evaluated: acupuncture therapy alone; acupuncture in combination with moxibustion; acupuncture in combination with cupping; acupuncture in combination with moxibustion and cupping; acupuncture in combination with tuina; acupuncture in combination with chiropractic therapy; and acupuncture in combination with fumigation.

Metaregression analyses were also performed with Stata software using restricted maximum likelihood method. Dependent variable percent cure rate was tested for its possible relationships with several independent variables including sample size, follow-up, age, gender, disease duration and the number of additional therapies.

RESULTS

Twenty-seven studies were selected for data acquisition. The overall population of this meta-analysis was 2,853 patients. The average patient age was 46.2 ± 9.5 (range 15–70) years, and $51.7 \pm 12.5\%$ were males; average disease duration was 3.8 ± 2.7 (0.1–53) years.

In the overall population of this meta-analysis, acupuncture treatment either alone or in combination with other related therapies achieved a cure rate (95% confidence interval) of 42.47% (36.22, 49.73). However, acupuncture alone was associated with 33.41% (25.50, 41.24) cure rate whereas cure rate with acupuncture in combination with other related therapies was 53.36% (41.9, 64.8). Effect sizes of various combinations are given in Fig. 1.

In the overall meta-analysis, the acupuncture treatment either alone or in combination with other related therapies achieved a total effectiveness rate of 90.1% (87.31, 31.89). Acupuncture alone was associated with 87.01% (83.40, 90.62) total effectiveness rate, whereas with acupuncture in combination with other TCM therapies it was 93.62% (89.85, 97.38). Effect sizes of various combinations are given in Fig. 2.

In the overall meta-analysis, the acupuncture treatment alone or in combination with other related

therapies was associated with a failure rate of 10.1% (8.60, 11.80). Acupuncture alone was associated with 11.36% (9.02, 13.70) whereas in combination with other TCM therapies acupuncture had 8.29% (6.30, 10.29) failure rate. Effect sizes of the failure rate with acupuncture-moxibustion combination was 3.39% (1.74, 5.05), and with acupuncture-cupping combination was 11.00% (5.63, 16.37). For other combinations the number of studies was not adequate for achieving reliable effect size/s.

In metaregression analyses, the age was inversely

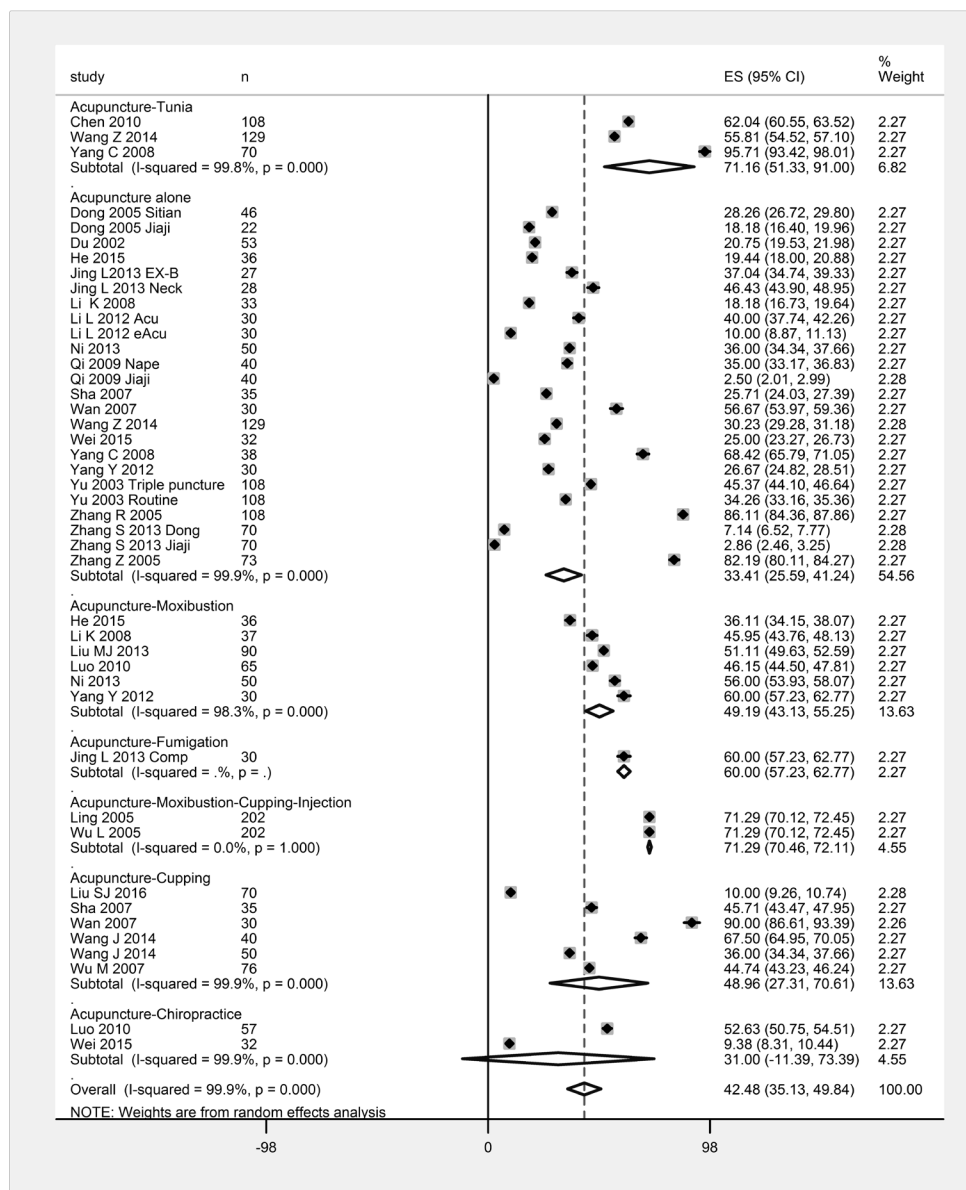


Fig. 1. Forest graph showing the percent cure rate of individual studies and overall/sub-groups-wise effect sizes.

associated with the percent cure rate [coefficient = -1.167 ($-2.206, -0.129$); $p=0.029$; Fig. 3a]. However, this inverse relationship failed to attain statistical significance in the metaregression analysis either with the acupuncture only data [coefficient -1.148 ($-2.587, 0.292$); $p=0.111$] or with acupuncture in combination with other related therapies [coefficient -0.930 ($-2.347, 0.486$); $p=0.184$].

The number of combination therapies with

acupuncture was positively associated with the cure rate [coefficient 10.569 ($2.679, 18.459$); $p=0.010$; Fig. 3b].

DISCUSSION

This meta-analysis observed that acupuncture therapy alone can provide cure rate and total effectiveness rate was 33% and 87%, respectively.

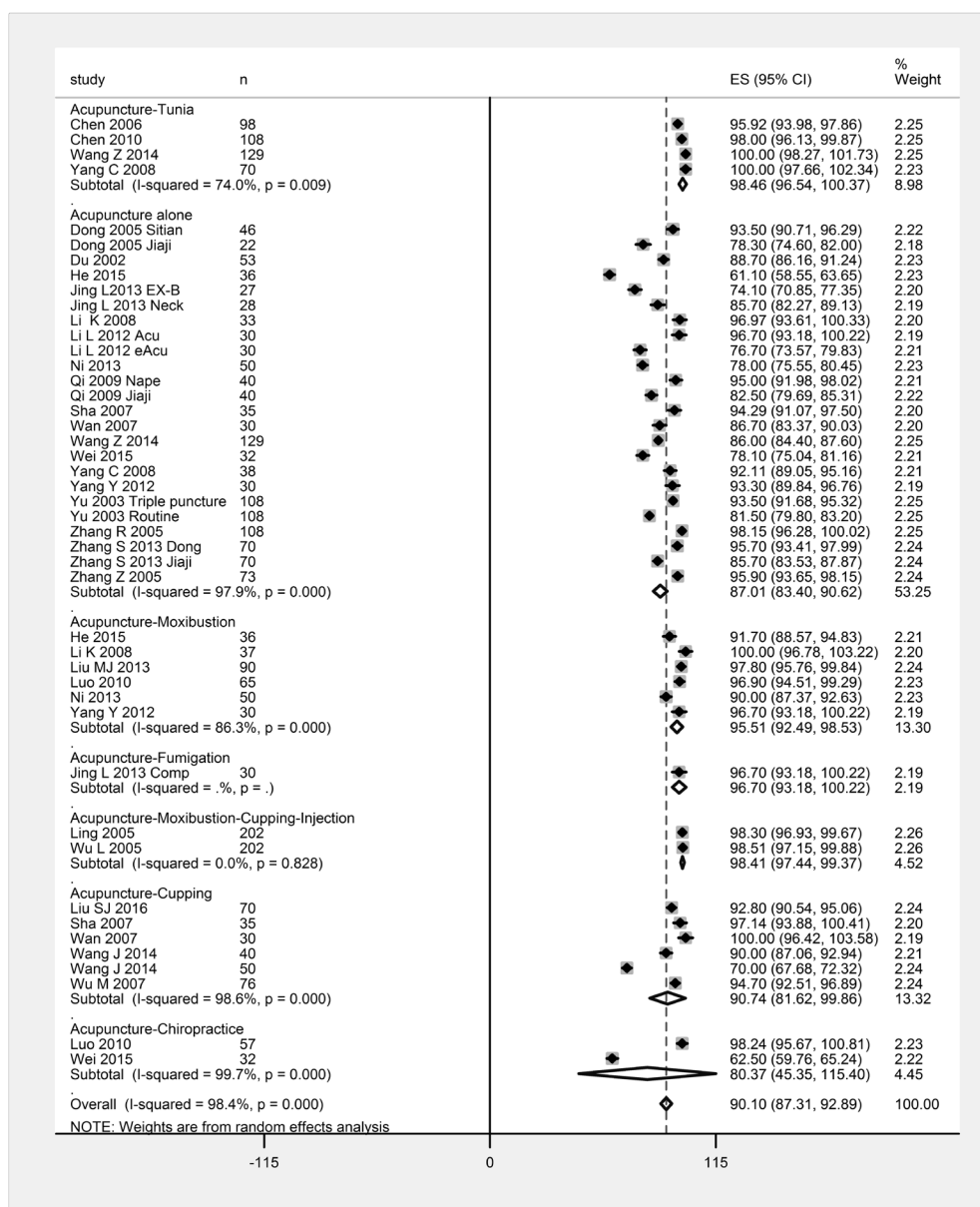


Fig. 2. Forest graph showing the total effectiveness rate of individual studies and overall/sub-groups-wise effect sizes.

Additional therapies with acupuncture can improve the cure rate and total effectiveness rate to 53% and 94%, respectively. Moreover, the number of additional therapies was positively associated with the efficacy. However, age was found to be inversely associated with efficacy.

Advantages of acupuncture treatment for cervical pathologies include high efficacy, low cost, and less adverse reactions. Acupuncture can improve local

blood circulation, relieve muscle spasm and resorb nerve root edema to release local adhesions and nourish nerves (12). In traditional Chinese medicine philosophy, an acupoint is a special position where qi of human viscera and meridians is infused and spread to the body surface. It is not only a special place where qi and blood converge, transfer and pass in and out but also a stimulation point for promoting flow of qi and blood supply to enhance visceral

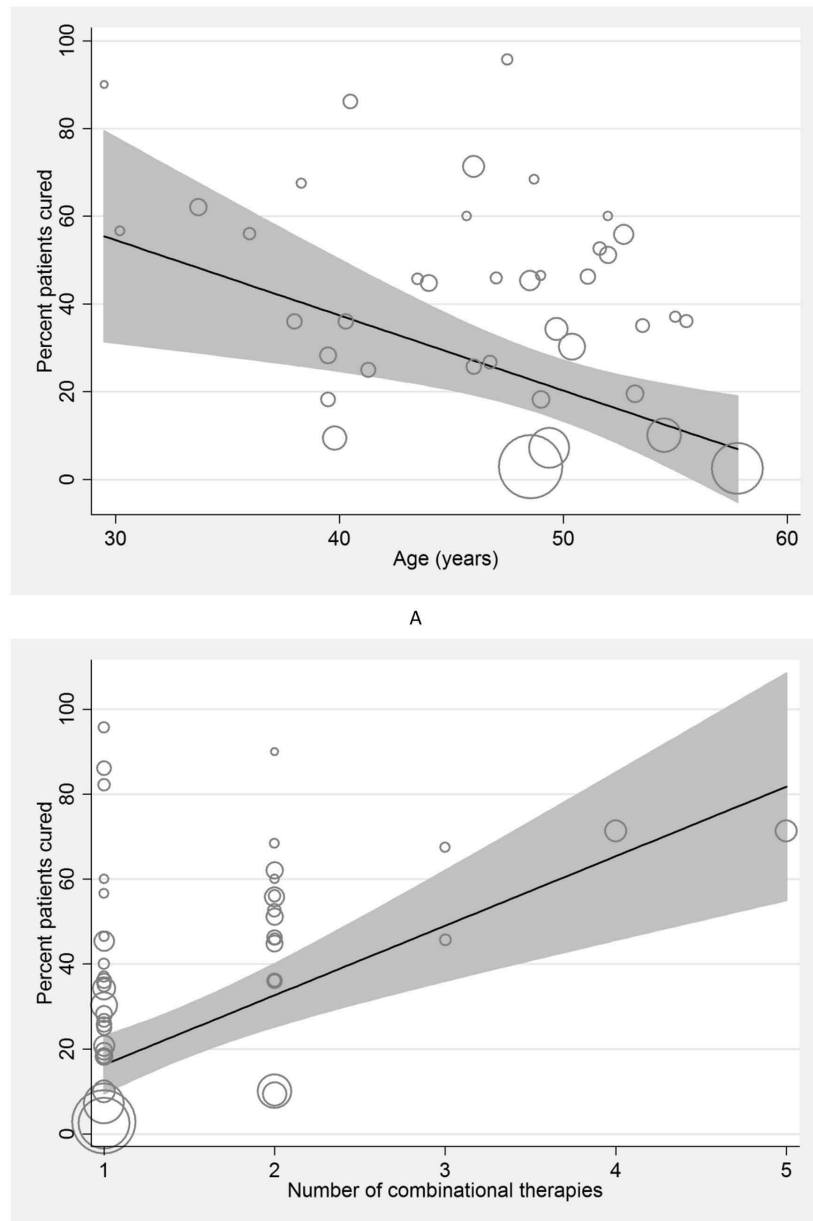


Fig. 3. Meta-regression scatterplot showing the relationship between the cure rate and **a)** age of the patients, and **b)** the number of additional therapies used.

functions (13). It has effects of dredging meridians, expelling wind and cold, and stimulating Qi and blood circulation towards other acupoints as well (12).

However, even though the use of acupuncture is growing, evidence regarding its efficacy as a therapeutic strategy is not considered adequately convincing based on the methodological grounds. The vast majority of systematic reviews attempted to evaluate the efficacy of acupuncture objected to the quality of the trials in their conclusion(14). Flaws included small sample size, short duration, lack of follow-up, and variation in the composition of acupuncture (15). Also in the present study insufficient follow-up information was available in the respective articles. Keeping in mind these shortcomings, many trial protocols are now published depicting better designs (for example ChiCTR-TRC-12002206 and ChiCTR-TRC-13003422). Thus, in coming years with the availability of the outcomes of these better designed randomized trials, the state of evidence will improve.

REFERENCES

1. Fu WB, Liang ZH, Zhu XP, Yu P, Zhang JF. Analysis on the effect of acupuncture in treating cervical spondylosis with different syndrome types. *Chin J Integr Med* 2009; 15:426-30.
2. Rao RD, Currier BL, Albert TJ, Bono CM, Marawar SV, Poelstra KA, Eck JC. Degenerative cervical spondylosis: clinical syndromes, pathogenesis, and management. *J Bone Joint Surg Am* 2007; 89:1360-78.
3. Ferrara LA. The biomechanics of cervical spondylosis. *Adv Orthop* 2012; 2012: 493605.
4. Zhang XF, Gao YB, Luo CH, Sun LY, Yang H. The application of X-ray on the diagnosis of cervical spondylosis. *Xian Dai Yi Yong Ying Xiang Xue* 2008; 17:329-31.
5. Matsumoto M, Fujimura Y, Suzuki N, Nishi Y, Nakamura M, Yabe Y, Shiga H. MRI of cervical intervertebral discs in asymptomatic subjects. *J Bone Joint Surg Br* 1998; 80:19-24.
6. Zhu AJ. The applied anatomy research in adult cervical spondylosis in age changes with multi-slice spiral CT. *Med Information*. 2010; 23:1319-21.
7. Borenstein DG. Chronic neck pain: how to approach treatment. *Curr Pain Headache Rep* 2007; 11:436-39.
8. Xu HR, Wang WJ. Effect observation of cervical spondylotic radiculopathy with radian draught gauge. *Xian Dai Zhong Xi Yi Jie He Za Zhi* 2010; 2:182-83.
9. Huang JQ. Effect observation of 300 case reports of cervical spondylotic radiculopathy with Manipulation mainly. *Zhong Guo Dang Dai Yi Yao* 2010; 17:89-90. Ren JL. Fifty-eight case reports of cervical spondylotic radiculopathy treated with the decoction of Jing Jian Bi Tong. *Si Chuan Zhong Yi Za Zhi* 2010; 4:111-12.
10. Dong WK, Lin XH. Clinical Observation on Cervical Spondylosis of Neck Type Treated With Acupuncture at Original and Terminal Points of Trapezius. *Zhongguo Zhen Jiu* 2012; 32:211-14.
11. Wang J, Zhang J. Clinical observation on acupuncture combined with hyperbaric oxygenation in treating patients with cervical spondylosis of nerve root type in acute phase. *J Acupunct Tuina Sci* 2014; 12:236-40.
12. Yang L, Huang Q, Wu H, et al. Analysis of the frequency of clinical acupoint use based on 'traditional chinese medical acupuncture-moxibustion information databank'. *J Acupunct Tuina Sci* 2014; 12:80-8.
13. Manheimer E, Wieland S, Kimbrough E, Cheng K, Berman BM. Evidence from the Cochrane Collaboration for Traditional Chinese Medicine therapies. *J Altern Complement Med* 2009; 15:1001-14.
14. Sun ZR, Yue JH, Zhang QH. Electroacupuncture at Jing-jiaji points for neck pain caused by cervical spondylosis: a study protocol for a randomized controlled pilot trial. *Trials* 2013; 14:360.

LETTER TO THE EDITOR

ASSOCIATION OF BCL-2 WITH OXIDATIVE STRESS AND TOTAL ANTIOXIDANT STATUS IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

I.M. TAHIR¹, T. IQBAL¹, A. JAMIL² and M. SAQIB³

¹Pharmaceutical Research Lab, Department of Biochemistry, University of Agriculture, Faisalabad-Pakistan; ²Molecular Biochemistry Lab, Department of Biochemistry, University of Agriculture, Faisalabad-Pakistan; ³Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad-Pakistan

Received May 5, 2017 – Accepted October 5, 2017

B-Cell Lymphoma protein-2 (BCL-2) is one of the most studied proteins with substantial regulatory potential for both apoptosis and autophagy. BCL-2 confer chemoresistance through influencing cancer pathophysiology. Serum level of lactate dehydrogenase (LDH) predicts increased anaerobic glycolysis and is associated with metabolic modulation in cancer cells. In the present research, the interplay of BCL-2, total oxidative status (TOS) and LDH was investigated in patients with acute lymphoblastic leukemia (ALL). The studied parameters, BCL-2 protein ($p < 0.001$), TOS ($p < 0.001$) and LDH ($p < 0.001$) were significantly elevated in the ALL group compared to the normal group (N-group). However, the total antioxidant status (TAS) was reduced significantly ($p < 0.01$) in ALL patients. In the ALL group, the TOS had significant negative correlation with TAS ($p < 0.01$). Furthermore, non-significant positive correlations were found between BCL-2 and LDH, BCL-2 and TAS and LDH and TAS (each with; $p > 0.05$). However, a negative non-significant correlation was observed between BCL-2 and TOS and LDH and TOS (each with; $p > 0.05$).

To the Editor,

BCL-2 is an oncoprotein with a well-established anti-apoptotic potential (1). It also possesses the ability to counteract the autophagy through a beclin-dependent mechanism (2). Apoptosis, being widely accepted as an adopted mechanism of a variety of chemotherapeutic agents, is receiving substantial attention for chemotherapy concerns (3). The tumor microenvironment with high reactive oxygen species (ROS) is attributed to the oxidative stress leading towards the worsening of cancer progression and poor outcome of anticancer therapy (4). Generally, BCL-2 contributes to chemoresistance through counterbalancing the apoptosis induced by anticancer drugs (5).

Apoptosis ensures the integrity and conservative recycling of the tissue components throughout the cellular origin, differentiation and the stress conditions leading towards aging.

The specific activity of evolutionarily conserved BCL-2 homology domains (BH1, BH2, BH3, and BH4) are attributed to confer the particular, pro- or anti-apoptotic characteristics to the BCL-2 protein family (6). BCL-2-mediated regulation of apoptosis is predominantly based on unloading and activation of proactive caspases and cytochrome c through permeabilization of mitochondrial membrane (7).

The overwhelmed *in-vivo* oxidation state is appreciated as “oxidative stress”, predisposing

Key words: apoptosis, childhood leukemia, free radicals, lactate dehydrogenase (LDH), oxidative stress

Mailing address:

Imtiaz Mahmood Tahir,
Pharmaceutical Research Lab,
Department of Biochemistry,
University of Agriculture, Agriculture University Road,
Faisalabad, Zip Code; 38000, Pakistan
Tel.: +923327209967
e-mail: imtiazmlt@hotmail.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties

DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

to a variety of diseases such as cancer, infertility, diabetes and other neurological, cardiac and pulmonary diseases (8, 9). Reactive oxygen species (ROS) or oxidants are reactive chemical byproducts of basal metabolism potentially harmful to the cytoskeleton and macromolecules (10). Natural antioxidants are the functional antagonist of ROS (11, 12). In the present research, the association of BCL-2 with oxidative stress (TOS) and LDH activity was studied in childhood ALL.

MATERIALS AND METHODS

Study population

A total of 49 (n=49) patients diagnosed with acute lymphoblastic leukemia confirmed by bone marrow examination were enrolled. The enrolled subjects' ages ranged from 1.6 to 13 years with median 5.5 years and male to female ratio was 1.6: 1. A group of twenty (n=20) healthy volunteers (50% male) was included as a normal group (N-group). All patients being treated with a standard

chemotherapy protocol receiving the same drug combination (induction chemotherapy: comprising daunorubicin, vincristine, prednisone and asparaginase) were enrolled in the present study. During the course of the aforesaid therapeutic regimen, the patients representing the remission phase were presented with chemotherapy-induced pancytopenia. Hence the inclusion criteria for current research was set at the preceding bone marrow findings with more than 25% lymphoblast count with diagnostic morphology. The patients with viral hepatitis, diabetes or any type of cancer diagnosed other than ALL were excluded.

The research work was conducted as per the institutional bioethics and research standards. Written informed consent was given by each patient/attendant, followed by the detailed description of the research protocols and requirements in an understandable manner.

Collection of blood samples

Blood samples were collected from the study subjects/normal group and processed to obtain separated cell-free plasma and was stored at -20°C until further analysis.

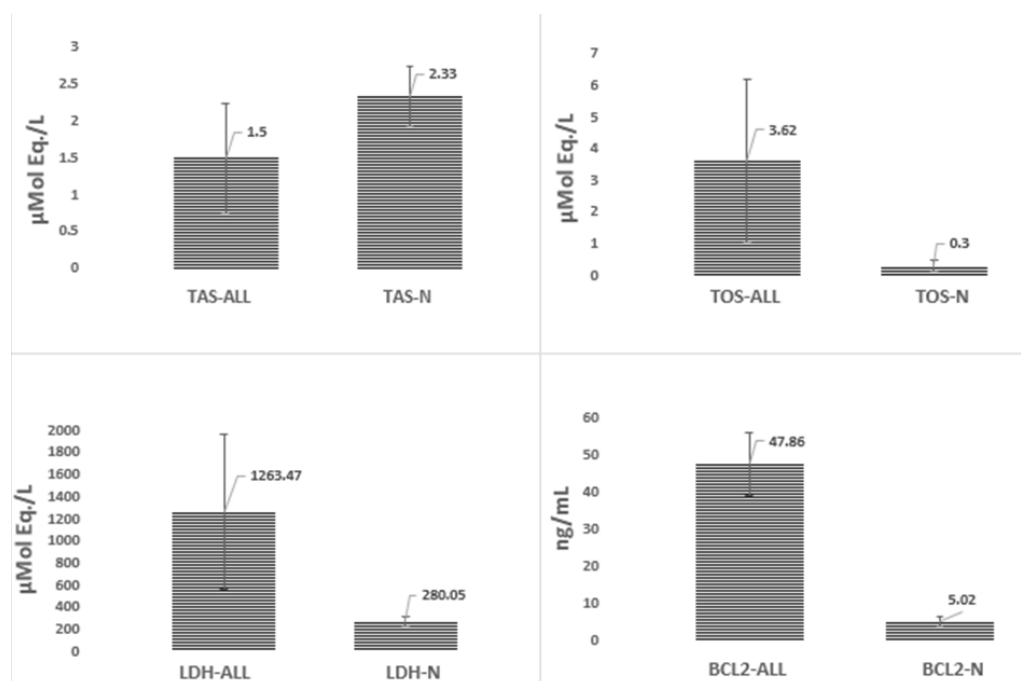


Fig. 1. Bar charts representing the mean $\pm$ SD values of TAS-ALL and TAS-N; Total antioxidant status in ALL patient and in normal group, p -value <0.001 (Upper left), TOS-ALL and TOS-N; Total oxidant status in ALL patient and in normal group, p -value <0.001 (Upper right), LDH-ALL and LDH-N; Lactate dehydrogenase in ALL patient and in normal group, p -value <0.001 (Lower left), BCL2-ALL and BCL2-N; B-cell lymphoma protein in ALL patient and in normal group, p -value <0.001 (Lower right).

Measurement of serum LDH

Serum LDH activity (IU/mL) was determined spectrophotometrically on semi-automated clinical chemistry analyzer Microlab 300™ (Merck, Germany) following DGKC guidelines.

Measurement of total oxidant status

Plasma total oxidant status was measured by a method validated by Erel (2005); a modified FRAP [Ferric reducing ability of plasma] assay. Measurement of total oxidant status (TOS) in plasma samples was based on the cumulative oxidative potential of a variety of oxidant species against ferrous ions. At acidic pH, the xylenol orange concentration proportional to the conversion of ferrous to ferric ions was measured spectrophotometrically at 560 nm.

Measurement of total antioxidant status

The total antioxidant capacity of leukemia blood samples was measured by a direct method by Erel (2004) based on ABTS [2,2V-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] discoloration by all potential antioxidants present in plasma measured at 660 nm.

Measurement of BCL-2 protein

Highly specific double antibody ELISA (Elabscience Biotech, USA) was used for the quantitative analysis of plasma BCL-2 level on automated ELISA reader (BioTek, μ -Quant™ USA). Lyophilized reference standard (10 ng/ml) provided by the manufacturer, was serially diluted with standard diluent to make concentrations of 5, 2.5, 1.25, 0.625, 0.312, and 0.156 ng/ml, and the diluent itself was used as a zero standard.

Table I. Comparison of listed parameters between ALL and control group.

Variables	Normal Group (n=20) Mean $\pm$ SD	ALL Patients (n=49) Mean $\pm$ SD	95 % CI	p- Value
BCL-2 ng/mL	5.02 $\pm$ 1.33	47.86 $\pm$ 8.56	(40.32, 45.37)	<0.001
TOS H ₂ O ₂ μ Mol Eq./L	0.301 $\pm$ 0.18	3.62 $\pm$ 2.58	(2.577, 4.067)	<0.001
TAS Vit C μ Mol Eq./L	2.33 $\pm$ 0.41	1.49 $\pm$ 0.76	(-1.104, -0.543)	<0.001
LDH IU/L	280 $\pm$ 44	1263 $\pm$ 696	(782, 1184)	<0.001

Student's t-test statistics for studied parameters among ALL group and normal group. The p-value ≤ 0.05 is considered of significant. (BCL-2; B-Cell Lymphoma-2 Protein, TOS; Total oxidant status, TAS; Total antioxidant status, LDH; Lactate dehydrogenase, CI; Confidence interval).

Table II. Correlation between listed variables in ALL group.

Variables	BCL-2	LDH	TOS	TAS
BCL-2	1			
LDH	0.19	1		
TOS	-0.09	-0.25	1	
TAS	0.08	0.2	-0.45*	1

Correlation matrix for studied parameters in ALL patients. The minus symbol (-) of r-values shows a negative correlation. Asterisk (*) shows a significant negative correlation between TOS and TAS ($p < 0.01$).

A linear standard curve ($R^2=0.994$) was achieved by the reference standards of BCL-2.

Statistical analysis

Student's *t*-test was performed through Minitab™ statistical software (version 17) for statistical analysis of data. At 95% CI for the differences between means, the significance was obtained at the p -value ≤ 0.05 . Results for the studied parameter in both ALL and N-group were presented as mean $\pm$ SD.

RESULTS

All studied parameters were found significantly different among the ALL group and the N-group (Table I). The Mean $\pm$ SD values of TOS, TAS, LDH and BCL-2 in both groups are presented in Fig. 1. A significantly higher protein expression of BCL-2 ($p<0.001$) was found in the ALL group as compared to the N-group. The TOS ($p<0.001$) and LDH activity ($p<0.001$) was also recorded significantly increased in the ALL group. However, the total antioxidant status (TAS) was found significantly reduced ($p<0.01$) in the ALL patients.

In the ALL group, a non-significant positive correlation was found between BCL-2 and LDH, BCL-2 and TAS and LDH and TAS ($p>0.05$). A negative non-significant correlation was observed between BCL-2 and TOS and LHD and TOS ($p>0.05$). A significant negative correlation was recorded between TOS and TAS ($p<0.01$). The correlation coefficients for the studied parameters are given in Table II.

DISCUSSION

In our study, a significantly elevated level of BCL-2 associated with oxidative stress in acute lymphoblastic leukemia patients was recorded as compared to the normal group. Generally, the altered apoptotic pathway, as a consequence of hyperexpression of BCL-2 and associated proteins, contributes to MDR and sensitivity (15). The higher level of BCL-2 was found to be associated with poor prognosis in various hematological malignancies such as follicular lymphoma, CLL and AML.

Contrarily, Coustan-Smith, et al. (1996) reported that the hyper-expression of BCL-2 was associated with better survival and reduced chances of recurrence in childhood ALL, which is supported by the fortunate fact of approximately 90% survival and remission rate of ALL cases (17).

The higher level of total oxidative stress (TOS) recorded in our research work is attributed to a variety of cancer modulations in energy metabolism and administration of anticancer drugs in ALL patients (18). These research findings are also strengthened by the work of Mazor, et al. (2008). However, this mutual interaction needs to be elucidated further on the molecular basis. Leukemia cells are subjected to relatively higher oxidative stress because of their enhanced dependence on oxidative respiration rather than glycolysis. The oxidative stress-triggered cell death may result in an improved therapeutic sensitivity and prognosis in childhood ALL (10). A Higher level of LDH in ALL patients also entails the metabolic switching of glycolysis to its anaerobic pathway even in non-hypoxic conditions; appreciated as Warburg effect (20).

ACKNOWLEDGEMENTS

The Authors highly acknowledge the contributions of the Jinnah Hospital (Oncology), Lahore, Dr. Tahir Bashir and Dr. Ali Raza (Medical oncologists), and Prime care Hospital (Pathology), Faisalabad, regarding patient recruitment and the clinical assessment.

REFERENCES

1. Vervliet T, Parys JB, Bultynck G. Bcl-2 proteins and calcium signaling: complexity beneath the surface. *Oncogene* 2016; 35:5079-92.
2. Pattingre S, Tassa A, Qu X, et al. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* 2005; 122:927-39.
3. Zhivotovsky B, Kroemer G. Apoptosis and genomic instability. *Nat Rev Mol Cell Biol* 2004; 5:752-62.
4. Al-Tonbary Y, Al Hasan SA, Zaki M, Hammad A, Kandil S, Fouda A. Impact of antioxidant status and apoptosis on the induction phase of chemotherapy in childhood acute lymphoblastic leukemia. *Hematol-*

- ogy 2011; 16:14-19.
5. Srinivas G, Kusumakumary P, Nair MK, Panicker KR, Pillai MR. Bcl-2 protein and apoptosis in pediatric acute lymphoblastic leukemia. *Int J Mol Med* 1998; 1:755-60.
 6. Borner C, Andrews D. The apoptotic pore on mitochondria: are we breaking through or still stuck? *Cell Death Differ* 2014; 21:187-91.
 7. Gross A. BCL-2 family proteins as regulators of mitochondria metabolism. *Biochim Biophys Acta Bioenergetics* 2016; 1857:1243-46.
 8. Valko M, Rhodes C, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem-Biol Interact* 2006; 160:1-40.
 9. Riaz M, Mahmood Z, Shahid M, et al. Impact of reactive oxygen species on antioxidant capacity of male reproductive system. *Int J Immunopathol Pharmacol* 2016; 29:421-5.
 10. Testa U, Labbaye C, Castelli G, Pelosi E. Oxidative stress and hypoxia in normal and leukemic stem cells. *Exp Hematol* 2016; 44:540-60.
 11. Locatelli M, Ferrante C, Carradori S, et al. Optimization of aqueous extraction and biological activity of harpagophytum procumbens root on ex vivo rat colon inflammatory model. *Phytother Res* 2017.
 12. Menghini L, Ferrante C, Leporini L, et al. An hydroalcoholic chamomile extract modulates inflammatory and immune response in HT29 cells and isolated rat colon. *Phytother Res* 2016; 30:1513-18.
 13. Erel O. A new automated colorimetric method for measuring total oxidant status. *Clin Biochem* 2005; 38:1103-11.
 14. Erel O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin Biochem* 2004; 37:277-85.
 15. Reed JC, Miyashita T, Takayama S, et al. BCL2 family proteins: Regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. *J Cell Biochem* 1996; 60:23-32.
 16. Coustan-Smith E, Kitanaka A, Pui C-H, et al. Clinical relevance of BCL-2 overexpression in childhood acute lymphoblastic leukemia. *Blood* 1996; 87:1140-46.
 17. Inaba H, Greaves M, Mullighan CG. Acute lymphoblastic leukaemia. *Lancet* 2013; 381:1943-55.
 18. Mantovani G, Macciò A, Madeddu C, et al. Antioxidant agents are effective in inducing lymphocyte progression through cell cycle in advanced cancer patients: assessment of the most important laboratory indexes of cachexia and oxidative stress. *J Mol Med* 2003; 81:664-73.
 19. Mazar D, Abucoider A, Meyerstein N, Kapelushnik J. Antioxidant status in pediatric acute lymphocytic leukemia (ALL) and solid tumors: the impact of oxidative stress. *Pediatr Blood Cancer* 2008; 51:613-15.
 20. Dromparis P, Sutendra G, Michelakis ED. Metabolic modulation as a novel cancer treatment. *Heart Metab* 2011:20-26.

LETTER TO THE EDITOR

T1 MAPPING COMBINED WITH Gd-EOB-DTPA-ENHANCED MAGNETIC RESONANCE IMAGING IN PREDICTING THE PATHOLOGIC GRADING OF HEPATOCELLULAR CARCINOMA

C.Y. CHEN, J. CHEN, C.C. XIA, Z.X. HUANG and B. SONG

*Department of Radiology, West China Hospital, Sichuan University, Chengdu, China**Received August 24, 2017 – Accepted October 5, 2017*

The aim of this study was to investigate the value of Gd-EOB-DTPA-enhanced MRI on hepatobiliary phase (HBP) imaging and T1 mapping sequence in the differentiation of hepatocellular carcinoma (HCC). A total of 45 patients with HCC who were to undergo a resection were enrolled in this study. Gd-EOB-DTPA-enhanced magnetic resonance examination was performed prior to resection. T1 mapping was performed before and 20 min after injection of Gd-EOB-DTPA. T1 values of the lesions were measured on pre-contrast (T1p) and during HBP (T1-HBP) on T1 maps. The signal intensity, the diameter and the margin of HCC lesions on HBP images were analyzed. The reduction in T1 value (T1d) and the reduction rate ($\Delta T1\%$) of T1 mapping between pre-contrast and HBP were calculated. The Edmondson-Steiner classification of each lesion was made after surgery. The SPSS software package was used for statistical analysis and the analysis of receiver operator characteristic (ROC) curve and area under the curve (AUC) were carried out by using MedCalc software package. Mean values of T1p and T1-HBP were 1935.4 ± 730.8 ms and 1257.1 ± 529.1 ms, respectively. T1p accuracy ($AUC = 0.685, p = 0.037$) in predicting pathological grading was similar to that of T1-HBP ($AUC = 0.751, p = 0.005$). A T1p of 1648.2 ms or greater had a sensitivity and specificity of 85.19% and 61.11%, respectively. A T1-HBP of 1006 ms or greater had a sensitivity and specificity of 81.84% and 61.11%, respectively. The number of HCCs with a non-smooth tumor margin was 20 (44.4%), and a non-smooth tumor margin correlated moderately with the Edmondson-Steiner grade (Spearman $r = 0.491, p = 0.041$). There was no significant correlation between T1d, $\Delta T1\%$, HCC signal intensity on HBP image and lesion diameter with pathologic grading. T1 mapping in pre-contrast and HBP of Gd-EOB-DTPA-enhanced MRI, a non-smooth tumor margin in the HBP of Gd-EOB-DTPA-enhanced MRI, are useful in predicting the pathologic grading of HCC.

To the Editor,

Primary liver cancer is one of the most common malignancies in the world. More than 780,000 cases were diagnosed all over the world, and nearly 750,000 patients died during 2012 (1). Early diagnosis of hepatocellular carcinoma (HCC) and accurate assessment of the malignancy degree are the key points to successful treatment and a favorable prognosis (2). Histological and immunohistochemical

examinations and/or cytology are gold standards for the assessment of malignant HCC, however, the shortcomings are obvious (3, 4). These are invasive and the following limitations should be considered before surgery: immunosuppressed patients with infections of resistant bacterial germs (5, 6); vitamin K-dependent coagulation disorders (7); association with other liver diseases such as nonalcoholic fatty liver (8); and hepatitis with C virus infection (9).

Key words: hepatocellular carcinoma, Gd-EOB-DTPA, T1 mapping, Edmondson-Steiner grade, magnetic resonance imaging

Mailing address:

Dr Bin Song,

No. 37 GuoXue Xiang,

Chengdu, Sichuan, 610041, P.R. China

Tel.: +86 18980601592 - Fax: 028 85592944

e-mail: anicesongbin@163.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties

DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

Currently, T1 mapping is more frequently used for diagnosis of cardiovascular diseases, especially in myocardial fibrosis and myocardial amyloidosis (10), and is less frequently used in the research of liver diseases. The GOLD technique allows dynamic sampling of 2D axial T1 maps of the rat abdomen with 6-second temporal resolution, enabling simultaneous and robust pharmacokinetic modeling of HCC, normal liver, and spinal muscle (11). Both pre-contrast and gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid (Gd-EOB-DTPA)-enhanced MRI T1 mapping images can predict the pathological grading of HCC. The quantitative analysis of T1 mapping in Gd-EOB-DTPA-enhanced MRI hepatobiliary phase (HBP) can help to differentiate liver hemangioma from liver metastases. T1 mapping cannot only observe the morphological characteristics of the nodule, but can also obtain the T1 value quickly, directly and quantitatively, and obtain information regarding Gd-EOB-DTPA uptake and organizational characteristics. Gd-EOB-DTPA is a type of hepatocyte-specific MR contrast medium, with diphasic enhanced function for dynamic enhancement and hepatobiliary phase, which can be used as non-specific extracellular gap contrast medium to observe the blood supply situation (12). Hepatocyte with normal function can specifically uptake Gd-EOB-DTPA during HBP, while HCCs cannot, due to the absence of normal hepatocytes, which helps the detection of HCCs.

We propose the use of T1 mapping on Gd-EOB-DTPA-enhanced MRI and other image parameters that might predict the pathologic grading of HCC.

MATERIALS AND METHODS

Patients

The study was approved by the Sichuan University Ethics Committee, and all patients who participated in the trial signed informed consent. A total of 52 adult patients (age > 18 years) who were admitted to the West China Hospital (the second most popular class 3A hospital located in Chengdu Sichuan province of China) suspected as HCC by ultrasonography, CT or laboratory tests and proposed resection were prospectively included, between May 2015 to March 2016.

The following patients were excluded: patients aged under 18 years; patients who had previously undergone surgery for HCC, including resection or interventional treatment; patients with biliary tract surgery history; patients with severe systemic disease or severe renal and hepatic function insufficiency; patients who had claustrophobia or other reasons that could not endure MRI examination or patients who had MRI contraindication; patients who are allergic to the gadolinium contrast medium; poor breath-hold patients whose image quality was poor and could not meet the diagnostic and evaluation requirements.

Of the 52 patients, 4 were intrahepatic cholangiocarcinoma (ICC) proved by surgery and pathology, 1 was focal nodular hyperplasia (FNH) proved by surgery and pathology, 2 had poor breath-hold whose image quality could not meet diagnostic requirements, therefore all 7 were excluded. Consequently, 45 patients were included in this study. Thirty-four of the enrolled patients were male (75.6%) and 11 were female (24.4%), and the average age was 53.9 ± 12.1 years (range: 28- 83 years).

MRI technique and imaging data

All patients underwent Gd-EOB-DTPA-enhanced MRI examination 7 days prior to surgery. All patients were examined on a Siemens MAGNETOM Skyra 3T scanner (Siemens Healthcare, Erlangen, Germany) with an 18-channel phased-array body coil and spine coil. T1 mapping was acquired using a dual flip-angle 3D gradient-echo sequence with volumetric interpolated breath-hold examination (VIBE) before and 20 minutes after injection of Gd-EOB-DTPA with the following parameters: TE/TR = 1.29/4.00 ms, FA = 2° and 14°; FOV = 380 380 mm², matrix = 192 154, slice thickness = 4 mm, iPAT = 2. Breath training before examination was carried out with each patient. The scan was taken while patients held their breath at the end of natural inspiration.

All patients received a body weight-adjusted dose of Gd-EOB-DTPA (Primovist®, Schering AG, Berlin, Germany, 0.1ml/kg body weight) administered as bolus injection with a flow rate of 2 ml/s, flushed with 20 ml of normal saline at the same rate.

All images were transferred to the Picture Archiving and Communication System (PACS). Image analysis was performed blindly by two experienced radiologists who

independently reviewed all MR images on the Syngo Imaging Workplaces workstation (Version VX91B, Siemens AG, Berlin and Munchen 2013, Germany) and recorded the relevant image data. When the MR findings observed by both readers were inconsistent, the images were reanalyzed and a definitive result was reached by consensus. The final image data were the average of the two measured data.

Tumor margins in the HBP images were classified. If a tumor had a minute budding portion at its periphery protruding into the liver parenchyma, the tumor margin was considered to be non-smooth (Fig. 1). The tumor signal intensity on HBP images were observed. Hypointensity represented lower signal intensity (SI) compared with SI of the liver parenchyma, whereas iso- or hyperintensity represented the same or higher SI, respectively. T1 values of HCCs were measured before (T1p) and during HBP (T1-HBP). The diameter of tumor (accuracy 0.1 cm, D)

was measured on the maximal transverse-section of the maximal tumor on HBP images. T1 difference (T1d) and T1 value reduction rates ($\Delta T1$) were calculated as follows: $T1d = T1p - T1-HBP$; $\Delta T1 (\%) = (T1p - T1-HBP) / T1p < 100$.

The lesions were measured by using the method of drawing the circular regions of interest (ROI). ROIs were drawn on the maximum section of the maximal nodule, to avoid nodular hemorrhage, liquefaction or necrotic areas as much as possible to draw the largest ROI range. Each object was measured on three sections separately and the average of three values was applied for final calculation. ROI drawings on different sequence images were copied at the same level in order to ensure consistent rendering of ROI.

Laboratory, pathological and clinical data

All basic patient information, including gender and

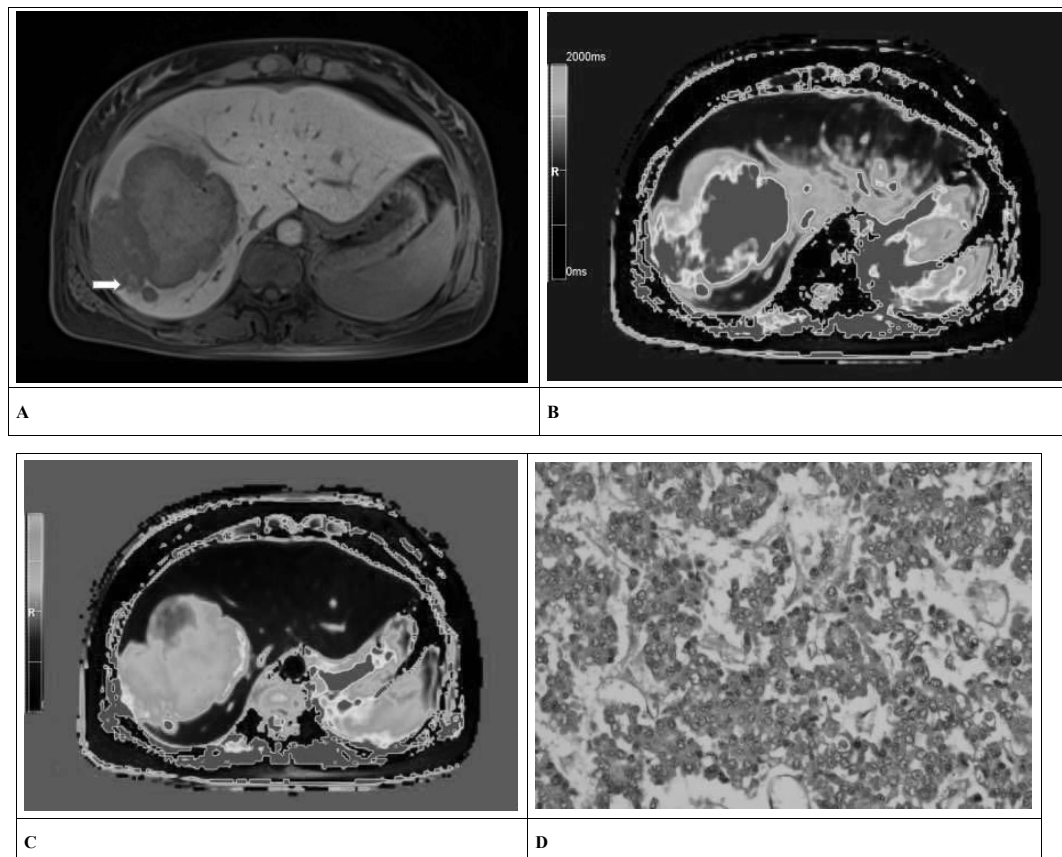


Fig. 1. *A) Hepatobiliary phase image; B) T1 mapping of pre-contrast image; C) T1 mapping of Gd-EOB-DTPA-enhanced MRI on hepatobiliary phase image; D) corresponding pathological picture. A tumor (Edmondson-Steiner grade III) shows heterogeneous low intensity with a minute budding portion at its periphery protruding into the liver parenchyma on the hepatobiliary phase image on Gd-EOB-DTPA-enhanced MRI. This tumor margin was considered to be non-smooth (Awhite arrow).*

age, was recorded. The maximum node section was selected for hematoxylin and eosin (HE) staining.

The results of HE biopsy of the lesion were analyzed by two pathologists who had more than 5 years' experience. If the lesion was HCC, the pathological grade was performed. When the pathologic findings observed by both readers were inconsistent, the two pathologists reanalyzed the biopsy and a definitive result was reached by consensus.

Statistical analysis

All statistical analyses were performed in the SPSS software package (version 19.0, IBM Corporation, Armonk, NY, USA), with the exception of ROC curve analyses which were performed in the MedCalc software package (version 11.4.2.0, MedCalcSoftware bvba, Belgium). Continuous variables were compared with Student's *t*-tests or Mann-Whitney U tests. Categorical variables were compared with Spearman rank correlation tests. ROC curves were plotted to assess the ability to predict pathologic grading. The AUC and 95% confidence interval (CI) for each of these scoring systems was

calculated, and the AUCs were compared. An AUC value of 0.50–0.69 was defined as low accuracy, 0.70–0.90 was defined as moderate accuracy, and >0.90 was defined as good accuracy. Differences with a *P* value < 0.05 were considered statistically significant. A Spearman correlation coefficient with an absolute value in the range of 0.090–0.099 was defined as indicating no correlation, those in the range of 0.10–0.29 were defined as indicating a weak correlation, those in the range of 0.30–0.49 were defined as indicating a moderate correlation, and those in the 0.50–1.0 range were defined as indicating a strong correlation.

RESULTS

Imaging characteristics of the entire cohort, HCCs well differentiated and poorly differentiated are shown in Table I. The mean diameter of the maximal lesion of each patient was 5.5 cm±2.7 cm. The number of patients with a non-smooth margin in the axial HBP was 20 (44.4%). In terms of SI on HBP images, 6 patients had an HCC showing iso-to hyperintensity

Table I. Characteristics of entire cohort and patient differentiation.

Characteristic	All patients(<i>n</i> =45)	Edmondson-Steiner grade	
		well(<i>n</i> =18)	poorly(<i>n</i> =27)
Age ^a (years)	54(28–83)	56(36–71)	50(28–83)
Male ^b	34(75.6)	15(83.3)	19 (70.4)
Image parameters			
Diameter ^a (cm)	5.3(1.5–13.0)	5.6(2.2–13.0)	5.1(1.5–12.3)
Non-smooth tumor margin ^b	20(44.4)	3(16.7)	17(63.0)
Signal intensity on HBP image ^b	6(13.3)	3(16.7)	3(11.1)
T1p ^a (ms)	1880.9(666.7–3588.3)	1914.1(880.0–3588.3)	1849.1(666.7–3510.8)
T1-HBP ^a (ms)	1260.0(326.6–3364.7)	1296.5(727.4–3364.7)	1214.4(326.6–1842.2)
T1d ^a	640.0–339.3–1906.5	709.4–339.33–1522.43	640.0–15.8–1906.5
ΔT1 ^a (%)	35.6–0.9–82.3	39.9–10.9–82.3	29.80.9–62.1

^a Values are expressed as the median (range). ^b Values are expressed as the number of patients (percentage). HBP: hepatobiliary phase; T1p: T1 value of the lesion measured on pre-contrast T1 map; T1-HBP: T1 value of the lesion measured on HBP T1 map; T1d: reduction in T1 value; ΔT1: T1 value reduction rate.

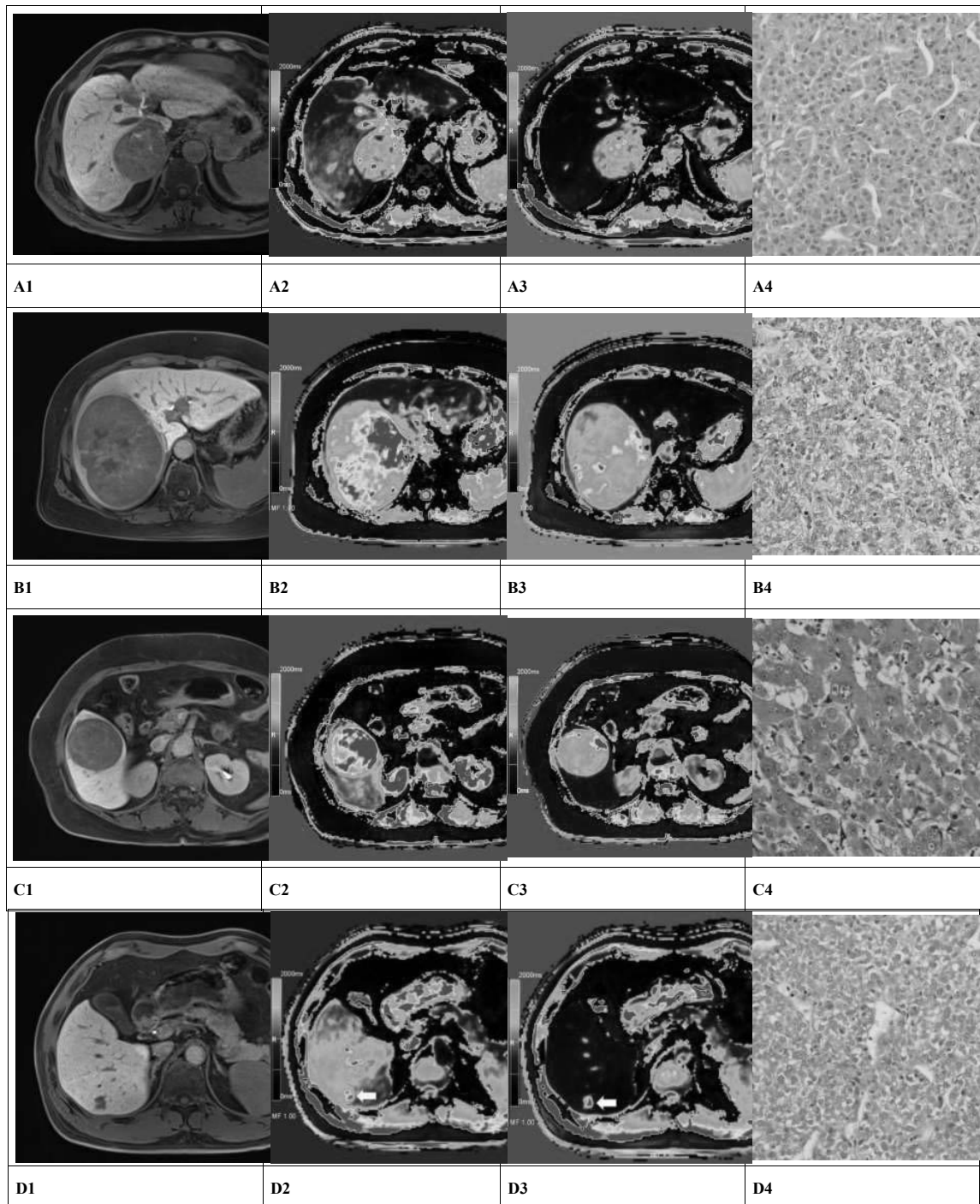


Fig. 2. Row 1) hepatobiliary phase image; Row 2) T1 mapping of pre-contrast image; Row 3) T1 mapping of Gd-EOB-DTPA-enhanced MRI on hepatobiliary phase image; Row 4) corresponding pathological picture. Rows A to D, HCCs from Edmondson-Steiner grade I to IV. The higher the Edmondson-Steiner grade, the higher T1 value of lesions on pre-contrast T1 mapping images and hepatobiliary phase T1 mapping images, which performed as larger percentage of red. Lesions were more conspicuously different from background liver parenchyma on hepatobiliary phase T1 mapping images than on pre-contrast T1 mapping images.

and the remaining 39 patients had HCC manifesting as hypointensity.

The Edmondson-Steiner grade of HCCs of the 45 patients was as follows: 8 (17.8%) with grade I; 10 (22.2%) with grade II; 11 (24.4%) with grade III; 16 (35.6%) with grade IV. Grade I and grade II of HCCs were classified into the well differentiated group ($n = 18$), grade III and grade IV of HCCs were classified into the poorly differentiated group ($n = 27$) (Table I).

Spearman's correlation coefficient analysis showed that age was significant negatively correlated with Edmondson-Steiner grade, Spearman $r_{45} = -0.306$, $p = 0.041$. An on-smooth tumor margin in the HBP image was significant positively correlated with Edmondson-Steiner grade, Spearman $r_{45} = 0.491$, $p = 0.041$. T1p was significant positively correlated with Edmondson-Steiner grade, Spearman $r_{45} = 0.400$, $p = 0.006$. T1-HBP was significant positively correlated with Edmondson-Steiner grade, Spearman $r_{45} = 0.481$, $p = 0.001$ (Fig. 2). There were no significant correlations between T1d (Spearman $r_{45} = 0.050$, $P = 0.743$), $\Delta T1\%$ (Spearman $r_{45} = -0.080$, $P = 0.599$), HCC signal intensity on HBP image (Spearman $r_{45} = -0.165$, $P = 0.279$) and lesion diameter (Spearman $r_{45} =$

-0.050 , $P = 0.742$) with Edmondson-Steiner grade.

ROC curves of the T1p and T1-HBP for predicting Edmondson-Steiner grade in HCC patients are depicted in Fig. 3. The mean values of T1p and T1-HBP were 1935.4 ± 730.8 ms and 1257.1 ± 529.1 ms respectively. The AUCs of the T1p and T1-HBP were 0.685 ($P = 0.037$, 95%CI, 0.519–0.851) and 0.751 ($P = 0.005$, 95%CI, 0.610–0.892), respectively. A T1p cutoff of 1648.2 ms or greater had a sensitivity and specificity for predicting pathologic grading of 85.19% (95%CI, 66.3–95.8) and 61.11% (95%CI, 35.7–82.7), respectively. A T1-HBP cutoff of 1006.0 ms or greater had a sensitivity and specificity for predicting pathologic grading of 81.84% (95%CI, 61.9–93.7) and 61.11% (95%CI, 35.7–82.7), respectively. The T1p exhibited a trend toward lower accuracy compared to the T1-HBP in predicting pathologic grading, but these differences were not significant ($P = 0.270$).

DISCUSSION

We found that T1p and T1-HBP had a significant positive correlation with Edmondson-Steiner grading.

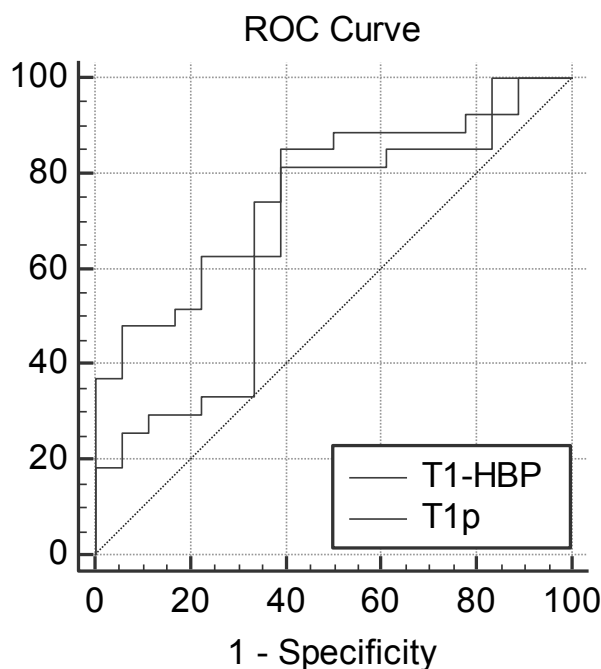


Fig. 3. ROC curves of T1p and T1-HBP for predicting Edmondson-Steiner grade in HCC.

Peng and colleagues (12) found that there was no statistical significance of T1-precontrast and T1-HBP between Edmondson-Steiner grades I, II and III, but both T1d and $\Delta T1\%$ showed statistical differences between the groups. $\Delta T1\%$ had the best correlation with Edmondson-Steiner grade, with a Spearman correlation coefficient index -0.676. Both their study and ours indicated that T1 mapping combined with Gd-EOB-DTPA-enhanced MRI was valuable in evaluating the degree of HCC differentiation. However, our studies suggested that T1p and T1-HBP were associated with Edmondson-Steiner grading, whereas T1d and $\Delta T1\%$ were not.

Gd-EOB-DTPA improves the hepatobiliary phase in MRI. Parenchymal cells with normal function can absorb the contrast medium by Organic Anionic Carrier (OATP) polypeptides on the sinusoidal surface of the cell membrane that performed the hyperintensity and then it is excreted by multi-drug resistance (MRP) protein on the small surface of the bile duct of hepatic cells that form hepato-biliary phase specific imaging. The decrease of OATP and MRP of the lesion lead to the abnormal uptake and excretion of Gd-EOB-DTPA, which result in hypointensity on HBP images. T1 mapping could be used to quantify the Gd-EOB-DTPA uptake information and tissue properties which can be quantified directly on the T1 maps. HCCs which showed iso-to hyperintensity on hepatobiliary phase indicated less aggressive, with a lower level of tumor markers, and had a better prognosis than hypointensity HCCs (13). Choi and colleagues (14) found that HCCs with atypical enhancement pattern and iso-to hyperintensity on HBP images had smaller tumor size, lower histologic grade than hypointensity HCCs. And HCCs with iso-to hyperintensity on HBP images had significantly longer time to tumor recurrence after surgery. However, in our study, HCC signal intensity on HBP imaging was not associated with Edmondson-Steiner grading. This might be due to the small sample size, as only 6 patients (13.3%) showed the iso- to hyperintensity on HBP images.

In conclusion, using T1 value on T1 mapping pre-contrast and Gd-EOB-DTPA-enhanced hepatobiliary phase images the tumor margins were valuable for preoperative assessment of HCC pathological grading.

ACKNOWLEDGEMENTS

This work was supported by Science and Technology Support Program of Sichuan Province (2017SZ0003); Research Grant of National Nature Science Foundation of China (81471658).

REFERENCES

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012, CA. Cancer J Clin 2015; 65(2):87-108.
2. de Lope CR, Tremosini S, Fomer A, Reig M, Bruix J. Management of HCC. J Hepatol 2012; 56 (Suppl 1):S75-87.
3. Docea AO, Mitrut P, Grigore D, Pirici D, Călina CD, Gofiță E. Immunohistochemical expression of TGF beta (TGF- β), TGF beta receptor 1 (TGFB β 1), and Ki67 in intestinal variant of gastric adenocarcinomas. Rom J Morphol Embryol 2012; 53(3 Suppl):683-92.
4. Zlatian OM, Comănescu MV, Roșu AF, et al. Histochemical and immunohistochemical evidence of tumor heterogeneity in colorectal cancer. Rom J Morphol Embryol 2015; 56(1):175-81.
5. Călina D, Docea, AO, Roșu L, et al. Antimicrobial resistance development following surgical site infections. Mol Med Rep 2017; 15(2):681-88.
6. Călina D, Roșu L, Roșu AF, et al. Etiological diagnosis and pharmacotherapeutic management of parapneumonic pleurisy. Farmacia 2016; 64(6):946-52.
7. Wojciechowski VV, Călina D, Tsarouhas K, et al. A guide to acquired vitamin K coagulopathy diagnosis and treatment: the Russian perspective. Daru 2017; 25(1):10.
8. Cioboată R, Găman A, Trașcă D, et al. Pharmacological management of non-alcoholic fatty liver disease: Atorvastatin versus pentoxifylline. Exp Ther Med 2017; 13(5):2375-281.
9. Docea AO, Gofiță E, Călina D, Zaharie SI, Vâlcea DI, Mitruț P. Autoimmune disorders due to double antiviral therapy with Peginterferon and Ribavirin in patients with hepatitis C virus infection. Farmacia 2016; 64(4):605-11.
10. Mewton N, Liu CY, Croisille P, Bluemke D, Lima JA. Assessment of myocardial fibrosis with cardiovascular magnetic resonance. J Am Coll

- Cardiol 2011; 57(8):891-903.
11. Braren R, Curcic J, Remmele S, Altomonte J, Ebert O, Rummeny EJ, Steingoetter A. Free-breathing quantitative dynamic contrast-enhanced magnetic resonance imaging in a rat liver tumor model using dynamic radial T(1) mapping. *Invest Radiol* 2011; 46(10):624-31.
 12. Peng Z, Jiang M, Cai H, et al. Gd-EOB-DTPA-enhanced magnetic resonance imaging combined with T1 mapping predicts the degree of differentiation in hepatocellular carcinoma. *BMC Cancer* 2016; 16: 625.
 13. Yuan SX, Yang F, Yang Y, et al. Long noncoding RNA associated with microvascular invasion in hepatocellular carcinoma promotes angiogenesis and serves as a predictor for hepatocellular carcinoma patients' poor recurrence-free survival after hepatectomy. *Hepatology* 2012; 56(6):2231-41.
 14. Choi JW, Lee JM, Kim SJ, Yoon JH, Baek JH, Han JK, Choi BI. hepatocellular carcinoma_ imaging patterns on gadoxetic acid-enhanced MR images and their value as an imaging biomarker. *Radiology* 2013; 267(3):776-86.

LETTER TO THE EDITOR

LINK BETWEEN CUTANEOUS INFECTION, STRESS AND DEPRESSION

T. JAGMAG¹, M. TIRANT² and T. LOTTI²¹The Dent-All Clinic, Mumbai, India; ²Department of Dermatology, University of Rome "G.Marconi", Rome, Italy*Received May 22, 2017 – Accepted October 30, 2017*

Depression and mood disorders often develop after dermatological conditions which could be primary or secondary to dermatological pathology. The oxidative and psychological stress cause physiological changes in the body. Shift in the methylation pathway, elevated cortisol, lowered neurotransmitter levels and lowered immune system allow infection to penetrate the body and lead to anxiety and depression. Here, a case report of a 20 year old male patient is presented to show how infectious skin lesions, unresponsive to the usual treatment plan, were treated after using a multipronged approach of addressing systemic infection of *Escherichia coli*, elevated cortisol levels and nutritional imbalances.

To the Editor,

Dermatological conditions are associated with psychiatric illness; with an estimation of about 30% of patients suffering with psychiatric comorbidity (1). A literature review article showed level III evidence of patients with conditions such as acne, atopic dermatitis, or psoriasis suffering from anxiety and depression (2). Assessing these coexisting conditions is important while treating the patients with dermatological disorders, whether the psychiatric condition is primary or secondary to the dermatological pathology (3). The severity of psychiatric conditions may not always be proportional to the clinical severity of the dermatologic disorder (4), hence taking a detailed case history along with clinical examination is of the utmost importance as many patients visiting a dermatologist may not feel the necessity to share their psychiatric history.

Depression and mood disorders often develop after infections; links between viral infection and depression have been shown in a recent review article

(5) and a preclinical study (6). A prospective cohort study on 3.56 million people with over 30 years of follow-up has shown that patients with history of infections and autoimmune diseases have 45% increased chance of developing mood disorders (7). Thus, infections should be considered as one of the risk factors while treating patients with depression and vice-a-versa. The case report presented here shows how a 20-year-old patient with infectious skin lesions, unresponsive to any treatment, developed depression with a suicidal tendency. Although depression can be caused by other possible risk factors, such as genetic predisposition, stress, altered neurotransmitter levels (8) and low grade inflammation (9), the present case report explores the connection between infection and depression.

Case report

A 20-year-old, Asian male patient presented with skin lesions in the periorbital area and lateral side of the left cubital fossa, and nasal sinusitis for the

Key words: lymphocutaneous sporotrichosis, oxidative stress, cutaneous infection, depression, phosphatidylserine

Mailing address:

Dr Tariq Jagmag,
The Dent-All Clinic,
Paras Niketan, Shop no 11, Chedda Nagar,
Chembur, Mumbai, 400089, India
Tel.: +91 9869116308
e-mail: tariqjagmag@gmail.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

previous 2 months. Clinical signs and symptoms of his skin lesions included red itchy lesions with classical ringworm pattern, red bordered lesions with oozing of transparent fluid and crusting; constant itching and flaking of the skin which would bleed slightly after scratching (Fig. 1). His complaints of sinusitis included runny nose and headache which he thought was probably due to pollen allergy. He also had axillary lymphadenopathy with no probable cause. He developed sudden onset of depression and suicidal thoughts.

Past history included stammering issues since childhood, suggesting an underlying psychological stress. There was no past history or family history of skin disorders, anxiety, depression, mental disorders, chronic allergic sinusitis or other allergies. He had recently moved to another country for further studies.

The past treatment included topical steroid cream for skin lesions and oral antihistamines with oxymetazoline nasal spray for sinusitis. In the meantime he took on a part-time job of cleaning utensils in a restaurant and within 2 weeks his depression started. When he showed no improvement after 2 months of treatment, an oral antibiotic course was prescribed for a week. His skin lesions still persisted and his signs and symptoms of depression worsened.

All other previous medications were stopped and phosphatidylserine (PS) [300 mg per os (PO) twice a day for 3 months] was started to lower his cortisol levels. Laboratory investigations were prescribed to further investigate. The complete blood count report revealed increased red blood cell count with a slightly elevated red cell distribution width; indicating infection, hypoxia and low iron. The high lymphocyte percentage (with a normal leucocyte count) and elevated Immunoglobulin E indicated presence of parasites, allergy and infection. His plasma amino acid levels were also abnormal. Urine examination showed pus cells with negative culture. Stool examination showed presence of *Entamoeba histolytica* (0-1/hpf) and culture showed *Escherichia coli* (*E. Coli*) which was sensitive to Intravenous (IV) Cefoperazone/Sulbactam.

The treatment plan included supplementation of amino acids, vitamins, minerals (all PO for 3

months) and IV antibiotics along with probiotics. After 1 month of supplementation, a 10-day IV antibiotic course (Cefoperazone/Sulbactam) was given to eradicate the *E. Coli* infection. To prevent the imbalance in the gut flora after antibiotics which might have worsened the condition; targeted IV antibiotic treatment along with probiotics was prescribed.

RESULTS

The periorbital lesions showed remarkable improvement within 3 days of starting PS. After 1 month of PS and oral supplements, the lesions around the cubital fossa and the symptoms of depression improved. The patient's symptoms of depression completely lifted after the course of IV antibiotics. The lymphadenopathy also resolved after antibiotics. However, after 2 months there was relapse of depression. The repeat stool culture showed presence of *Klebsiella*, sensitive to Cefoperazone/sulbactam. The 10-day IV antibiotic course was repeated which resolved the depression. Follow-up of around 1 year showed no relapse or recurrence of either the depression or the skin lesions.

DISCUSSION

The differential diagnosis for skin lesions included bacterial infection, fungal infection and allergic lesion. Since there was no improvement in the lesion after steroid and antihistamines, possibility of the autoimmune and allergic lesion was ruled out. Also the depression worsened after an oral antibiotic course, which increased the likelihood of fungal infection causing secondary depression. The provisional diagnosis was lymphocutaneous sporotrichosis (painless ulcerating pustules and axillary lymphadenopathy) (10).

Infections can cause both oxidative stress (11) and psychological stress. Sporotrichosis infection causes inflammatory response and oxidative stress in the host as shown in a recent preclinical study (12). Altered oxidant/antioxidant levels may be secondary to the fungal infection or could be associated with pathogenesis (13). The body uses antioxidants to

remove the “burn” produced by infections, leading to oxidative stress. Oxidative stress shifts the body’s methylation pathway to the trans-sulfuration pathway leading to anxiety and psychological stress (14). Psychological stress elevates cortisol, which lowers secretory Immunoglobulin A (sIGA) and reduces the body’s immune system (15). This allows infections to penetrate the mucosal defense systems and then persist in the body.

Prolonged neurotransmitter depletion in patients with infections leads to mental disorders including depression, and anxiety disorders. This often turns into a vicious cycle where infections and depression persist unmitigated. During systemic infection the activated peripheral immune system has been shown to increase the symptoms of depression (16). In the above case report, the skin lesions and sinusitis were accompanied by chronic systemic infection of *E. Coli*. It was postulated that the elevated cortisol and lowered sIGA enabled the *E. Coli* to grow in the biofilm of the intestine, which caused the cascade leading to depression.

Treatment of sporotrichosis usually requires long-term administration of itraconazole, potassium iodide, or amphotericin B, usually for a period of 3-6 months, depending on the severity and location of the lesions (17). However, the side effects associated with long-term treatment and emergence of drug-resistant strains (18) necessitates exploring

an alternative treatment approach. Although provisional diagnosis of sporotrichosis was made, considering the patient’s concurrent infections and depression with increased stress levels, the first line of treatment to give PS was considered. Studies have shown that PS supplementation lowers serum cortisol (19) and adrenocorticotrophic hormone levels and salivary cortisol responses to the Trier Social Stress Test when compared to placebo (20). PS can be used as a safe oral effective supplementation for combating stress by lowering serum cortisol levels and promoting brain functions (21-23).

Within 3 days of PS, the periorbital skin lesions and sinusitis symptoms showed remarkable improvement. The patient’s depression and lesions around cubital fossa persisted although there was an improvement in both conditions. Lesions on his arm and depression healed completely only after the IV antibiotics course to eradicate the systemic infection. Thus the treatment plan included elimination of infection and supplementation of the body with the required nutrition to restore the balance.

The systemic *E. Coli* infection may not have been the cause of either of the conditions but it augmented their severity and prevented their treatment with the traditional piecemeal approach. PS supplementation reduced cortisol levels and showed improvement in his conditions. But his immune system was not able to eradicate the systemic infection; only the IV



Fig. 1. Pre-treatment photograph of skin lesions on lateral side of left cubital fossa.

antibiotics were able to eradicate it. As the systemic infection was treated, the cycle broke and all the skin lesions, sinusitis and depression were treated.

Skin conditions may not always cause psychological stress leading to depression. Systemic infection causes physiological changes in the body such as neurotransmitter depletion, inability to methylate, elevated cortisol etc.; these changes lead to mental disorders. Thus having only a linear approach to treat skin infection and/or mental disorders separately cannot always treat patients. The treating physicians should broaden their outlook and look at the missing links while treating patients who are not responding to the traditional treatment.

ACKNOWLEDGEMENTS

We acknowledge Dr. Anuradha Kulkarni for providing the medical writing services.

We gratefully acknowledge the patient for providing informed consent for the treatment and permission to publish his case report.

REFERENCES

- Gupta MA, Gupta AK, Ellis CN, Koblenzer CS. Psychiatric evaluation of the dermatology patient. *Dermatol Clin* 2005; 23(4):591-99.
- Barankin B, DeKoven J. Psychosocial effect of common skin diseases. *Can Fam Physician* 2002; 48:712-16.
- Sambhi R, Lepping P. Psychiatric treatments in dermatology: an update. *Clin Exp Dermatol* 2010; 35(2):120-25.
- Gupta MA, Gupta AK. Psychiatric and psychological co-morbidity in patients with dermatologic disorders: epidemiology and management. *Am J Clin Dermatol* 2003; 4(12):833-42.
- Coughlin SS. Anxiety and depression: linkages with viral diseases. *Public Health Rev* 2012; 34(2):92.
- Hampton T. How depressed mood may develop after viral infection. *JAMA* 2016; 315(21):2267.
- Benros ME, Waltoft BL, Nordentoft M, Østergaard SD, Eaton WW, Krogh J, Mortensen PB. Autoimmune diseases and severe infections as risk factors for mood disorders: a nationwide study. *JAMA Psychiatry* 2013; 70(8):812-20.
- Hasler G. Pathophysiology of depression: do we have any solid evidence of interest to clinicians? *World Psychiatry* 2010; 9(3):155-61.
- Ekinci O, Ekinci A. The connections among suicidal behavior, lipid profile and low-grade inflammation in patients with major depressive disorder: a specific relationship with the neutrophil-to-lymphocyte ratio. *Nord J Psychiatry* 2017; 71(8):574-80.
- Mahajan VK. Sporotrichosis: An overview and therapeutic options. *Dermatol Res Pract* 2014; 2014:272376.
- Ivanov AV, Bartosch B, Isagulians MG. oxidative stress in infection and consequent disease. *Oxid Med Cell Longev* 2017; 2017:3496043.
- Castro VS, Da Silva AS, Thomé GR, et al. Oxidative stress in rats experimentally infected by *Sporothrix schenckii*. *Microb Pathog* 2017; 107(6):1-5.
- Beigh SA, Soodan JS, Singh R, Khan AM, Dar MA. Evaluation of trace elements, oxidant/antioxidant status, vitamin C and b-carotene in dogs with dermatophytosis. *Mycoses* 2014; 57:358e365.
- Salim S. Oxidative stress and psychological disorders. *Curr Neuropharmacol* 2014; 12(2):140-47.
- Campos-Rodríguez R, Godínez-Victoria M, Abarca-Rojano E, et al. Stress modulates intestinal secretory immunoglobulin A. *Front Integr Neurosci* 2013; 7:86.
- Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat Rev Neurosci* 2008; 9(1):46-56.
- Kauffman CA, Bustamante B, Chapman SW, Pappas PG. Clinical practice guidelines for the management of sporotrichosis: 2007 update by the Infectious Diseases Society of America. *Clin Infect Dis* 2007; 45(10):1255-65.
- Gremião IDF, Miranda LHM, Reis EG, Rodrigues AM, Pereira SA. Zoonotic epidemic of sporotrichosis: cat to human transmission. *PLoS Pathog* 2017; 13(1):e1006077.
- Starks MA, Starks SL, Kingsley M, Purpura M, Jäger R. The effects of phosphatidylserine on endocrine response to moderate intensity exercise. *J Int Soc Sports Nut* 2008; 5:11.
- Hellhammer J, Fries E, Buss C, Engert V, Tuch A, Rutenberg D, Hellhammer D. Effects of soy lecithin

- phosphatidic acid and phosphatidylserine complex (PAS) on the endocrine and psychological responses to mental stress. *Stress* 2004; 7 (2):119-26.
21. Monteleone P, Beinat L, Tanzillo C, Maj M, Kemali D. Effects of phosphatidylserine on the neuroendocrine response to physical stress in humans. *Neuroendocrinology* 1990, 52 (3):243-48.
22. Jäger R, Purpura M, Geiss K-R, et al. The effect of phosphatidylserine on golf performance. *J Int Soc Sports Nutr* 2007; 4:23.
23. Moré MI, Freitas U, Rutenberg D. Positive effects of soy lecithin-derived phosphatidylserine plus phosphatidic acid on memory, cognition, daily functioning, and mood in elderly patients with Alzheimer's disease and dementia. *Adv Ther* 2014; 31:1247-62.

LETTER TO THE EDITOR

p16 GENE EXPRESSION IN PANCREATIC CANCER TISSUE AND ITS IMPORTANCE IN DIAGNOSISH. MOU¹, L. YU¹, X. ZHENG², Q. LIAO¹, X. HOU¹ and Y. WU¹¹Department of Medical Oncology, Zhejiang University International Hospital, Shulan (Hangzhou) Hospital, Hangzhou, China; ²Department of Pathology, Zhejiang University International Hospital, Shulan (Hangzhou) Hospital, Hangzhou, China*Received August 29, 2017 – Accepted October 31, 2017*

The aim of this study was to assess the importance of p16 gene expressions in pancreatic cancer tissue for early diagnosis and treatment. Two groups were included in the study: an experimental group of 35 pancreatic cancer tissue specimens and a control group of 35 pancreatic cancer adjacent tissues collected during surgery. The expression of p16 gene in the two groups was observed using the immunohistochemical streptavidin peroxidase conjugated (S-P) method. The results were statistically interpreted using SPSS22.0 software. The positive expression rate of p16 in the experimental group was lower than that of the control group ($p < 0.05$). Comparing the intensity of p16 gene expression, differentiation degree, clinical stage and metastases of nearby lymph nodes the differences had statistical significance ($p < 0.05$). The intensity of p16 gene expression in high and medium differentiated pancreatic cancer group was higher than that of the low differentiated group. p16 gene expression in stages III and IV groups was lower than that in stages I and II groups. Differentiation degree, clinical stage, metastases of nearby lymph nodes and distant metastases were negatively related with p16 gene expression ($p < 0.05$). There was no correlation between age or gender and p16 gene expression. The decreased expression of p16 gene in pancreatic cancer tissue was negatively correlated with differentiation degree and clinical stage. Our results indicated that p16 can be used as a cue signal for diagnosing advanced pancreatic cancer.

To the Editor,

Studies show that pancreatic cancer mortality ranks 8th in the world causing 200,000 deaths annually (1). The progression of the disease is rapid and there are no specific symptoms in the early stages, therefore misdiagnosis or improper dealing with the disease is the main cause of treatment failure (2). Many studies have been carried out aiming to determine a connection between pancreatitis, pancreatic cancer morphological characteristics and tumor markers (3, 4). The unbalance between cell proliferation and cell apoptosis was shown to be one of the main causes

of all malignant tumors (5). The decrease in the cell apoptosis process could lead to increased survival of abnormal cells leading, in some cases, to tumor genesis. Studies show that some oncogenes and tumor suppressor genes play important roles in the development of malignant tumors and their changes are closely related with tumors. For a correct clinical diagnosis of tumors, histochemical and immunohistochemical determinations (6) as well as immunohistochemical expression of some receptors play an important role (7). p16 gene expression is correlated with cell cycle and cell proliferation.

*Key words: p16, pancreatic cancer, cancer diagnosis, immunohistochemical method**Mailing address:*

Dr Haibo Mou,
Department of Medical Oncology,
Zhejiang University International Hospital,
Shulan (Hangzhou) Hospital, #848 Dongxin Road,
Hangzhou, Zhejiang Province, 310022, P.R. China
e-mail: haibomouoncology@163.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

The decrease of p16 gene expression is correlated with the occurrence and development of tumors (8). This study aimed to detect p16 gene expression in pancreatic cancer tissue and its adjacent tissues and to evaluate the association with pancreatic cancer differentiation, stage and metastasis. These findings could provide a new basis for the diagnosis and treatment of pancreatic cancer.

MATERIALS AND METHODS

Study subjects

Two groups were included in the study, an experimental group (PC group) composed of 35 patients diagnosed with pancreatic cancer and treated in Zhejiang University International Hospital, Shulan (Hangzhou) Hospital, Hangzhou city, from November 2014 to November 2016 and a control group (ANT group) which included 35 patients whose lymph nodes had been removed after surgery. The experimental group consisted of 19 men and 16 women with an average age of 42 ± 38.9 years, while the control group consisted of 21 men and 14 women with an average age of 42 ± 38.9 years. The control group excluded patients with other malignant or chronic diseases. The experiment was approved by the ethics committee of Zhejiang University International Hospital, Shulan (Hangzhou) Hospital. All the patients included in the study signed informed consent to participate in the study.

Experimental methods

We collected pancreatic specimens from both groups and peripheral adjacent tissue specimens only from the experimental group. The specimens were fixed in 10% formalin for 24 h and paraffin embedded. Paraffin blocks were fixed in the microtome and each specimen was sliced into 4- μ m pieces. Sections not used in the main study were used as negative control in the experiment. The smoother surface of the wax was dipped into 40–45°C water, keeping the section spread over water. Uniform thickness wax slices were attached in the slide glass and put in 69°C warm box for 1–3 h.

Every specimen was consecutively sliced into 4 pieces, 3 of which were used for p16 detection by immunohistochemistry staining and the remaining one was used as negative control when primary antibody was replaced by phosphate-buffered saline (PBS).

Immunohistochemical detection was carried out for p16 expression with a p16 staining kit (Fujiang Maisheng Biotechnology Company, China) according to the manufacturer's instructions. Paraffin wax was processed by enzyme digestion and then hydrated, washed with 0.01mol/L pH 7.2–7.4 PBS for 5 min, dried with cold air and then placed into a wet box. After that, diluted fluorescent antibody was added for 30–60 min 37°C or overnight at 4°C, and then washed twice with PBS and once with distilled water. The pieces were sealed with 50% glycerin buffer solution. Then they were examined with a fluorescence microscope and staining level was counted. Dyed slices were then dehydrated and examined by microscope. Two senior pathologists evaluated the samples by the double blind method to ensure the accuracy of the results.

Cytometry was used for p16 analysis. For each specimen, ten high microscope power fields ($\times 400$) were randomly selected to measure and classify the positive cells according to the number of dyed cells and staining level. The intensity score was defined as: negative (-), the number of positive cells was less than 5%; weak positive (+), the number of positive cells was 5%–24%; moderate positivity (++), the number of positive cells was 25%–50%; strong positivity (+++), the number of positive cells was more than 50%.

SPSS22.0 software was used for statistical analysis of the obtained data. Differences in proportions in the two groups were checked by *Chi*-squared test on 2X2 table. The differences of the intensity of p16 expression between groups defined by the clinical pathological factors were checked by Kruskal-Wallis rank sum test; the ratio between clinical pathological factors and the intensity of p16 expression was analyzed using Spearman's rank correlation. Rank correlation analyses were used for interrelation between p16 expressions. A difference of $p < 0.05$ was considered to have statistical significance.

RESULTS

p16 expression in pancreatic cancer tissue and adjacent tissue

In pancreatic cancer tissue, the p16 positive expression rate was 40% and in adjacent tissues was 88.57%. The positive expression rate of p16 in the pancreatic cancer group was lower than that in the

control group ($p < 0.05$). Details are shown in Table I. Immunohistochemical staining of the two groups showed that p16 positive cells were cytoplasmic stained with dark grey (Fig. 1).

Classification of clinical-pathologic factors of pancreatic patients and the comparison of p16 expression

Classification of clinical-pathologic factors of pancreatic cancer and the comparison of p16 expression, checked by Kruskal-Wallis rank sum, showed that the expression of p16 was correlated with differentiation degree, clinical stage and metastasis of nearby lymph gland ($p < 0.05$). p16 expression was not correlated with the distant metastasis group. The intensity of p16 expression in the high and medium differentiated pancreatic cancer group was higher than that of the low differentiated group. p16

expression in stage III and IV groups was lower than that in stages I and II groups; p16 expression in the adjacent lymph node metastasis group was higher than that of non-metastasis group (Table II).

The relationship between clinical-pathological factors of pancreatic cancer and p16 expression intensity

Spearman's correlation analysis was used to analyze the relationship between clinical-pathological factors of pancreatic cancer and p16 expression intensity. Differentiation degree, clinical stage, metastasis of nearby lymph gland and distant metastasis were negatively related with p16 expression ($p < 0.05$). R values were -0.669, -0.386, -0.548, and -0.312, respectively. There was no correlation between age, gender and p16 expression ($p < 0.05$) (Table II).

Table I. p16 expression and comparison in experimental and control groups.

Index		Experimental group					Control group				
		Negative	Positive				Negative	Positive			
		-	+	++	+++	sum	-	+	++	+++	sum
p16	Number of Cases	21	5	8	1	14	4	11	18	2	31
	Percentage	60	14.29	22.86	2.86	40	11.43	31.43	51.43	5.71	88.57
	χ^2	16.89									
	P value	0.002									

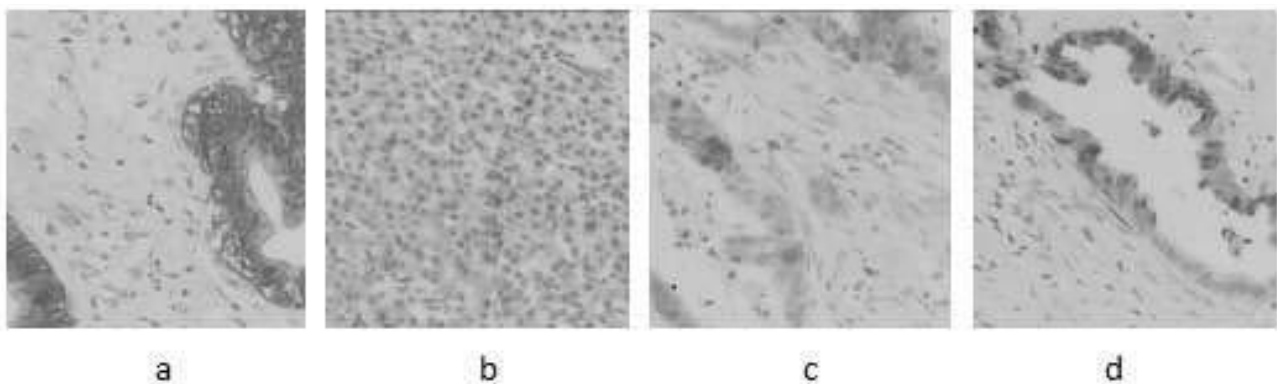


Fig. 1. Immunohistochemical staining results of p16 in the two groups ($\times 200$). Hematoxylin staining: **a)** strong positive expression of p16 in adjacent pancreatic cancer tissue; **b)** negative (-); **c)** weak positive (+); **d)** positive (++)

Table II. Comparison and correlation analysis between clinical-pathologic factors of pancreatic cancer and p16 expression intensity.

Clinical case characteristics		Number of cases	p16				Intensity comparison p	Correlation analysis R p	
			-	+	++	+++			
Gender	Male	19	13	2	4	0	0.563	0.025	0.565
	Female	16	8	3	4	1			
Age (years)	≤60	23	15	4	3	1	0.115	-0.247	0.117
	>60	12	6	1	5	0			
Differentiation degree	Low	28	21	4	3	0	0.001	-0.669	0.003
	Medium/high	7	0	1	5	1			
Clinical stage	Stage I+ II	20	10	2	7	1	0.002	-0.386	0.016
	Stage III+ IV	15	11	3	1	0			
Metastasis of nearby lymph gland	Yes	23	18	4	1	0	0.003	-0.548	0.001
	No	12	3	1	7	1			
Distant metastasis	Yes	8	7	1	0	0	0.047	-0.312	0.037
	No	27	14	4	8	1			

DISCUSSION

Currently, many studies are investigating pancreatic cancer etiology and pathogenesis. Researchers have found that the abnormal expression of genes is most closely related with the development of the disease. Genic mutation, such as the mutation of anti-oncogene Cdkn2a and oncogene KRAS2, are important markers of the occurrence of pancreatic cancer (9). The role of genes in the occurrence of malignant tumors and the relationship between them and specific malignant tumors is a hot topic in current tumor research. The results of this study showed that p16 gene expression in pancreatic cancer tissue was correlated with the differentiation degree, infiltration degree, clinic stage and other clinical-pathologic factors. The lack of p16 expression in pancreatic cancer tissue may be the leading cause of cancer proliferation which is in agreement with results of other similar studies (10). Clinical manifestation, biological characteristics and prognosis of pancreatic cancer are different according to histological type. The chemotherapeutic treatment of these tumors is based on their type and stage and has multiple severe side effects. Due to

immunosuppression, most patients may suffer from severe infections by bacterial germs (11, 12) or fungi (*Aspergillus* or *Fusarium* genus) (13) highly resistant to treatment, with a negative impact on their clinical condition. Surgical removal techniques are the only curative therapeutic option, especially in cases of pancreatic ductal adenocarcinoma (14).

In our study, p16 positive expression rate in the experimental group was lower than that in the control group and in correlation with several clinical-pathological factors. These results are consistent with what Demokan et al. (15) had shown for other types of cancers, so we conclude that p16 could be a marker for the malignancy of pancreatic cancer.

In conclusion, this study demonstrated that low expression of p16 may be considered as an important indicator for the degree of malignancy of pancreatic tumors.

ACKNOWLEDGEMENTS

This work was supported by the Department of Health of Zhejiang province (2013KYB101), Department of Education of Zhejiang Province

(Y201636437) and Bureau of Traditional Chinese Medicine of Zhejiang Province (2012ZB085).

REFERENCES

1. Hariharan D, Saied A, Kocher H M. Analysis of mortality rates for pancreatic cancer across the world. *HPB(Oxford)* 2008; 10(1):58-62.
2. Agarwal B, Abuhamda E, Molke KL, Correa AM, Ho L. Endoscopic ultrasound-guided fine needle aspiration and multidetector spiral CT in the diagnosis of pancreatic cancer. *Am J Gastroenterol* 2004; 99(5):844-50.
3. Koomen JM, Shih LN, Coombes KR, et al. Plasma protein profiling for diagnosis of pancreatic cancer reveals the presence of host response proteins. *Clin Cancer Res* 2005; 11(3):11101-8.
4. Lei XF, Jia SZ, Ye J, Qiao YL, Zhao GM, Li XH, Chang H. Application values of detection of serum ca199, ca242 and ca50 in the diagnosis of pancreatic cancer. *J Biol Regul Homeost Agents* 2017; 31(2):383-88.
5. Johansson G, Mahller YY, Collins MH, et al. Effective in vivo targeting of the mammalian target of rapamycin pathway in malignant peripheral nerve sheath tumors. *Mol Cancer Ther* 2015; 7(5):1237-45.
6. Zlatian OM, Comănescu MV, Roșu AF, et al. Histochemical and immunohistochemical evidence of tumor heterogeneity in colorectal cancer. *Rom J Morphol Embryol* 2015; 56(1):175-81.
7. Stănculescu D, Mărgăritescu C, Stepan A, Mitruț AO. E-cadherin in gastric carcinomas related to histological prognostic parameters. *Rom J Morphol Embryol* 2011; 52(3 Suppl):1107-12.
8. Chen J, Li D, Wei C, et al. Aurora-A and p16 polymorphisms contribute to an earlier age at diagnosis of pancreatic cancer in Caucasians. *Clin Cancer Res* 2007; 13(10):3100-104.
9. Elnemr A, Ohta T, Yachie A, et al. Human pancreatic cancer cells disable function of Fas receptors at several levels in Fas signal transduction pathway. *Int J Oncol* 2001; 18(2):311-16.
10. Feng L. Aberrant methylation of CpG island in 5' promoter region of p16 gene and its role in diagnosis of pancreatic cancer. *Academic Journal of Second Military Medical University* 2007; 28(6):629-32.
11. Călina D, Docea, AO, Roșu L, et al. Antimicrobial resistance development following surgical site infections. *Mol Med Rep* 2017; 15(2):681-88.
12. Călina D, Roșu L, Roșu AF, et al. Etiological diagnosis and pharmacotherapeutic management of parapneumonic pleurisy. *Farmacia* 2016; 64 (6):946-52.
13. Tănase A, Coliță A, Ianoși G, et al. Rare case of disseminated fusariosis in a young patient with graft vs. host disease following an allogeneic transplant. *Exp Ther Med* 2016; 12(4):2078-82.
14. Petrou A, Moris D, Tabet PP, Richards BDW, Kourounis G. Ablation of the locally advanced pancreatic cancer: an introduction and brief summary of techniques. *J BUON* 2016; 21(3):650-58.
15. Demokan S, Chuang A, Suoğlu Y, Ulasan M, Yalnız Z, Califano JA, Dalay N. Promoter methylation and loss of p16 INK4a, gene expression in head and neck cancer. *Head*.

LETTER TO THE EDITOR

MECHANISM OF ACTION OF MINOXIDIL IN THE TREATMENT OF ANDROGENETIC ALOPECIA IS LIKELY MEDIATED BY MITOCHONDRIAL ADENOSINE TRIPHOSPHATE SYNTHASE-INDUCED STEM CELL DIFFERENTIATION

A. GOREN^{1,2}, T. NACCARATO¹, M. SITUM³, M. KOVACEVIC², T. LOTTI²
and J. McCOY¹

¹Applied Biology, Inc., Irvine, CA, USA; ²Department of Dermatology and Venereology, University of Rome "G.Marconi", Rome, Italy; ³Department of Dermatology and Venereology, University Hospital Center "Sestre milosrdnice", Zagreb, Croatia

Received October 13, 2017 – Accepted November 3, 2017

Topical minoxidil is the only topical drug approved by the US Food and Drug Administration (FDA) for the treatment of androgenetic alopecia. However, the exact mechanism by which minoxidil stimulates anagen phase and promotes hair growth is not fully understood. In the late telogen phase of the hair follicle growth cycle, stem cells located in the bulge region differentiate and re-enter anagen phase, a period of growth lasting 2-6 years. In androgenetic alopecia, the anagen phase is shortened and a progressive miniaturization of hair follicles occurs, eventually leading to hair loss. Several studies have demonstrated that minoxidil increases the amount of intracellular Ca^{2+} , which has been shown to up-regulate the enzyme adenosine triphosphate (ATP) synthase. A recent study demonstrated that ATP synthase, independent of its role in ATP synthesis, promotes stem cell differentiation. As such, we propose that minoxidil induced Ca^{2+} influx can increase stem cell differentiation and may be a key factor in the mechanism by which minoxidil facilitates hair growth. Based on our theory, we provide a roadmap for the development of a new class of drugs for the treatment of androgenetic alopecia.

To the Editor,

The hair follicle is a sheath of cells and tissue that surround the hair root and is comprised of 3 parts: the papilla, matrix, and root sheath. Each hair follicle also contains a "bulge" of slow-cycling undifferentiated stem cells located in the outer root sheath that form during embryonic skin development (1). To regulate hair growth the hair follicle goes through three distinct cycles: the anagen phase (growth), catagen phase (transition), and the telogen phase (rest). During the late telogen phase, hair follicle stem cells in the bulge differentiate into

matrix cells and enter the anagen phase (2). The anagen phase typically lasts two to six years, and 90-95% of hair follicles in the scalp are in anagen phase during normal hair cycling (3, 4). Disruption or change in the dynamics of the hair growth cycle can lead to hair loss. Androgenetic alopecia, the most common form of hair loss in both men and women, is characterized by a measured miniaturization of the hair follicle and shortening of the anagen phase. The normal anagen to telogen ratio is 12:1, but a ratio of 5:1 is common in patients with androgenetic alopecia (5). Eventually, the anagen phase is reduced

Key words: androgenetic alopecia, minoxidil, ATP synthase

Mailing address:

Andy Goren, MD
Applied Biology, 17780 Fitch,
Suite 192, Irvine, CA 92614, USA
Tel.: +1 (949) 387-4526
e-mail: andyg@appliedbiology.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

to such an extent that emerging hairs do not reach the skin surface and the increase in telogen hairs, which are more loosely anchored, results in an increase of hair shedding.

During hair follicle stem cell differentiation, mitochondria undergo a change in structure. Anagen matrix cells contain elongated mitochondria with more abundant and organized cristae compared to telogen phase undifferentiated bulge cells (6). Teixeira et al. (7) demonstrated that a knockdown of specific adenosine triphosphate (ATP) synthase subunits disrupted the mitochondrial inner membrane morphology, resulting in mitochondria with fewer cristae that more closely resemble stem cell mitochondria. ATP synthase is an enzyme most commonly associated with its function during ATP synthesis, converting ATP from adenosine diphosphate in the mitochondrial matrix. However, Teixeira et al. (7) demonstrated that ATP synthase plays a critical role in stem cell differentiation, independent of its role in ATP synthesis. These results suggest that ATP synthase acts as a differentiation factor and is required for cristae development during stem cell differentiation.

Minoxidil is a strong vasodilator and potassium channel opener, which, to date, has been the leading theory presented to explain its mechanism of action (8, 9). In addition, several studies have demonstrated that minoxidil increases intracellular Ca^{2+} levels *in vitro* (10, 11). Chen et al. (11) demonstrated that minoxidil at concentrations of 200 to 800 μM caused intracellular levels of Ca^{2+} to rise in a concentration-dependent manner, with a maximum net increase of approximately 230%. While high Ca^{2+} have been reported to be cytotoxic, increases in Ca^{2+} induced by minoxidil would likely not damage stem cells, owing to the fact that minoxidil increases stem cell proliferation and results in longer anagen phase hair growth.

Furthermore, Das and Harris (12) demonstrated that increases in intracellular Ca^{2+} levels directly leads to mitochondrial ATP synthase up-regulation. At 5 μM Ca^{2+} , a 130% increase in ATP synthase capacity compared to control was reported (12). Interestingly, the use of calcium channel antagonists (e.g., nifedipine) have been noted to cause telogen

effluvium (i.e., hair loss), while the use of calcium channel agonists has been suggested to enhance hair re-growth (13).

CONCLUSION

Topical minoxidil is the only FDA approved drug for the treatment of androgenetic alopecia in men and women. The mechanism by which minoxidil promotes hair growth is not fully understood. We propose that an up-regulation of ATP synthase due to a minoxidil-induced increase in intracellular Ca^{2+} levels is likely to increase bulge stem cell differentiation and promote hair growth through a shift in the anagen to telogen cycle ratio. This suggests a pathway for the development of a new treatment for androgenetic alopecia: the identification of modifiers of calcium channels expressed in the stem cells of the hair bulge. Once new molecules are identified, *in-vitro* screening of the molecules for increased mRNA expression of ATP synthase could yield candidates suitable for pre-clinical studies. Our group is now working on the development of one such candidate molecule.

REFERENCES

1. Greco V, Chen T, Rendl M, et al. A two-step mechanism for stem cell activation during hair regeneration. *Cell Stem Cell* 2009; 4:155-69.
2. Wilson C, Cotsarelis G, Wei Z-G, et al. Cells within the bulge region of mouse hair follicle transiently proliferate during early anagen: heterogeneity and functional differences of various hair cycles. *Differentiation* 1994; 55:127-36.
3. Malkud S. Telogen effluvium: a review. *J Clin Diagn Res* 2015; 9 9.
4. Burg D, Yamamoto M, Namekata M, Haklani J, Koike K, Halasz M. Promotion of anagen, increased hair density and reduction of hair fall in a clinical setting following identification of FGF5-inhibiting compounds via a novel 2-stage process. *Clin Cosmet Investig Dermatol* 2017; 10:71-85.
5. Sinclair R. Male pattern androgenetic alopecia. *BMJ* 1998; 317:865-69.
6. Tang Y, Luo B, Deng Z, et al. Mitochondrial aerobic

respiration is activated during hair follicle stem cell differentiation, and its dysfunction retards hair regeneration. *PeerJ* 2016; 4:e1821.

7. Teixeira F, Sanchez C, Hurd T, et al. ATP synthase promotes germ cell differentiation independent of oxidative phosphorylation. *Nat Cell Biol* 2015; 17(5):689-96.
8. Messenger AG, Rundegren J. Minoxidil: Mechanisms of action on hair growth. *Br J Dermatol* 2004; 150:186-94.
9. Headington JT. Hair follicle biology and topical minoxidil: possible mechanisms of action. *Dermatologica* 1987; 175: 19–22.
10. Li M, Marubayashi A, Nakaya Y, Fukui K, Arase S. Minoxidil-induced hair growth is mediated by adenosine in cultured dermal papilla cells: possible involvement of sulfonylurea receptor 2B as a target of minoxidil. *J Invest Dermatol* 2001; 117:1594-600.
11. Chen IS, Chou CT, Liu YY, et al. The investigation of minoxidil-induced $[Ca^{2+}]_i$ rises and non- Ca^{2+} -triggered cell death in PC3 human prostate cancer cells. *J Recept Signal Transduct Res* 2017; 37:1-7.
12. Das A, Harris D. Intracellular calcium as a regulator of the mitochondrial ATP synthase in cultured cardiomyocytes. *Biochem Soc Trans* 1990; 18:554-5.
13. Paus R, Schilli MB, Handjiski B, Menrad A, Henz BM, Plonka P. Topical calcitriol enhances normal hair regrowth but does not prevent chemotherapy-induced alopecia in mice. *Cancer Res* 1996; 56:4438-43.

LETTER TO THE EDITOR

EXPRESSION PROFILE OF EUKARYOTIC TRANSLATION INITIATION FACTOR AND MATRIX METALLOPROTEINASE 9 IN ENDOMETRIAL CANCER TISSUEZM. SHI¹, YN. LIU¹, B. FU¹, YF. SHEN² and LM. LI¹

¹Pathological Staff Room, School of Medicine and Affiliated Hospital of HeBei University of Engineering, Handan, HeBei, China; ²Oncology Department, School of Medicine and Affiliated Hospital of HeBei University of Engineering, Handan, HeBei, China

Received August 29, 2017 – Accepted November 3, 2017

The aim of the present study was to provide a novel method for the diagnosis, prevention and treatment of endometrial cancer by the determination of the characteristic expression of the eukaryotic translation initiation factor 4E (eIF4E) and the enzyme matrix metalloproteinase 9 (MMP9) in endometrial cancer tissue. Three types of endometrial tissue specimens were selected (including 20 cases of normal endometrial tissue specimens, 15 cases of hyperplastic endometrial tissue specimens and 45 cases of endometrial cancer tissue specimens). The expression of eIF4E and MMP9 in the specimens was examined by immunohistochemistry and their corresponding levels were statistically analyzed. The positive expression rates of eIF4E and MMP9 in endometrial cancer specimens were 64.44% and 66.67% respectively, which were higher than those noted in hyperplastic endometrial tissue specimens and normal endometrial tissue specimens ($p < 0.05$). The comparisons between the groups indicated that the expression levels of eIF4E and MMP9 in the endometrial cancer specimens were increased compared with those noted in the normal endometrial tissue specimens ($p < 0.0167$). In endometrial cancer specimens, the positive expression rates of eIF4E and MMP9 were related to the endometrial cancer stages as determined by the International Federation of Gynecology and Obstetrics (FIGO), tumor cell differentiation degree and lymphatic metastasis ($p < 0.05$) classifications. eIF4E expression was positively related to MMP9 expression in endometrial cancer specimens. High expression levels of eIF4E and MMP9 proteins were noted in endometrial cancer specimens, which were correlated with FIGO stages, histological grade and degree of lymphatic metastasis. Thus, endometrial cancer and malignant biological behavior may be connected to the high expression of eIF4E and MMP9. The positive correlation between eIF4E and MMP9 expression in endometrial cancer specimens suggests their potential up-regulation during carcinogenesis.

To the Editor,

Endometrial cancer is one of the most common gynecological malignant tumors that affect women's health. The symptoms include infertility, polycystic ovarian syndrome with treatment-resistant acne in women over 35 years, long-term estrogen effects in

the absence of progestational hormone resistance, insulin resistance, endometriosis, obesity and high blood pressure (1, 2). The morbidity that occurs due to endometrial cancer is rapidly increasing, therefore it is important to identify its pathogenesis in order to develop an effective diagnostic method. The

Key words: endometrial cancer, eIF4E, MMP9, immunohistochemical method

Mailing address:

Dr Lanmei Li,
Pathological Staff Room,
School of Medicine, Affiliated Hospital,
HeBei University of Engineering,
Handan 056000 Hebei, China
e-mail: shizhiminszm1980@163.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

underlying mechanism may involve the contribution of estrogen and progesterone receptors, as well as matrix metalloproteinase enzymes, as has been previously noted for other aggressive cancers, such as melanoma (3).

The eukaryotic translation initiation factor 4E (eIF4E) is a polypeptide that is involved in protein translation. The location of the eIF4E fragment is on chromosome 4 q21-4q25 and encompasses a genetic region of 50kb. eIF4E plays an important role in oncogenesis via the regulation of cancer-related mRNA transcripts. This transcription factor participates in proto-oncogene activation, the induction of apoptosis, angiogenesis, invasion and transfer mRNA (4). McClusky et al. (5) demonstrated that the expression levels of eIF4E were positively associated with the recurrence rate of breast cancer. Matrix metalloproteinase 9 (MMP9) is an important enzyme that belongs to the matrix metalloproteinase family of enzymes (MMPs), which participate in tumorigenesis in various aspects. MMP9 is implicated in tumor spreading by the degradation of the extracellular matrix (EEC) and the basement membrane of the vascular wall from the neighboring tumor surface, such as collagen (6). The expression levels of eIF4E and MMP9 in selected endometrial specimens are mainly detected by immunohistochemical methods. These methods are combined with the histological examination in order to provide accurate diagnosis of the majority of cancer types (7, 8). The present study aimed to analyze the role of eIF4E and MMP9 in the early diagnosis and prognosis of endometrial cancer.

MATERIALS AND METHODS

Experimental material

A total of 80 paraffin-embedded endometrial tissue specimens were randomly selected from Affiliated Hospital of HeBei University of Engineering from July 2014 to July 2016. A total of 20 out of 80 cases were classified as normal endometrial tissue specimens (9 cases were in the secretory phase and 11 cases were in the proliferating phase). The control group comprised 15 cases of hyperplastic endometrial tissue specimens (4 cases of simple endometrial hyperplasias, 3 cases of mild atypical hyperplasia of the endometrium

and 4 cases of moderately and severe atypical hyperplasia of the endometrium, respectively). The remaining 45 cases were classified as endometrial cancer cases. The pathology department examined the aforementioned specimens in order to ensure that the patients from whom the samples were collected had not received chemotherapy, radiotherapy or endocrine therapy prior to surgery. No statistically significant difference was noted with regard to the age of these patients. The classification of the 45 cases of endometrial cancer specimens was conducted according to the following criteria. The classification according to FIGO stages in 2009 (9) was as follows: a total of 28, 9, 7 and 1 cases were in stages I, II, III and IV, respectively. The classification according to the histological grade was as follows: a total of 16, 12 and 17 cases were in grades 1, 2 and 3, respectively. (3) The classification according to the depth of the myometrial invasion was as follows: a total of 32 cases were of superficial muscle (1/2 muscular layer) infiltration, whereas 13 cases were of deep muscle (1/2 muscle layer) infiltration.

Experimental method

Immunohistochemistry was used to examine the specimens (PV6000 two steps method). The optical cutting layers among the experimental tissues were selected and numbered. Subsequently, the paraffin blocks were selected and frozen according to the corresponding serial numbers. The sections were cut into 4 μ m layers, dried for 15 min and finally cooled prior to use. The paraffin blocks were processed according to the following steps:

Dewaxing and hydration for the paraffin section was carried out by soaking the cooled glass slides in xylene liquid I and II (Beijing Zhongshan Golden Bridge Bio-technology Co., Ltd) for 5 min, respectively. Subsequently, the slides were soaked in 95% and 85% anhydrous alcohol for 2 min and were washed twice with distilled water and an additional two times with tap water.

Antigen thermal remediation

The slides were put into ethylenediaminetetraacetic acid (EDTA) repair liquid (Beijing Dingguo Changsheng Biotechnology Co., Ltd) following hyperbaric heating, cooled and subsequently rinsed with phosphate-buffered saline (PBS) (Beijing Dingguo Changsheng Biotechnology Co., Ltd) solution. The peroxidase treatment for the inactivation of the tissue was conducted as follows: the slides

were soaked, retained in freshly prepared 3% H₂O₂ solution for 10 min and finally washed with PBS.

The primary antibodies were added by the dropping method. The eIF4E and MMP9 antibodies (Beijing Dingguo Changsheng Biotechnology Co., Ltd) were diluted at a ratio of 1:150. The diluent was added onto the slide by the dropping method and the slides were incubated for 2 h at room temperature and finally washed with PBS. The secondary antibodies were further added by the dropping method and incubated for 15 min at room temperature. Subsequently, the slides were washed with PBS buffer solution.

DAB staining was carried out with 3,3'-Diaminobenzidine (DAB) color developing agent (Beijing Bioss Biotechnology Co., Ltd) which was added to the specimens at room temperature and the samples were visualized by microscopy in order to observe the change in color. The samples were washed in order to finalize the coloration process. Counterstaining was carried out by staining the slides with Hematoxylin (Beijing Bioss Biotechnology Co., Ltd) following washing, after which the samples were washed with tap water.

For dehydration, drying and sealing, the processed slides were soaked and dehydrated in 1% hydrochloric acid alcohol and then were dried under electro-thermal constant temperature. The neutral balsam was used to seal the slides.

The positive expression levels of eIF4E and MMP9 proteins were shown on the slides in color. For example, the cytoplasm was in yellow, claybank and/or sepia. Thus, the double blind method was used. The processed slides were observed and scored by two independent pathologists. A high power lens of 400 x magnification (Olympus company) was used in order to observe the processed pathological section. A total of 5 images under the lens were randomly selected. The average number of positive cells in the five images was averaged and the percentage of the positive cells accounting for the total cell number in each image was estimated. The sections were scored according to the scoring method: positive cell percentage of 1-25%, 25-50%, 50-75% and 75% were scored as 1, 2, 3 and 4 points, respectively. The slides were further scored according to the coloring degree of the cytoplasm in the observed image as follows: without color, light yellow, claybank and sepia corresponding to 1, 2, 3 and 4 points, respectively. A sample was considered positive when the scores obtained by multiplying scores of the positive cell percentage and of the coloring degree were ≥ 3 .

Statistical software SPSS19.0 was used for statistical

analysis of the data. The expressions levels of eIF4E and MMP9 proteins at different FIGO stages, depths of myometrial invasion, histological grades, amount of lymphatic metastases and groups of endometrium were examined by the *chi*-squared test and the revised *chi*-squared tests and/or analyzed by exact probability testing according to the data. The correlation of the expression levels of eIF4E and MMP9 proteins with endometrial cancer were analyzed using the *chi*-square test (test criterion $\alpha=0.05$).

RESULTS

Expression levels of eIF4E and MMP9 proteins in endometrial tissues

The expression levels of eIF4E and MMP9 of three different endometrial specimens were detected by immunohistochemical staining and analyzed by statistical software in terms of their expression characteristics. The data indicated that the positive expression rate and the expression intensity of eIF4E and MMP9 of the three different endometrial tissues were increased (Figs. 1 and 2).

The positive expression rate of eIF4E in the normal endometrial specimens was 15%, whereas in the hyperplastic endometrium specimens it was 60% and in the endometrial cancer specimens 64.44%. The difference in the expression levels of eIF4E between the normal endometrial specimens and the endometrial cancer specimens was statistically significant ($p<0.0167$). The positive expression rate of MMP9 in the normal endometrial specimens was 10%, whereas in the hyperplastic specimens it was 46.67% and in the endometrial cancer specimens 66.67%. The expression levels of MMP9 exhibited a significant difference between normal endometrial specimens and endometrial cancer specimens ($p<0.05$, $p=0.0167$) (Table I).

Associations between the expression levels of eIF4E and MMP9 and the clinicopathological features of endometrial cancer

According to the FIGO stage classification [version 2009 (17)], the positive expression rate of eIF4E of the specimens that were classified as stage I was 57.14 %, whereas the corresponding positive expression rate of the stage II-IV specimens was

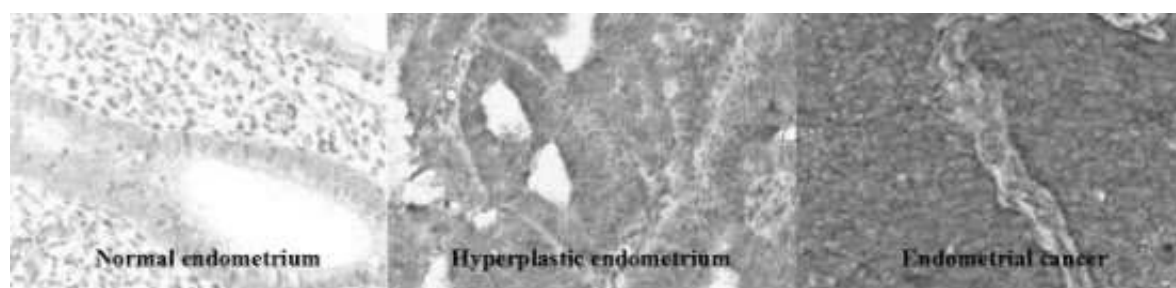


Fig. 1. *EIF4E* expression in endometrial tissue ($\times 400$).



Fig. 2. *MMP9* expression of endometrial tissue ($\times 400$).

76.47 %. The positive expression rate of eIF4E increased according to the FIGO stage ($p < 0.05$). With regard to the depth of myometrium invasion, the positive expression rate of eIF4E of the superficial muscle (1/2 muscular layer) infiltration specimens was 56.25%, whereas that of the deep muscle (1/2 muscle layer) infiltration was 84.62%. No significant difference was noted between the two groups. The histological grade exhibited positive expression rate of eIF4E of 50% for the grade G_1 - G_2 specimens, whereas the positive expression rate of the grade G_3 specimens was 88.24% ($p < 0.05$). As regards lymphatic metastasis, the positive expression rate of eIF4E in the specimens without lymphatic metastasis was 51.51%, whereas the corresponding positive expression rate of the specimens with lymphatic metastasis was 100% ($p < 0.05$).

According to the FIGO stage classification (2009 version), the positive expression rate of MMP9 in the endometrial cancer specimens that were classified as stage I was 57.14%, whereas the positive expression

rate of the stage II-IV specimens was 82.35% ($p < 0.05$). With regard to the depth of myometrium invasion, the positive expression rate of MMP9 of the endometrial cancer specimens with superficial muscle infiltration was 62.50%, whereas the positive expression rate of the deep muscle infiltration specimens was 76.92% ($p < 0.05$). The parameter histological grade exhibited positive expression rate of MMP9 protein in the endometrial cancer specimens with grades G_1 - G_2 of 46.42%, whereas the positive expression rate of the specimens with grade G_3 was 100% ($p < 0.05$). For the lymphatic metastasis, the positive expression rate of MMP9 of specimens without lymphatic metastasis was 54.54%, whereas that of specimens with lymphatic metastasis was 100% ($p < 0.05$) (Table II).

Expression levels of eIF4E and MMP9 proteins in endometrial cancer tissues

The expression levels of the eIF4E and MMP9 proteins were analyzed in endometrial cancer tissues

Table I. Expression of eIF4E and MMP9 in endometrial tissues.

Groups	Cases	eIF4E				MMP9			
		Positive	Negative	χ^2	p value	Positive	Negative	χ^2	p value
Normal endometrium	20	3	17		0.23a	2	18		0.017a
Hyperplastic endometrium	15	9	6	0.001b	1.02	7	8	0.379b	0.539
Endometrial cancer	45	29	16	12.382c	0.00	30	15	16.928c	0.000

a: Comparison between hyperplastic endometrium and normal endometrium with the Fisher exact probability method, $p > 0.0167$; b: Comparison between endometrial cancer and hyperplastic endometrium (revise), $p > 0.0167$; c: Comparison between endometrial cancer and normal endometrium, $p < 0.0167$.

Table II. Associations between expression levels of eIF4E and MMP9 in endometrial tissues and clinicopathological features.

Groups		Cases	eIF4E				MMP9			
			Positive	Negative	χ^2	p value	Positive	Negative	χ^2	p value
FIGO stages	Stage I	28	16	12	4.795e	0.027	16	12	7.269e	0.008
	Stage II-IV	17	13	4			14	3		
Depth of myometrium invasion	<1/2	32	18	14	1.623e	0.210	20	12	0.128e	0.709
	>1/2	13	11	2			10	3		
Histological grade	G ₁ -G ₂	28	14	14	6.058d	0.016	13	15	12.837e	0.001
	G ₃	17	15	2			17	0		
Lymphatic metastasis	Yes	12	12	0	6.426e	0.010	12	0	6.418e	0.011
	No	33	17	16			18	15		

d: chi-squared test; e: chi-squared test (revise)

using the χ^2 test. A positive association was noted between the expression levels of eIF4E and MMP9 proteins and the presence of endometrial cancer (Table III).

DISCUSSION

Previous studies have shown that eIF4E is overexpressed in head and neck squamous cell carcinoma, while in normal tissues its expression is decreased (10). The results of the present study

indicated an increased expression of eIF4E in endometrial cancer tissues, which was significantly different to the positive expression rate of eIF4E in normal endometrium ($p < 0.0167$). The present study demonstrated that the eIF4E expression level was associated with the FIGO stages for patients with endometrial cancer. eIF4E is an important biomarker for the assessment of the conditions of patients with endometrial cancer. The expression levels of eIF4E in endometrial cancer may further provide insight into the selection of chemotherapy treatment. The eIF4E

Table III. Correlation analysis on eIF4E and MMP9 expression levels in endometrial tissues.

eIF4E	MMP9		n	R
	Positive	Negative		
Positive	29	5	34	0.715
Negative	3	12	15	

positive expression in the patients without lymphatic metastasis was significantly lower compared with that of the patients with lymphatic metastasis ($p < 0.05$). The detection of eIF4E expression in endometrial specimens prior to surgery is important for the prediction and the treatment of the patients with lymphatic metastasis. The treatment is difficult, since chemotherapy often leads to immunosuppression and the development of infections caused by bacterial germs and fungi, namely *Aspergillus* and/or *Fusarium* which are extremely resistant to treatments (11, 12). Furthermore, the development of vitamin K-dependent coagulopathies is an additional side effect of the chemotherapy treatment in the patients with endometrial cancer (13).

The present study showed that the high expression of MMP9 might be related to the presence of endometrial cancer. Previous studies have shown that the expression of MMP9 is related to the malignant grade of ovarian cancer (14). The positive expression rate of MMP9 in the specimens without lymphatic metastasis was considerably lower than that noted in the patients with lymphatic metastasis ($p < 0.05$). Thus, the expression of MMP9 might be related to endometrial cancer.

In conclusion, the combined determination of eIF4E and MMP9 expression levels can be used as a new auxiliary examination index for the evaluation of endometrial cancer prognosis.

REFERENCES

1. ReIanoși, S, Ianoși, G, Neagoe D, et al. Age-dependent endocrine disorders involved in the pathogenesis of refractory acne in women. *Mol Med Rep* 2016; 14(6):5501-06.
2. Imperiale L, Manganaro L, Ticino A, et al. Endometriosis and Glanzmann's thrombasthenia. *J Biol Regul Homeost Agents* 2016; 30(3):877-82.
3. Caruntu C, Mirica A, Rosca AE, et al. The role of estrogens and estrogen receptors in melanoma development and progression. *Acta Endo (Buc)* 2016; 12:234-41.
4. Wendel HG, De SE, Fridman JS, et al. Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy. *Nature* 2004; 428(6980):332-37.
5. Mcclusky DR, Chu Q, Yu H, et al. A prospective trial on initiation factor 4E (eIF4E) overexpression and cancer recurrence in node-positive breast cancer. *Ann Surg* 2005; 242(4):590-92.
6. Jedryka M, Chrobak A, Chelmonska-Soyta A, Gawron D, Halbersztadt A, Wojnar A, Kornafel J. Matrix metalloproteinase (MMP)-2 and MMP-9 expression in tumor infiltrating CD3 lymphocytes from women with endometrial cancer. *Int J Gynecol Cancer* 2012; 22(8):1303-9.
7. Docea AO, Mitrut P, Grigore D, Pirici D, Călina CD, Gofiță E. Immunohistochemical expression of TGF beta (TGF- β), TGF beta receptor 1 (TGFR1), and Ki67 in intestinal variant of gastric adenocarcinomas. *Rom J Morphol Embryol* 2012; 53(3 Suppl):683-92.
8. Docea AO, Mitruț P, Cernea D, Georgescu CC, Olimid D, Mărgăritescu C, Dumitrescu D. Immunohistochemical expression of EGF, c-erbB-2 and EGFR in intestinal variant of gastric adenocarcinomas. *Rom J Morphol Embryol* 2013; 54(3):545-54.
9. Edey K, Murdoch J. FIGO staging in vulval and endometrial cancer. *Obstetric Gynaecol* 2010; 12(4):245-49.
10. Siegele B, Cefalu C, Holm N, et al. eIF4E-targeted suicide gene therapy in a minimal residual mouse model for metastatic soft-tissue head and neck squamous cell carcinoma improves disease-free survival. *J Surg Res* 2008; 148(1):83-89.
11. Călina D, Docea AO, Roșu L, et al. Antimicrobial resistance development following surgical site infections. *Mol Med Rep* 2017; 15(2):681-88.
12. Tănase A, Coliță A, Ianoși G, et al. Rare case of disseminated fusariosis in a young patient with graft vs. host disease following an allogeneic

- transplant. *Exp Ther Med* 2016; 12(4): 2078-82.
13. Wojciechowski VV, Călina D, Tsarouhas K, et al. A guide to acquired vitamin K coagulopathy diagnosis and treatment: the Russian perspective. *Daru* 2017; 25(1):10.
 14. Schmalfeldt B, Prechtel D, Härting K, et al. Increased expression of matrix metalloproteinases (MMP)-2, MMP-9, and the urokinase-type plasminogen activator is associated with progression from benign to advanced ovarian cancer. *Clin Cancer Res* 2001; 7(8):2396-404.

LETTER TO THE EDITOR

**TACROLIMUS INDUCED NEPHROTOXICITY
AND PULMONARY TOXICITY IN WISTAR RATS**

T. AKHTAR, N. SHEIKH, T. SHAN and R. GHAZANFAR

*Cell and Molecular Biology Lab, Department of Zoology, University of the Punjab, Q-A Campus,
Lahore, Pakistan**Received June 12, 2017 – Accepted November 6, 2017*

Transplantation has evolved into an accepted treatment for end-stage organ failure. The major limitation for solid organ transplantation is organ rejection, which is an adaptive immune response caused by the activation of T-cells. Immunosuppressant drugs are used to overcome this problem. Tacrolimus is a powerful immunosuppressive drug which is used to minimize the risk of organ rejection. The present study was designed to find the toxic effects of tacrolimus on lungs and kidneys. Wistar rats were divided into 4 experimental groups and one control group (n=9). Each rat of the experimental group was orally given the aqueous suspension of tacrolimus powder (3mg/ml) and dissected after 6, 12, 24 and 48 hours of tacrolimus suspension dose. Lungs and kidneys were excised and processed for histopathological and histochemical alterations. Kidney tissues presented signs of toxic potential on tissue architecture such as increased interstitial spaces, necrosis, especially acute tubular necrosis, glomerular shrinkage, dilated blood vessels and enlargement of Bowman's capsule. Lung sections also confirmed the toxic potential, characterized by bronchiolar wall thickening, alveolar cells necrosis, collapsing of alveolar spaces and interstitial round cell infiltrate. Results of Prussian blue iron staining showed no iron deposition in kidney architecture while in lung sections, iron accumulation was evident. Taken together, from these observations we can conclude that tacrolimus may induce toxicity to a certain extent with structural distortion of the kidneys and lungs.

To the Editor,

Transplantation is the ultimate treatment for end stage organ failure. The major limitation for solid organ transplantation is organ rejection, which is an adaptive immune response caused by the activation of T-cells (1, 2). Immunosuppressive drugs are used to overcome this problem. Tacrolimus (FK506) is an active immunosuppressive drug approved by the Food and Drug Administration (FDA) to minimize the probability of organ rejection after transplantation. Tacrolimus extracted from *Streptomyces tsukubaensis* for prevention of transplanted organ

rejection was introduced for the first time in Japan in 1993 (3). Tacrolimus binds to the cytoplasmic immunophilin, FK binding protein (FKBP) to immobilize Ca^{2+} /calmodulin-dependent phosphatase calcineurin. Inactivated calcineurin is unable to dephosphatase the resultant NF-AT, and it blocks the entry of NF-AT into the nucleus, consequently preventing the transcriptional activation of the IL-2 gene resulting inhibition of the T-lymphocyte proliferation and activation (4). Along with having immunosuppressive properties, tacrolimus can also cause toxicity because it increases oxidative stress

*Key words: antioxidant, histopathology, immunosuppressant, necrosis, reactive oxygen species**Mailing address:*

Dr. Nadeem Sheikh, Ph.D.
Cell and Molecular Biology Lab,
Department of Zoology, University of the Punjab,
Q-A Campus, Lahore, 54590, Pakistan
Tel.: +92 42 99231246/812
e-mail: s_nadeem77@yahoo.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
**DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF
INTEREST RELEVANT TO THIS ARTICLE.**

and TGF- β expression. Two mechanisms involved are: i) less expression of antioxidant defense gene, ii) higher expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit genes which result in increased production of reactive oxygen species (5, 6).

Kidneys are more susceptible to the toxic effects of medications because of their role in plasma filtration and sustenance of metabolic homeostasis. The vascular bed of kidneys is open to the quarter of resting cardiac output (7). Lungs are also susceptible to the lethal effects of oxygen and oxidative damage (8). Disturbance in oxidant-antioxidant balance is the main reason of the pathogenesis of acute respiratory diseases (9). The present experimental study was designed to find the toxic effects of tacrolimus on lung and kidney tissue architecture.

MATERIALS AND METHODS

Experimental animals

Forty-five Wistar rats were accommodated in the departmental animal house and maintained under set conditions with 12-h light-dark cycles at a temperature of $23\pm 2^{\circ}\text{C}$ with free access to rat chow and drinking water, in acquiescence with the institutional guidelines, approved by the ethics committee of the Department of Zoology, University of the Punjab, Lahore, Pakistan. All applicable institutional and/or national guidelines for the care and use of animals were followed.

The animals were divided into five equal sized groups ($n=9$): one group as control and four groups of experimental animals. The dose of tacrolimus was formed by diluting tacrolimus powder (3 mg) in distilled water (1 ml) and stirred well until the formation of a homogenous suspension. This suspension was given to the four groups of experimental rats using feeding catheters and they were sacrificed and dissected after 6, 12, 24 and 48 hours of dose administration.

Tissue sampling

Rats were anesthetized by an equal ratio of ketamine plus pyrogen free water intra-peritoneal injection, sacrificed, and dissected in aseptic conditions. The kidney and lung tissues were excised and fixed in 10% formalin in labeled glass vials.

Histopathological and histochemical analyses

Fixed tissues were dehydrated using ascending concentrations of ethanol (30%-100%), cleared by incubating in xylene at 25°C and infiltrated in molten paraffin wax followed by sectioning. Sections (5- μm thick) were stained with haematoxylin and eosin staining for histopathological analysis and with Prussian blue iron staining for histochemical changes. Stained slides were examined under an Optika B-150DBR microscope. The microphotographs of stained sections were taken at original magnification of 40x and 400x with the help of an Optika B-150DBR microscope built-in camera.

RESULTS

Histopathological alterations

Kidney sections of rats from the control group were free from any pathological abnormality, H&E stained sections were normal with two distinct regions: cortex and medulla. Cortex had renal corpuscles and numerous renal tubules. Renal corpuscles showed distinct Bowman's capsule and glomeruli. Other clearly visible structures were capsules, proximal convoluted tubules, distal convoluted tubules and collecting ducts (Fig. 1a). Kidney sections of rats with 6-h tacrolimus treatment revealed tubular necrosis (Fig. 1b). After the 12-h time point normal glomerular structure was disrupted and Bowman's capsular space increased as a result of its shrinkage. Tubular necrosis and epithelial changes were also obvious (Fig. 1c). Analysis after the 24-h time point revealed acute tubular necrosis glomerular shrinkage linked with increased capsular space (Fig. 1, c and d). Sections of rats with 48 h of exposure of tacrolimus showed necrosis of tubules in cortex as well as medulla, necrosis was also present in papillary side. Along with necrosis and epithelial damage, vacuole formation was also observed at the 48-h time point (Fig. 1e).

Lung sections of control rats were free from any pathological abnormality. H&E stained sections of lungs looked normal with regular cellular architecture. Alveoli were thin walled and the alveolar walls were composed of a single layer of squamous epithelium. These sections had prominent respiratory bronchiole, terminal bronchiole and

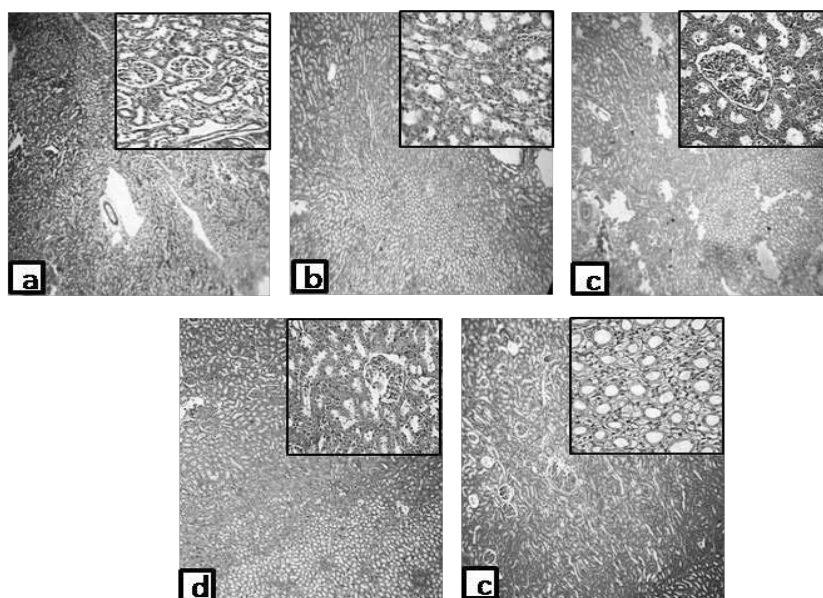


Fig. 1. *Acute tacrolimus induced nephrotoxicity, identified by H&E staining. Microphotographs were taken at 40x and 400x. a) Control group, (b) 6-h time point, (c) 12-h time point (d) 24-h time point, (e) 48-h time point.*

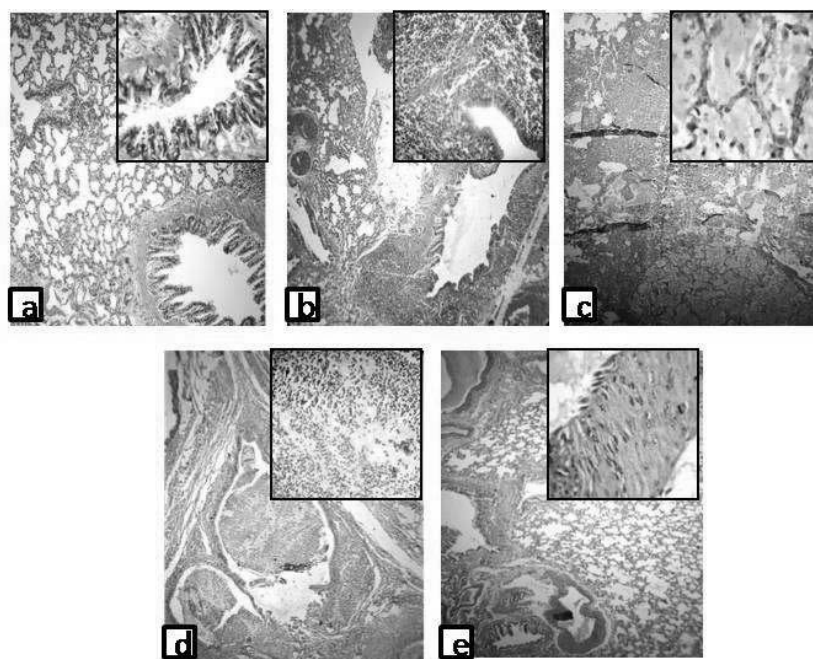


Fig. 2. *Acute tacrolimus induced pulmonary toxicity, identified by H&E staining. Microphotographs were taken at 40x and 400x. a) Control group, (b) 6-h time point, (c) 12-h time point (d) 24-h time point, (e) 48-h time point.*

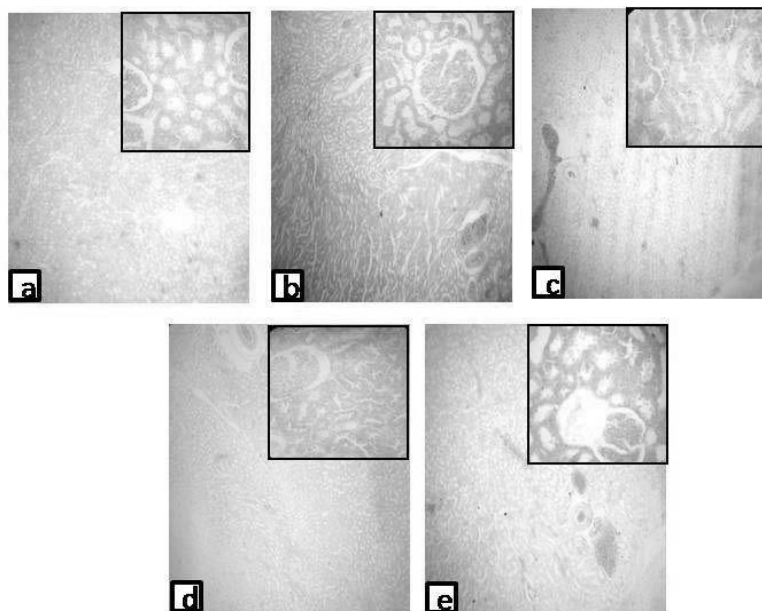


Fig. 3. Prussian blue iron stained sections of kidney. Microphotographs were taken at 40x and 400x. *a)* Control group, *(b)* 6-h time point, *(c)* 12-h time point, *(d)* 24-h time point, *(e)* 48-h time point.

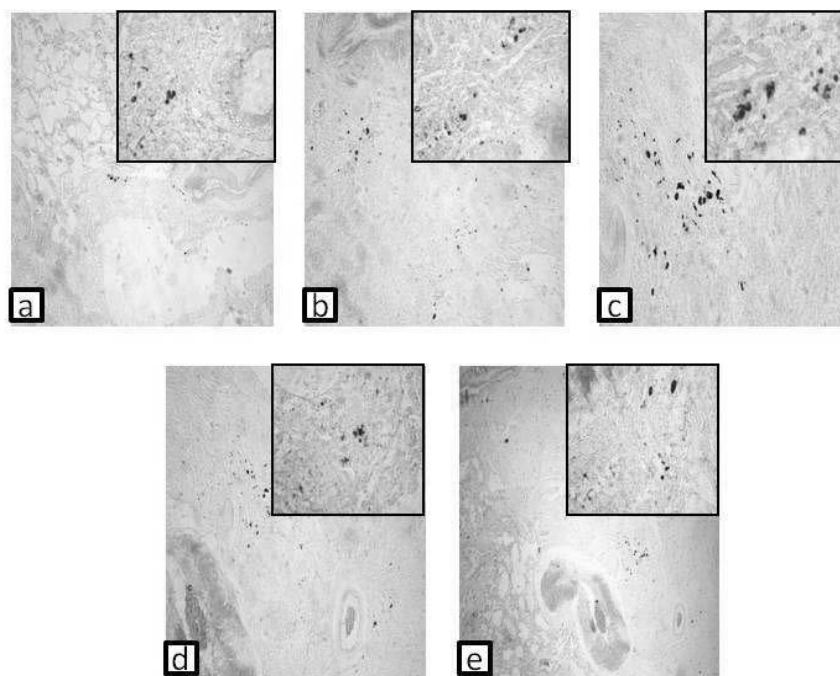


Fig. 4. Prussian blue iron stained sections of lung. Microphotographs were taken at 40x and 400x. *a)* Control group, *(b)* 6-h time point, *(c)* 12-h time point *(d)* 24-h time point, *(e)* 48-h time point.

intrapulmonary bronchus (Fig. 2a). Sections after 6-h tacrolimus treatment showed mild toxicity, having collapsed alveolar spaces (Fig. 2b). Section analysis after the 12-h time point revealed the diffuse filtration of plasma cells and the bronchial lamina was also filled with similar inflammatory cells (Fig. 2c). Pulmonary edema was observed after 24 h of tacrolimus dose. At this time point all the alveolar spaces were distended by the cellular infiltrates (Fig. 2d). Necrosis of myocytes and interstitial round cell filtration was also observed at the 48-h time point (Fig. 2e).

Histochemical analysis

Prussian blue staining was used to examine histochemical alterations. Presence of iron is identified by representative iron-containing hemosiderin. Iron deposition occurs in the form of ferrous ions (blue color) and, rarely, ferric ions (brown color). Prussian blue iron-stained kidney sections of the control and experimental rats revealed no hemosiderin, indicating the absence of iron deposition (Fig. 3).

Prussian blue iron-stained sections of lungs showed iron deposition in the alveoli at the 12-h and 24-h time points (Fig. 4, c and d). While at the 48-h time point a mild level of blue pigmentation of iron in the form of ferrous ions was observed (Fig. 4e). No significant hemosiderin granules were observed in the lung sections of control rats (Fig. 4a).

DISCUSSION

Tacrolimus is a powerful macrolide immunosuppressant and anti-T-lymphocyte agent. The present project was designed to study the acute toxic effect of tacrolimus on kidney and lungs of Wistar rats. Our findings pointed out that tacrolimus caused renal dysfunction, glomerular shrinkage linked with increased capsular space, and acute tubular necrosis. Kidokoro and colleagues in 2012 reported that tacrolimus increases reactive oxygen species (ROS), which may be created by NADPH oxidases. As a result, this increased ROS induces necrosis and cell death (10). This study also showed that tacrolimus induced glomerular injury by enhancing glomerular oxidative stress. Furthermore, tacrolimus increases TGF- β expression which causes

acute kidney injury (AKI) and necrosis (8, 20). In literature, vasodilation due to tacrolimus is reported which may be due to upregulation of vasoconstrictor factors that include thromboxane, endothelin and activation of the renin-angiotensin system (RAS), or by the reduction of vasodilator factors such as prostacyclin, and nitric oxide (NO) (12).

Due to deficiency of white blood cells and humoral agents, definite effects on lung were seen. Edema was observed at the 24-h time point. Endothelial damage is the early event in acute lung injury, and rupturing of the endothelium in capillaries gives rise to edema. Oxidative stress also plays an important role in apoptosis of both structural and inflammatory cells and alters the balance of the cytokine microenvironment (13). At different time points, inflammatory cell infiltration occurs which disturbs normal functioning of lungs and interferes with gas exchange. Iron is necessary in many biological processes and chemical reactions of metabolic pathways such as oxidation-reduction reactions (14). Iron deposition is responsible for the production of ROS, which may lead to damage at cellular and tissue levels (15).

Taking into consideration these findings, we can conclude that tacrolimus has toxic potential. It induces histopathological changes in kidneys and lungs of experimental animals; consequently it effects their normal functioning. In lungs, tacrolimus disturbs the normal mechanism of iron as iron deposits in lungs were revealed. Despite being vital on the one hand, this drug can also create damaging effects owing to its toxic potential. Thus we necessitate novel strategies for a healthier management of transplant receivers.

ACKNOWLEDGEMENTS

The authors are thankful to the Higher Education Commission (HEC) of Pakistan for providing financial support under Indigenous 5000 Fellowship Scheme (Phase-II) to carry out this project.

REFERENCES

1. Colvin RB, Smith RN. Antibody-mediated organ-allograft rejection. *Nat Rev Immunol* 2005; 5:807-17.
2. Yang HC. Tailoring tacrolimus-based immunotherapy

- in renal transplantation. *Nephrol Dial Transpl* 2003; 18:16-20.
3. Almawi WY, Melemedjian OK. Clinical and mechanistic differences between FK506 (tacrolimus) and cyclosporin A. *Nephrol Dial Transpl* 2000; 15:1916-18.
 4. Cook LG, Chiasson VL, Long C, Wu GY, Mitchell BM. Tacrolimus reduces nitric oxide synthase function by binding to FKBP rather than by its calcineurin effect. *Kidney Int* 2009; 75:719-26.
 5. Khanna AK, Pieper GM. NADPH oxidase subunits (NOX-1, p22phox, Rac-1) and tacrolimus-induced nephrotoxicity in a rat renal transplant model. *Nephrol Dial Transpl* 2007; 22:376-85.
 6. Provenzano A, Santeusano A, Mathis E, Notarbartolo M, Labbozzetta M, Poma P. Pharmacogenetic considerations for optimizing tacrolimus dosing in liver and kidney transplant patients. *WJG* 2013; 19:9156-63.
 7. Kristen KM, Rebecca AW. Renal stem cells: fact or science fiction? *Biochem J* 2012; 444:153-68.
 8. Schwaiblmair M, Behr W, Haeckel T, Mnrkl B, Foerg W, Berghaus T. Drug induced interstitial lung disease. *Open Respir Med J* 2012; 6:63.
 9. Bast A, Weseler AR, Haenen GR, den Hartog GJ. Oxidative stress and antioxidants in interstitial lung disease. *Curr Opin Pulm Med* 2010; 16:516-20.
 10. Kidokoro K, Satoh M, Nagasu H, et al. Tacrolimus induces glomerular injury via endothelial dysfunction caused by reactive oxygen species and inflammatory change. *Kidney Blood Press R* 2012; 35:549-57.
 11. Naesens M, Kuypers DR, Sarwal M. Calcineurin inhibitor nephrotoxicity. *Clin J AM Soc Nephro* 2009; 4:481-508.
 12. Berger M, Santi LI, Beys-da-Silva WO, et al. Mechanisms of acute kidney injury induced by experimental *Lonomia obliqua* envenomation. *Arch Toxicol* 2015; 89:459-83.
 13. Kuwano K, Nakashima N, Inoshima I, et al. Oxidative stress in lung epithelial cells from patients with idiopathic interstitial pneumonias. *Eur Respir J* 2003; 21:232-40.
 14. Lieu PT, Heiskala M, Peterson PA, Yang Y. The roles of iron in health and disease. *Mol Aspects Med* 2001; 22:1-87.
 15. Naz N, Malik IA, Sheikh N, Ahmad S, Khan S, Blaschke M, Schultze F, Ramadori G. Ferroportin-1 is a 'nuclear'-negative acute-phase protein in rat liver: a comparison with other iron-transport proteins. *Lab Invest* 2012; 92:842-56.

LETTER TO THE EDITOR

RELATIONSHIP BETWEEN TRANSMEMBRANE SERINE PROTEASE EXPRESSION AND PROGNOSIS OF ESOPHAGEAL SQUAMOUS CELL CARCINOMA

GT. LIU¹, C. SHEN², XH. REN², L. YANG³, YM. YU⁴, YX. XIU², RH. LI⁵,
L. JIANG⁵, CL. ZHANG³ and YW. LI⁵

¹Department of Thoracic Surgery, Affiliated Hongqi Hospital of Mudanjiang Medical University, MuDanjiang, China; ²Clinical Laboratory, The Second Affiliated Hospital of Mudanjiang Medical University, Mu Danjiang, China; ³Central Sterile Supply Department, Affiliated Hongqi Hospital of Mudanjiang Medical University, MuDanjiang, China; ⁴Western Language Department, Mudanjiang Normal University, MuDanjiang, China; ⁵Clinical Laboratory, Affiliated Hongqi Hospital of Mudanjiang Medical University, Mu Danjiang, China

Received October 4, 2017 - Accepted November 20, 2017

Esophageal squamous cell carcinoma is the most common type of esophageal cancer in Eastern Europe and Asia, being the 6th most common cause of cancer deaths worldwide. The aim of this study was to analyze the expression of transmembrane serine protein in esophageal squamous cell carcinoma, and to correlate it with the clinical biological features of esophageal cancer. The expression of transmembrane protease serine 4 (TMPRSS4) mRNA and protein in carcinoma tissues and corresponding adjacent tissues and non-tumorous esophageal tissues was determined using PCR (qRT-PCR). The results show that both TMPRSS4 mRNA and protein expression were remarkably lower in adjacent normal tissues than in tumorous tissues. TMPRSS4 protein expression in esophageal carcinoma was correlated with patient demographic characteristics, tumor type, high TNM stages and overall survival (OS). Based on the experimental results, we conclude that TMPRSS4 is closely related to the occurrence, development and metastasis of esophageal squamous cell carcinoma.

To the Editor,

Esophageal squamous cell carcinoma is the histological type of esophageal cancer most common in Eastern Europe and Asia, being the 6th most common cause of cancer deaths worldwide (1). Physical, histological and immunohistochemical examinations and imaging tests are the basis of a correct clinical diagnosis (2, 3).

Patients with stage I or II disease are less than fifty percent. Some patients can be cured, but the surgical treatment diminishes the quality of life and approximately 40-50% of patients who receive

it will eventually die from recurrent disease or by complications secondary to immunosuppression such as bacterial infections with severe germs resistant to antibiotic treatment, and fungal infections with the genera *Aspergillus* or *Fusarium* (4).

Chemotherapy treatment has modest outcomes, but has multiple undesirable side effects such as vitamin K-dependent coagulation disorders, psychiatric disorders, alopecia, nausea and vomiting (5).

New prognostic markers and therapeutic targets need to be discovered in order to improve the quality of life and the prognosis of these patients.

Key words: TMPRSS4, esophageal squamous cell carcinoma, esophageal squamous cells, prognosis

Mailing address:

Yanwei Li,
Tongxiang Road No. 5,
Affiliated Hongqi Hospital of Mudanjiang Medical University,
Mudanjiang City,
Hei Longjiang Province, China
e-mail: dryanweili_mdju@163.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

Transmembrane protease serine 4 (TMPRSS4) is part of a large family of II transmembrane serine proteases (TTSP) involved in a variety of biological and physiological processes whose malfunction leads to different diseases and disorders. TMPRSS4 is encoded by the TMPRSS4 gene. The encoded protein contains a hydrophobic N-terminal anchor sequence and a serine protease structure at the C-terminus, located or occurring outside a cell or cells, and a variable stem region in the middle. TMPRSS4 is differentially expressed in the subdivision of the squamous cell carcinoma. Initially, TMPRSS4 was purified and cloned from sputum collected from patients. It is overexpressed in the esophageal epithelium and expressed in the neck, prostate gland and lung cells (6). There are only few studies that investigated the role of TMPRSS4 in the carcinogenesis of human esophageal squamous cell carcinoma (7). In normal cervical and esophageal epithelia, TMPRSS4 protein is expressed only in differentiated epithelial cells.

In this study, through quantitative reverse transcription polymerase chain reaction and tissue microarray immunohistochemistry analysis, we determined the messenger RNA and the expression of carcinogenic protein TMPRSS4 in esophageal squamous cell carcinoma neoplasms and matched adjacent normal tissues, and we correlated it with the clinical features and total survival.

MATERIALS AND METHODS

Human tissue specimens and patient clinical information

Three hundred forty-two esophageal squamous cell carcinoma patients treated in the Affiliated Hongqi Hospital of Mudanjiang Medical University during 2014 and 2015 were included in the study. The study was approved by the Ethics Committee of the Affiliated Hongqi Hospital of Mudanjiang Medical University and all patients gave written informed consent for participating in this study.

At the time of surgery, the age of the patients ranged between 30 and 81 years, with an average age of 62.5 years.

Expression of TMPRSS4 mRNA and protein

TMPRSS 11D mRNA level was measured by relative quantitative method based on the housekeeping gene GAPDH standardized quantitative reverse transcription PCR (qRT-PCR). Four groups primers were used: (5'-TGC ACC AAC TGC TTA GC-3'), GAPDH reverse primer (5'-TGC ACC AAC TGC TTA GC-3'), GAPDH reverse primer (5'-TGC AAC ACC TGC TTA GC-3'), GAPDH reverse primer (5'-GGC ATG GAC TGT CAT GAG-3'). The TMPRSS4 protein expression in tissue blocks was determined by tissue microarray immunohistochemistry (TMA IHC). Rabbit polyclonal anti-human TMPRSS4 antibody (GL Biochem Shanghai Ltd) were used in a dilution of 1:200. Considering the intensity of the staining and the percentage of cells, the semi-quantitative H-score method was used to grade the TMPRSS 11D IHC data, and its range was 0-300. In the next step, by using specific cutoff values, the continuous TMPRSS4 protein expression data were converted into binary data. The overall survival was evaluated by X-tile software program. The cutoff value for TMPRSS 11D expression was considered 100 so a value between 101 and 300 was considered high TMPRSS 11D expression.

We analyzed the expression of TMPRSS4 and the relationship with patients' demographic and pathological characteristics, such as histologic type and cancer Tumor Nodules Metastasis [TNM] stage. The patients' characteristics were collected from medical records.

Statistical analysis

The data were analyzed using statistical software and STATA 12.0. The comparison between the TMPRSS4 mRNA and protein expression levels in the carcinoma specimen and ordinary tissue specimens was made using Student *t*-test. Pearson's χ^2 test was used to correlate the TMPRSS4 expression with clinical pathologic parameters. Univariate and multivariate Cox regression models were used to evaluate the prognostic factors for esophageal squamous cell carcinoma. The survival curve was calculated by Kaplan Meier method. *p* value below 0.05 was considered to be statistically significant.

RESULTS

TMPRSS4 mRNA expression and protein level in tumor and normal tissue

The expression of TMPRSS4 mRNA in the esophageal squamous cell carcinoma tumor tissue samples was higher compared with adjacent normal tissues. The levels of TMPRSS4 mRNA were measured in 342 snap-frozen esophageal squamous cell carcinoma and in adjacent normal tissues samples. PCR results showed that the level of TMPRSS4 messenger RNA in esophageal squamous cell carcinoma formations and adjacent tissues was 2.541 ± 0.2832 , and in the adjacent normal tissues was 1.146 ± 0.1532 ($p < 0.001$) (Fig.1).

The measurement of TMPRSS4 protein expression showed that in 43.85% of tumor tissues the expression of TMPRSS4 was high compared with only 11.99% in normal esophageal tissues that presented low protein expression (Pearson's $\chi^2 = 86.300$, $p < 0.001$) (Fig. 2).

The correlation between TMPRSS4 protein expression and clinical stage of esophageal squamous cell carcinoma

TMPRSS4 protein expression was correlated with clinical pathological characteristics of esophageal squamous cell carcinoma patients (including sex, age, histologic type and TNM stage). The high TMPRSS4 protein expression was correlated with the TNM stage of the carcinoma (Pearson's $\chi^2 = 7.2712$, $P = 0.0263$) (Table I).

Correlation of TMPRSS4 protein expression with total survival of esophageal squamous cell carcinoma patients

Single factor and multi-factors survival analysis was used to analyze the prognostic implications of TMPRSS4 protein expression in esophageal squamous cell carcinoma patients. Single factor analysis showed a significant association between high TMPRSS4 expression (Hazard Ratio, HR: 2.315, 95% CI: 1.643-2.864; $p < 0.001$), sex (compared to

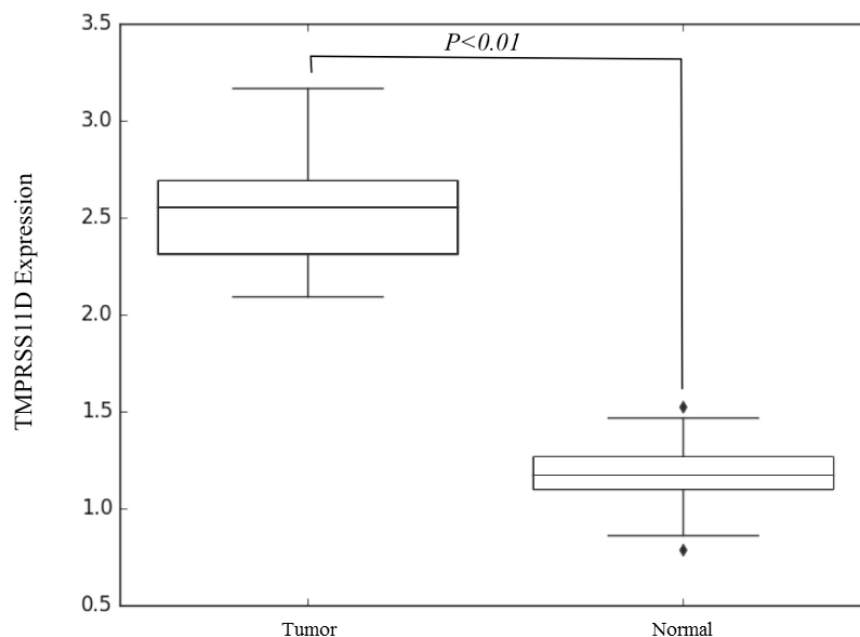


Fig. 1. *TMPRSS4 mRNA level was notably higher in esophageal squamous cell carcinoma tissues than in neighbor regular tissues.*

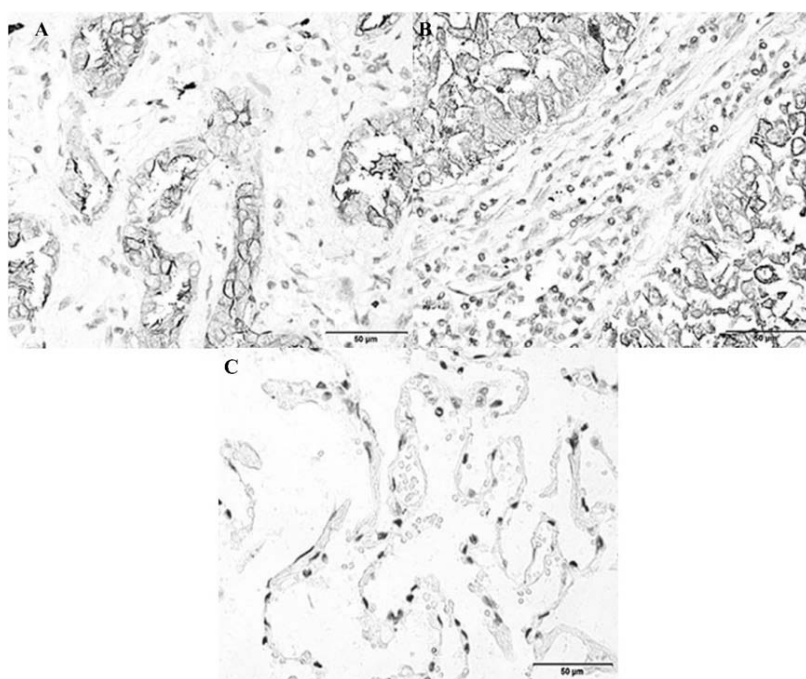


Fig. 2. *TMPRSS4* protein expression in esophageal squamous cell carcinoma and adjacent normal tissues determined by immunohistochemistry. **A)** *TMPRSS4* staining was positive in adenocarcinoma tissue; **B)** *TMPRSS4* staining was positive in squamous cell carcinoma tissue; **C)** *TMPRSS4* staining was negative in adjacent normal esophageal tissue.

Table I. *The correlation between the expression of TMPRSS4 and clinical pathological characteristics of esophageal squamous cell carcinoma.*

Characteristic	n	Low expression n (%)	High expression n (%)	Chi-squared test		Univariate survival analysis			Multivariate survival analysis		
				χ^2	p	HR	p	95% CI	HR	p	95% CI
Sex				0.75	0.386	1.534	0.020*	[0.454,2.174]	1.412	0.014*	[1.263,1.632]
Male	171	93(54.39)	78(45.61)								
Female	171	85(49.71)	86(50.29)								
Age				1.66	0.1970	1.037	0.645	[0.743,2.832]			
<60	173	96(55.49)	77(44.51)								
≥60	169	82(48.52)	87(51.48)								
Tissue type				86.30	<0.001*						
Normal	342	41(11.99)	301(88.01)								
Tumoral	342	150(43.86)	192(56.14)								
Differentiation				2.27	0.132	0.843	0.091	[0.153,1.432]			
Low grade	173	97(56.07)	75(43.93)								
Middle and High grade	169	81(47.93)	88(52.07)								
TNM stage				7.2712	0.0263*	1.845	<0.01*	[0.986,2.016]	1.626	<0.01*	[1.123,1.843]
I	168	98(58.33)	70(41.77)								
II	92	47(51.09)	45(48.91)								
III+IV	82	33(40.24)	49(79.76)								
TMPRSS4 expression	342	178 (52.05)	164 (47.95)	-	-	2.315	<0.01*	[1.643,2.864]	2.262	<0.01*	[1.533,2.531]

* $p < 0.05$

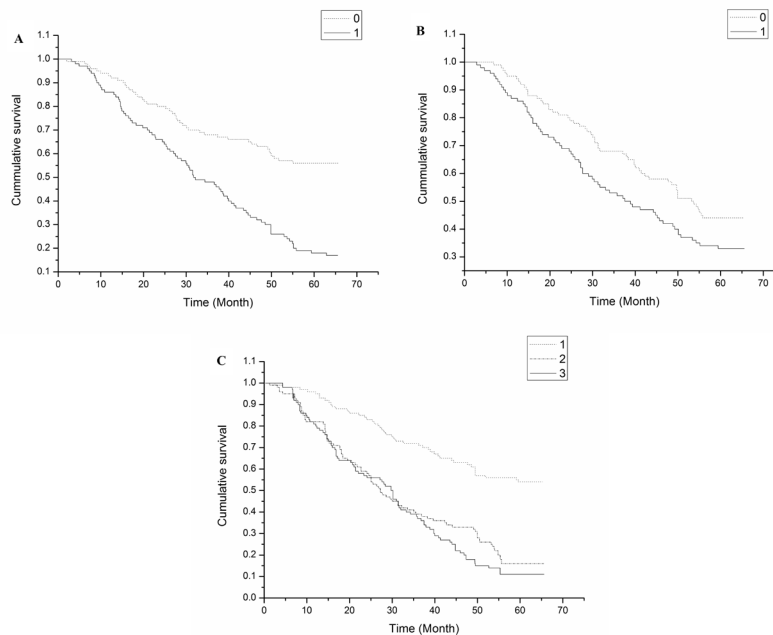


Fig. 3. Kaplan-Kaplan-Meier method and log-rank test for survival curves of patients with esophageal squamous cell carcinoma. **A)** The total survival rate of esophageal squamous cell carcinoma patients with low or no TMPRSS4 expression (line 0) was notably higher than esophageal squamous cell carcinoma patients with high TMPRSS4 expression (line 1). **B)** the overall survival of female esophageal squamous cell carcinoma patients (line 0) is notably higher than male esophageal squamous cell carcinoma patients (line 1). **C)** the total survival rate (line 3) of stage III-IV esophageal squamous cell carcinoma patients is lower than stage II patients (line 2) and stage 0-I esophageal squamous cell carcinoma patients (line 1).

female) (HR: 1.534, 95% CI: 0.454-2.174; $p=0.020$), TNM stage (HR: 1.845, 95% CI: 0.986-2.016; $p < 0.01$) and the overall survival. Multivariate survival analysis was performed through a Cox regression model that included TMPRSS4 expression, sex, and TNM stage, and showed a significant association between high TMPRSS4 expression (HR: 2.262, 95% CI: 1.533-2.531; $p < 0.01$), male sex (HR: 1.412, 95% CI: 1.263-1.632; $p=0.014$), TNM stages (HR: 1.626, 95% CI: 1.123-1.843; $p < 0.01$) and poor overall survival (Table I). The Kaplan-Meier survival curves showed similar results (Fig. 3).

DISCUSSION

We measured the messenger RNA and protein performance levels of TMPRSS4 in tumorous tissues; TMPRSS4 mRNA and protein levels

were notably high. In adjacent normal formations, TMPRSS4 mRNA and protein levels were low. A significant association was observed between high TMPRSS4 protein level and the TNM staging. For esophageal squamous cell carcinoma patients, high TMPRSS4 protein expression is a self-reliant prognostic indicator for poor total survival.

Comparably, in cervix and lung cancer, the TMPRSS show approximately identical characteristic (7). During the development of cervical and non-small cell lung cancer, the expression of TMPRSS4 protein is reduced or undetectable when the epithelial cells are differentiated (8, 9). In head and neck cancer, studies observed a similar expression pattern, as well as HATL5 (TMPRSS11B) in esophageal, cervical cancer, head and cervix cancer (10). In contrast to the condition of the cervix and lung, studies observed the over-expression of TMPRSS4 in non-small cell lung

cancer (NSCLC) compared with adjacent normal tissues, which suggests that TMPRSS4 play an important role in the carcinogenesis of NSCLC (11). When compared with healthy tissues, TMPRSS4 is over-expressed in pancreatic cancer (12). In thyroid gland, lungs, liver, cancer of the colon, ovarian cancer, and gastric cancer, its expression is also very high (13). Metastatic capacity of a cancer toward other tissues (such as clear cell carcinoma towards thyroid) could also be related to common over-expression of TMPRSS4 (14).

In summary, our findings indicate that TMPRSS4 play a key and prominent role in the advancement of esophageal squamous cell carcinoma, and that TMPRSS4 overexpression is an independent prognostic marker of esophageal squamous cell carcinoma. Future studies should focus on investigating the effect of other HAT/DESC subfamily members in esophageal squamous cell carcinoma.

REFERENCES

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA: Cancer J Clinicians* 2015; 65(2):87-108.
2. Zlatian OM, Comănescu MV, Roșu AF, et al. Histochemical and immunohistochemical evidence of tumor heterogeneity in colorectal cancer. *Rom J Morphol Embryol* 2015; 56(1):175-81.
3. Docea AO, Mitrut P, Grigore D, Pirici D, Călina CD, Gofiță E. Immunohistochemical expression of TGF beta (TGF- β), TGF beta receptor 1 (TGFB β 1), and Ki67 in intestinal variant of gastric adenocarcinomas. *Rom J Morphol Embryol* 2012; 53(3 Suppl):683-92.
4. Kang X, Chen K, Li Y, Li J, D'Amico TA, Chen X. Personalized targeted therapy for esophageal squamous cell carcinoma. *World J Gastroenterol* 2015; 21(25):7648-58.
5. Chi YK, Chen Y, Li XT, Sun YS. Prognostic significance of the size and number of lymph nodes on pre and post neoadjuvant chemotherapy CT in patients with pN0 esophageal squamous cell carcinoma: a 5-year follow-up study. *Oncotarget* 2017; 8(37):61662-73.
6. De Aberasturi AL, Calvo A. TMPRSS4: an emerging potential therapeutic target in cancer. *Br J Cancer* 2015; 112(1):4-8.
7. Mukai S, Oue N, Oshima T. Overexpression of transmembrane protein BST2 is associated with poor survival of patients with esophageal, gastric, or colorectal cancer. *Ann Surg Oncol* 2017; 24(2):594-602.
8. Ha SY, Kim KY, Lee NK, Kim MG, Kim SH. Overexpression of matriptase correlates with poor prognosis in esophageal squamous cell carcinoma. *Virchows Arch.* 2014; 464(1):19-27.
9. Cheng MF, Lin LH, Huang MS. Downexpression of Matriptase-2 correlates with tumor progression and clinical prognosis in oral squamous-cell carcinoma. *Appl Immunohistochem Mol Morphol* 2017; 25(7):481-88.
10. Conti C, Caraffa A, Kritas SK, Ronconi G, Fulcheri M. Alexithymia and its relationships with inflammatory response mediated by IL-1 family members. *J Biol Regul Homeost Agents* 2017; 31(1):21-28.
11. Li X, Li L, Wang W, Yang Y, Zhou Y, Lu S. Soluble purified recombinant C2ORF40 protein inhibits esophageal cancer cell proliferation by inducing cell cycle G1 phase block. *Oncol Lett* 2015; 10(3):1593-96.
12. Li SM, Jiang P, Xiang Y. Protease-activated receptor (PAR)1, PAR2 and PAR4 expressions in esophageal squamous cell carcinoma. *Zool Res* 2014; 35(5):420-25.
13. Kanamoto A, Ninomiya I, Harada S. Valproic acid inhibits irradiation-induced epithelial-mesenchymal transition and stem cell-like characteristics in esophageal squamous cell carcinoma. *Int J Oncol* 2016; 49(5):1859-69.
14. Gheorghiu ML, Iorgulescu R, Vrabie CD, Tupea CC, Ursu HI. Thyroid metastasis from clear cell carcinoma of the kidney 16 years after nephrectomy. *Acta Endocrinologica-Bucharest* 2016; 12(1):80-84.

LETTER TO THE EDITOR

QUICK REHABILITATION NURSING IMPROVES THE RECOVERY OF COLON CANCER PATIENTS AFTER LAPAROSCOPYJ. WANG¹, L.J. REN², X.L. CHEN³, L. MA³, B.J. CHEN³, S.J. RAN¹ and S. LU¹

¹Department of General Surgery, The Third Affiliated Hospital of Qiqihar Medical University, Qiqihar, China; ²Department of Nuclear Medicine, The Third Affiliated Hospital of Qiqihar Medical University, Qiqihar, China; ³Department of Nursing, The Third Affiliated Hospital of Qiqihar Medical University, Qiqihar, China

Received October 12, 2017 - Accepted November 28, 2017

Colon cancer is a common malignant tumor with particularly high morbidity and mortality. The aim of this study was to compare the effect of quick rehabilitation nursing and routine nursing in postoperative recovery of patients with colon cancer after laparoscopic surgery. Two hundred forty patients with colon cancer were classified into four random groups (A, B, C and D, with 60 patients in each group). All patients underwent surgery to remove the colon tumor by laparoscopy under general anesthesia. Patients in groups A and B received quick rehabilitation nursing for post-surgery recovery. In group C patients, local anesthesia associated with quick rehabilitation nursing for post-surgery recovery was used. Group D was used as control group and the patients were treated based on routine nursing. Time to get out of bed, first bowel movement time and the average time of hospitalisation in group A was lower than group D ($p<0.05$), postoperative leukocyte level as well as the occurrence rate of nausea and vomiting, ankylosis and pelvic adhesion was decreased in group A compared to group D ($p<0.05$), but the postoperative albumin and total protein level was higher than group D ($p<0.05$). The serum level of C-Reactive Protein (CRP) and interleukin 6 (IL-6) in group A was decreased compared to group D several days after surgery ($p<0.05$); group B had 4 cases of intestinal obstruction after surgery that could be cured through conservative treatment, while group D had 10 cases of intestinal obstruction, 8 of which could be cured through conservative treatment and two needed surgery ($p<0.05$); VAS for pain degree of group C in active state was clearly lower at 1h, 5h, 7h, 15h, 30h and 42h after surgery, and side effects of postoperative analgesia were clearly reduced. Time to get out of bed was obviously decreased, while there was no evident effect on postoperative dosage, chronic pain and complications. Adopting quick rehabilitation nursing can effectively reduce occurrence of complications and postoperative pain, speed up the recovery of gastrointestinal function, shorten the length of stay, and improve patients' satisfaction.

To the Editor,

Colon cancer is a common malignant tumor with particularly high morbidity and mortality. For a correct diagnosis of different types of cancers,

histological and immunohistochemical examinations are fundamental (1). Treatment with cytostatics has severe adverse effects including a decrease in the number of leukocytes with immunosuppression

Key words: quick rehabilitation, colon cancer; laparoscope, intestinal obstruction, local anesthesia

Mailing address:

Jing Wang,
Department of General Surgery,
The Third Affiliated Hospital of Qiqihar Medical University,
27 Taishun Street, Tiefeng District,
Qiqihar 161000, Heilongjiang, China
e-mail:surgeryjingwang@163.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

(2), increased infection susceptibility to resistant bacteria and fungus from *Aspergillum* or *Fusarium* genes (3, 5) and vitamin K-dependent coagulation disorders (5). Alternative therapies with extracts of some plants with cytotoxic effect (6) or the use of extracorporeal shock waves (7) have been tried but the studies are still at the beginning. Although there is progress in the diagnosis and treatment of colon cancer, the postoperative morbidity rate is still high, and the analgesia used is very strong and has many side effects. Application of various molecular targeted drug and gene therapeutic agents makes the range of surgery and trauma smaller, therefore minimal invasive surgery, such as laparoscopy, could be applied.

Quick rehabilitation nursing is introduced to further reduce postoperative analgesia and side effects (8). This consists of multidisciplinary and multi-sector treatments, including preoperative psychological guidance, bowel preparation, choosing appropriate anesthesia pattern, minimally invasive technology and encouraging patients to eat after surgery as well as to do out of bed activity with

the cooperation of surgeon, anesthetist and nurses, so that trauma and the occurrence of complications are reduced and the patient's recovery is improved. Local anesthesia is one of the key factors in quick rehabilitation nursing, and is used for relieving pain of patients after abdominal operation.

The aim of this study was to evaluate the influence of quick rehabilitation nursing on the recovery of patients who had undergone colon cancer laparoscopic surgery. We analyzed the risk of postoperative complications and analgesia degree between quick rehabilitation nursing and routine nursing.

MATERIALS AND METHODS

Patients

In the study 240 colon cancer patients were included, 130 males and 110 females, aged between 35 and 65 years and diagnosed between June 2015 and April 2017. The patients were classified into 4 groups; 60 patients per group. Inclusion criteria: definite diagnosis of colon cancer as described by diagnostic criteria of colon cancer,

Table I. Basic information of patients from the 4 groups.

Parameters	Group A	Group B	Group C	Group D	χ^2/t	p
Age(x±s, years)	42.37±5.73	45.52±5.62	43.75±6.27	43.66±6.85	0.673	>0.05
BMI(x±s, kg/m²)	23.78±1.68	24.02±1.79	24.82±2.03	24.57±2.03	0.735	>0.05
Surgical Area						
Right Hemicolon	15	21	16	18	-0.857	>0.05
Left Hemicolon	17	19	17	19		
Transverse Colon	28	20	27	23		
Tumor Size						
<2cm	28	24	27	26	0.817	>0.05
2.0~3.5cm	32	36	33	34		
Pathological Type						
Phase 1	31	30	27	30	-0.775	>0.05
Phase 2	21	25	17	21		
Phase 3	8	5	16	9		

chemotherapy and radiotherapy, patients with clear consciousness and steady vital signs. Exclusion criteria: patients in a critical condition; patients not suitable for laparoscopic surgery; patients with complications like serious intestinal obstruction; patients with organ dysfunction or complete dysfunction. The research was approved by the Medical ethics committee. Patients signed an informed consent form in which they agreed with all the study procedures. There were no statistically significant differences between gender, age, surgical area, tumor size and pathological type in the four groups ($p>0.05$), Table I.

Laparoscopic surgery

All patients had undergone laparoscopic surgery for colon cancer under the supervision of the same group of physicians. The surgery technique was carried out

according to the Guidance for Radical Operation of Rectal Cancer under Laparoscope (9).

Nursing methods in the four groups

The control group D was treated with routine nursing methods. Before surgery polyethylene glycol-electrolyte solution (Jiangxi Hengkang Pharmaceutical Co. Ltd, China) was administered orally and skin was prepared. The patients followed 6 hours of fasting and water deprivation and then the stomach tube and ureter were inserted. During surgery the vital indexes of patients were measured under general anesthesia. After surgery the patients received painkillers through a patient-controlled analgesia pump with an oral nonsteroidal anti-inflammatory agent. The stomach tube was removed when the intestines exhausted. The patients received liquid diets during postoperative recovery till the dietary mode was changed to normal life mode.

Table II. Comparison of different indexes from Group A and Group D.

Comparison of patient recovery after surgery ($x\pm s$)				
Group	Patients' Number	Time to Get Out of Bed(h)	Bowel movement Time(h)	The Average Time of Hospitalization(d)
A	60	17.85 $\pm$ 10.56	13.52 $\pm$ 8.11	5.37 $\pm$ 2.85
D	60	45.21 $\pm$ 16.54	22.55 $\pm$ 6.72	10.78 $\pm$ 4.28
<i>P</i>		0.025	0.0312	0.021
Comparison of patients' white blood cells, albumin, total protein in the pre and postoperative stages ($x\pm s$)				
		Group A(n=60)	Group D(n=60)	<i>p</i>
White Blood Cells($\times 10^9$)	Preoperative	5.84 $\pm$ 1.28	5.85 $\pm$ 0.75	>0.05
	Postoperative	10.35 $\pm$ 1.57	14.82 $\pm$ 1.65	<0.05
Albumin(g/L)	Preoperative	70.85 $\pm$ 5.62	70.65 $\pm$ 5.68	>0.05
	Postoperative	63.72 $\pm$ 3.37	44.82 $\pm$ 3.12	<0.05
Total Protein(g/L)	Preoperative	42.67 $\pm$ 4.34	41.75 $\pm$ 3.87	>0.05
	Postoperative	40.54 $\pm$ 4.14	24.98 $\pm$ 2.57	<0.05
Comparison of patients' surgery time, bleeding volume, loss of weight and cost of treatment ($x\pm s$)				
Parameter	Group A (n=60)	Group D (n=60)	<i>t</i>	<i>p</i>
Surgery Time(h)	3.25 $\pm$ 0.84	3.67 $\pm$ 0.88	1.923	0.047
Bleeding Volume(ml)	224.52 $\pm$ 54.35	218.85 $\pm$ 52.39	0.481	0.631
Loss of Weight (kg)	3.21 $\pm$ 0.58	4.22 $\pm$ 1.12	8.324	0.0029
Cost of Treatment (¥)	4.88 $\pm$ 0.77	5.87 $\pm$ 0.88	3.074	0.0021

Quick rehabilitation nursing methods were applied for groups A and B. Before surgery the patients took drugs orally to clean the gastrointestinal tract. Surgery took place under general anesthesia, and their vital indexes were observed in real-time. Blood pressure was stabilized and temperature changes were observed. An abdominal drainage tube was inserted, depending on the patient's physical condition. One day after surgery, the ureter was removed and they were allowed to drink water. Painkillers were given instead of opioids to reduce stimulation of intestines. When a painkiller was taken, the medical staff prepared for the patient's analgesia nursing, and a real-time detection of the patient's coagulation conventional index was determined to observe any appearance of anaphylaxis. Two or more days after surgery, patients were assisted to get off the bed and carry out some general activities and were encouraged to return to a normal diet.

Before laparoscopic surgery, group C patients were injected with 30 ml of 0.75% ropivacaine (Tiantai Mountain Chengdu Pharmaceutical Co. Ltd, China). The postoperative analgesia method used was the same as that

used in the control group. The only differences were the quick rehabilitation nursing and the local anesthesia.

Observation and test methods

After surgery, a comparison of bedtime activity, bowel movement time, average hospitalization time, hospitalization costs and satisfaction between Groups A and D were made.

Pain degree was evaluated by Visual Analogue Scale (VAS). Blood leukocyte, albumin, total albumin, serum level of C-reactive protein (CRP) and IL-6, side effects and complications after surgery were evaluated. Latex particle reinforcement immuno-turbidimetry was used for CRP detection, and ELISA was used for IL-6 detection. Morbidity of adhesive intestinal obstruction, as well as its occurrence time, symptoms and outbreak times of complications were compared between groups B and D. 1h, 5h, 7h, 15h, 30h and 42h post-surgery coagulation conventional index, VAS, and analgesia degree in resting and active conditions of groups C and D were compared.

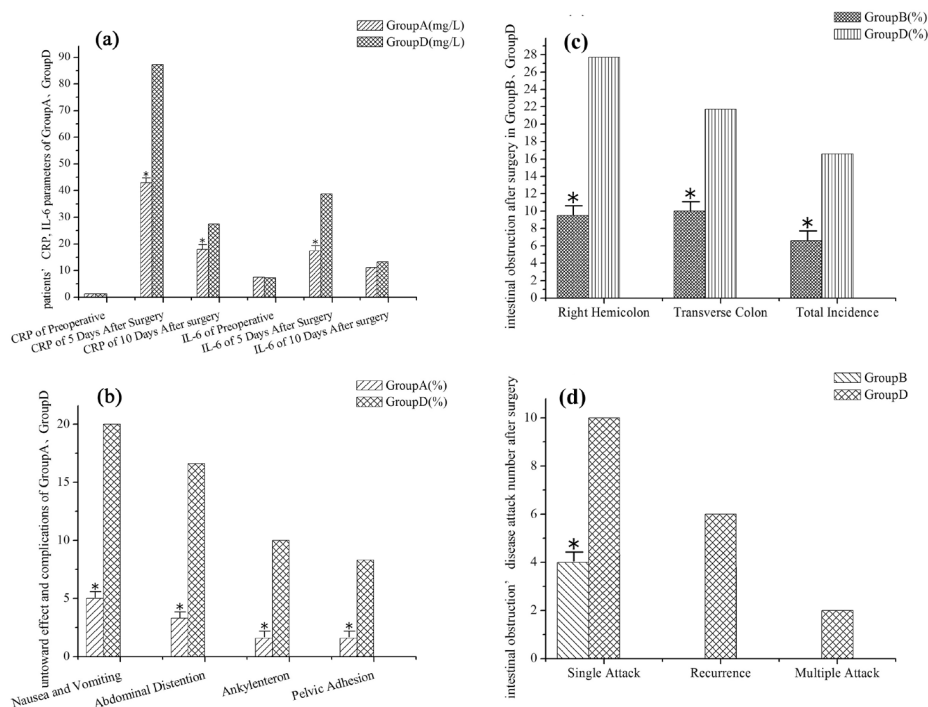


Fig. 1. Statistical analysis of untoward effects and complications between groups. **a)** Comparison of CRP, IL-6 parameters. **b)** Statistical analysis of untoward effects and complications. **c)** Incidence of intestinal obstruction after surgery. **d)** Comparison of number of intestinal obstruction disease attacks after surgery. * $p < 0.05$

Statistical analysis

SPSS18.0 software was used to statistically analyze the data expressed by mean $\pm$ standard deviation ($\bar{x} \pm s$). Group differences were compared by two independent sample *t* tests, enumeration data were revealed by % and the difference between groups was compared by χ^2 ($p < 0.05$ means that the difference was statistically significant).

RESULTS

The impact of quick rehabilitation nursing for group A on patients' recovery, physical condition and related vital indexes was compared with the control group D.

As shown in Table II, time to get out of bed, bowel movement time and the average time of hospitalization in group A patients were decreased compared with group D ($p < 0.05$). Time to get out of bed is associated with postoperative recovery, and a prolonged time is unbeneficial for postoperative recovery. A shorter bowel movement time means that gastrointestinal functions have better recovery. Fast recovery after surgery is correlated with a shorter average time of hospitalization.

It was observed that postoperative recovery of group A was faster than in group D. In group A patients, the level of leukocytes decreased compared to group D, signifying that group A had a lower chance of inflammation. Compared to group D, declining range of albumin and total protein in group A were increased, demonstrating that quick rehabilitation nursing was less harmful ($p < 0.05$). Thus, adopting quick rehabilitation nursing can reduce postoperative side effects on patients, speed their recovery and save on the cost of treatment ($p < 0.05$).

Inflammatory activity was evaluated by CRP and IL-6 levels and is presented in Fig. 1a. There was no difference in preoperative inflammatory indexes between groups A and D, but 5 days after surgery, IL-6 and CRP in group A were significantly decreased compared to group D ($p < 0.05$), and this significant difference remained for CRP levels also 10 days post-surgery ($p < 0.05$).

The effect of quick rehabilitation nursing on the incidence of side effects and possible complications

after surgery is shown in Fig. 1b. The patients from group A had a decreased occurrence of nausea and vomiting, abdominal distension, ankyloenteron and pelvic adhesion compared to the control group ($p < 0.05$). These facts suggest that quick rehabilitation nursing can reduce the risk of discomfort and complications arising after surgery.

Using laparoscopy for colon cancer leaves marks on the patient's body. During the recovery process, medical staff need to carefully accompany patients to reduce post-surgery complications. Intestinal obstruction of long duration commonly appears after laparoscopic surgery, which generates a serious threat to recovery, leading to inflammation swelling since mononuclear macrophages are stimulated to generate inflammatory cytokines. Group B was compared with the control group D for intestinal obstruction outbreak. As shown in Fig. 1c, there was no incidence of intestinal obstruction in groups B and D after left hemicolon surgery, but this appeared after right hemicolon and transverse colon surgery. Compared with group D, group B had a much lower incidence of intestinal obstruction after right hemicolon and transverse colon surgery ($p < 0.05$). Also the total incidence of intestinal obstruction in group B was lower than in group D ($p < 0.05$).

Intestinal obstruction may reappear after surgery. A comparison between group B, where quick rehabilitation nursing was applied, and group D was made and is shown in Fig. 1d. Group B had a lower incidence with rare relapse that needed only conservative treatment; group D had 10 patients with intestinal obstruction; 6 of these patients relapsed after treatment. Two patients from group D manifested multiple attacks of intestinal obstruction that requested surgery.

Quick rehabilitation nursing could be applied in all stages of surgery and calls for a close collaboration between physicians, nurses and patients. Postoperative pain always manifests in an uncomfortable recovery after surgery, at the same time, influencing the patient's cardiovascular, respiratory, endocrine and nervous system. Local anesthesia with 0.75% ropivacaine administered extra-peritoneally can be used for postoperative pain, decreasing side effects on the organism determined

by the pain. Group C, on which quick rehabilitation nursing intervention and local anesthesia before surgery were applied, was compared with group D with regard to pain degree VAS score after surgery and incidence of adverse reactions (Fig. 2).

Group C had a lower VAS score under local anesthesia compared with group D 1h, 5h, 30h and 42h post-surgery ($p<0.05$). The incidence of adverse reactions in group C was lower compared to group D at any level ($\chi^2=2.831, p=0.038$) (Fig. 2b).

DISCUSSION

Colon cancer is a very common malignant tumor that affects the intestinal tract-stomach system. Its has increased morbidity, and even after

treatment changes may occur. Quick rehabilitation nursing brings a new perspective for recovery after surgery in patients suffering from colon cancer. Laparoscopic surgery can improve the recovery of patients, decrease the recovery period and surgery trauma, reduce the adverse reactions determined by the postoperative use of analgesia and, finally, decrease the rate of complications as well as the cost of hospitalization.

Generally, surgery procedures include preoperative, intraoperative and postoperative stages. The patients' recovery levels are compared by adopting quick rehabilitation nursing using different indexes. The basic index includes time to get out of bed, time to first bowel movement, and the average time of hospitalization. Group A, which was treated with quick rehabilitation nursing, showed better indexes compared to the control group. After tumor resection surgery, protein in the patient's body clearly decreases due to malnutrition (10). Comparison of blood leukocytes, albumin and total proteins between groups A and D showed that in group A the decrease was lower than in the control group, demonstrating that quick rehabilitation nursing leads to a significant decrease in the side effects after surgery. This also applies in the case of inflammatory markers, CRP (11) and IL-6 (12), which are known to increase after surgery due to inflammatory reactions. Therefore, quick rehabilitation nursing also has positive impacts on decreasing inflammatory reactions and on the development of postoperative complications. When recovery is combined with local anesthesia, pain degree of the is reduced and the side effects determined by high doses of analgesics are decreased.

In conclusion, it is feasible to apply quick rehabilitation nursing to colon cancer patients as it can reduce the use of postoperative analgesia and speed the recovery of physical and gastrointestinal functions. Compared with routine nursing, it has an positive effect on recovery with less adverse reactions.

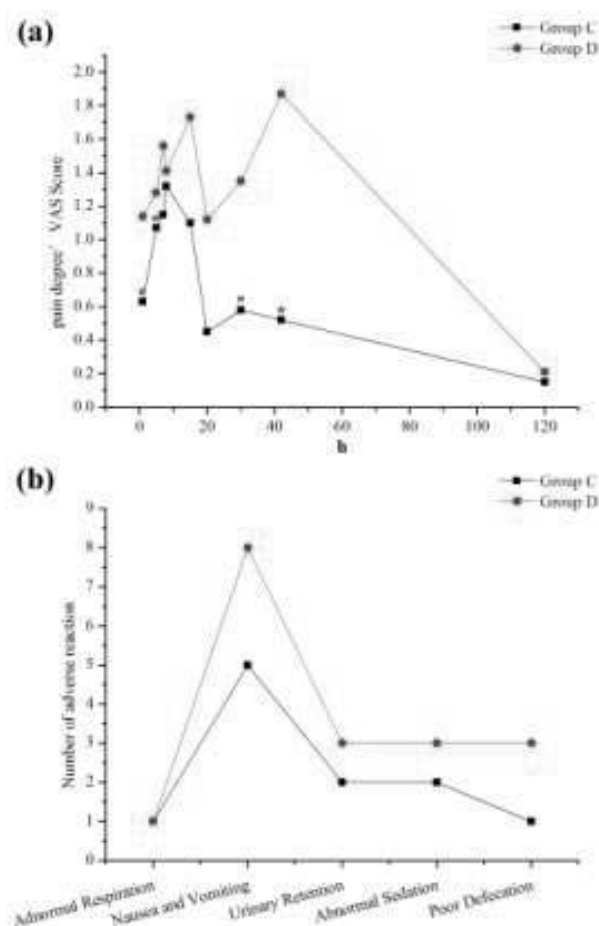


Fig. 2. Pain degree VAS score after surgery and incidence of adverse reactions of Group C and Group D. **a)** Comparison of pain degree VAS Score after surgery. **b)** Comparison of adverse reaction after surgery.

REFERENCES

1. Zlatian OM, Comănescu MV, Roșu AF, et al.

- Histochemical and immunohistochemical evidence of tumor heterogeneity in colorectal cancer. *Rom J Morphol Embryol* 2015; 56(1):175-81.
2. Ginghină O, Burcea Dragomiroiu GTA, Negrei C, Bârcă M. The importance of chemotherapy in the management strategies regarding hepatic metastases in colorectal cancer. *Farmacia* 2014; 62(3):429-35.
 3. Călina D, Docea, AO, Roșu L, et al. Antimicrobial resistance development following surgical site infections. *Mol Med Rep* 2017; 15(2): 681-88.
 4. Tănase A, Coliță A, Ianoși G, et al. Rare case of disseminated fusariosis in a young patient with graft vs. host disease following an allogeneic transplant. *Exp Ther Med* 2016; 12(4): 2078-2082.
 5. Wojciechowski VV, Călina D, Tsarouhas K, et al. A guide to acquired vitamin K coagulopathy diagnosis and treatment: the Russian perspective. *DARU* 2017; 25(1):10.
 6. Sahpazidou D, Geromichalos GD, Stagos D, et al. Anticarcinogenic activity of polyphenolic extracts from grape stems against breast, colon, renal and thyroid cancer cells. *Toxicol Lett* 2014; 230(2):218-24.
 7. Frairia R, Berta L, Catalano MG. Extracorporeal shock waves: perspectives in malignant tumor treatment. *J Biol Regul Homeost Agents* 2016; 30(3):641-8.
 8. Gillissen F, Ament SM, Maessen JM, Dejong CH, Dirksen CD, van der Weijden T, von Meyenfeldt MF. Sustainability of an enhanced recovery after surgery program (ERAS) in colonic surgery. *World J Surg* 2015; 39(2):526-33.
 9. ERAS Compliance Group. The impact of enhanced recovery protocol compliance on elective colorectal cancer resection: results from an international registry. *Ann Surg* 2015; 261(6):1153-59.
 10. Kirchhoff P, Clavien P-A, Hahnloser D. Complications in colorectal surgery: risk factors and preventive strategies. *Patient Safety in Surgery* 2010; 4(1):5.
 11. Krpata DM, Keller DS, Samia H, et al. Evaluation of inflammatory markers as predictors of hospital stay and unplanned readmission after colorectal surgery. *Pol Przegl Chir* 2013; 85(4):198-203.
 12. Knupfer H, Preiss R. Serum interleukin-6 levels in colorectal cancer patients -a summary of published results. *Int J Colorectal Dis* 2010; 25(2):135-40.

LETTER TO THE EDITOR

PERIOPERATIVE PLASMATIC PRESEPSIN LEVELS IN PATIENTS UNDERGOING TOTAL HIP OR KNEE REPLACEMENT: A PRELIMINARY STUDY

G. VICENTI, V. PESCE, D. BIZZOCA, V. NAPPI, F. PALMIOTTO, M. CARROZZO
and B. MORETTI

*School of Medicine, University of Bari Aldo Moro, AOU Consorziiale Policlinico,
Department of Basic Medical Sciences, Neuroscience and Sense Organs,
Orthopaedic & Trauma Unit, Bari, Italy*

Received April 8, 2017 – Accepted August 25, 2017

Presepsin (sCD14-ST) is an emerging biomarker in the diagnosis of sepsis. In the field of orthopaedics, it could be useful in the diagnosis and management of periprosthetic joint infections (PJI). The aim of this study is to define the normal perioperative plasmatic levels of presepsin in patients undergoing primary cementless total hip replacement (THR) or primary cemented total knee replacement (TKR). For this purpose, 50 patients (19 male, 31 female, mean age = 64.04 ± 8.88) were recruited. The patients were divided into two groups: Group A patients underwent cementless THR, whereas Group B patients underwent cemented TKR. On recruitment, anthropometric data, smoking status, osteoarthritis stage according to Kellgren and Lawrence, Harris Hip Score (HHS) for Group A patients and Knee Society Score (KSS) for Group B patients, drugs assumption and comorbidities were recorded. All the patients underwent serial blood tests, including complete blood count, presepsin (PS), C-reactive protein (CRP) and procalcitonin (PCT) 24 hours before arthroplasty (T0) and at 24 (T1), 48 (T2), 72 (T3) and 96 (T4) hours postoperatively. Body temperature (θ) was recorded every six hours in the time lapse T0-T4. Presepsin plasmatic concentration was comparable at baseline in both groups. After surgery, however, a significant increase of presepsin was observed in Group A, whereas in Group B no significant changes of presepsin were recorded. A comparable trend of this biomarker was found in the two groups, i.e. presepsin increased from T0 to T3, when it reached its maximum value, and its decrease started at T4. Finally, presepsin resulted more accurate than CRP in the evaluation of perioperative inflammatory response in patients undergoing THR or TKR. These data will be helpful in defining a reference interval for presepsin in patients with prosthetic joint implants, and a cut-off of this biomarker for the diagnosis of PJI.

To the Editor,

The soluble cluster of differentiation 14-SubType (sCD14-ST), also known as Presepsin, is an emerging biomarker for the diagnosis and evaluation of sepsis, originally described by Yaegashi et al. in 2004 (1).

Two different forms of the cluster of differentiation 14 (CD14) have been characterized: a membrane-

bound CD14 (mCD14) and a soluble CD14 (sCD14) (1-2).

mCD14 is mainly expressed on cellular membranes of monocytes, macrophages and neutrophils, where it acts as a high affinity receptor for the lipopolysaccharide-binding protein complex (LPS-LBP)(2, 3). It can also bind to peptidoglycan

Key words: presepsin, sCD14-ST, periprosthetic joint infections, cementless total hip replacement, total knee replacement

Mailing address:

Giovanni Vicenti M.D,
Department of Basic Medical Sciences, Neuroscience and Sense Organs,
Orthopaedic Clinic, School of Medicine, University of Bari "Aldo Moro",
AOU Consorziiale Policlinico, Piazza Giulio Cesare 11, 70100 Bari, Italy
Tel.: +39 0805593402
e-mail: dott.gvicenti@gmail.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
**DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF
INTEREST RELEVANT TO THIS ARTICLE.**

and other surface molecules, expressed in both Gram-negative and Gram-positive bacteria, thus triggering the acquired immune response (3, 4).

sCD14, on the other hand, enhances the immune and inflammatory responses, by mediating the activation of CD14-negative cells, namely epithelial and endothelial cells, in the presence of microbes. sCD14 is then cleaved in smaller fragments by plasma proteases; presepsin is a specific N-terminal 13 kDa glycoproteic fragment of CD14 (1–4).

Since 2004, several studies have investigated the role of presepsin in the diagnosis of systemic inflammatory response syndrome (SIRS), sepsis and neonatal sepsis. A recent metanalysis (5) has remarked that even if presepsin is a useful marker in the diagnosis of early sepsis, it should be carefully interpreted since it has shown a moderate accuracy in differentiating sepsis from non-sepsis.

Other papers have evaluated the role of presepsin in the prognosis of severe acute pancreatitis (6), in detecting infections in patients with haematological malignancies (7), in the risk stratification and diagnosis of severe community-acquired pneumonia (8), in the risk stratification in cardiac surgery patients (9) and in the diagnosis of pacemaker and implantable cardioverter defibrillator (ICD) pocket infections (10).

In the field of orthopaedic surgery, presepsin could be helpful in the diagnosis and management of patients with periprosthetic joint infections (PJI). PJI, indeed, is a fearsome complication of arthroplasty which accounts for 25% of failed TKR and 15% of failed THR in the USA (11). Decision-making in patients with a suspected PJI is never easy, so the search for a biomarker that, together with clinical and radiological findings, could improve the management of such of patients is currently a big challenge for orthopaedic surgeons.

The aim of this study is to define the normal perioperative plasmatic levels of presepsin, in patients undergoing primary cementless total hip replacement (THR) or primary cemented total knee replacement (TKR) for primary hip osteoarthritis of the hip or of the knee, respectively, in order to assess a reference interval for this marker in orthopaedic prosthetic surgery.

MATERIALS AND METHODS

From September 2016 to February 2017, 117 patients underwent arthroplasty at our institute. According to inclusion and exclusion criteria, 50 patients (19 male, 31 female; mean age = 64.04 ± 8.88) were enrolled in the present study. All the patients gave informed consent before enrolment.

Inclusion criteria were: primary cementless THR or primary cemented TKR; primary osteoarthritis of the hip or of the knee; urinary catheter time of permanence < 24 hours; peripheral venous cannulation time of permanence < 24 hours; no postoperative homologous blood transfusion administration and hospital stay < 5 days.

Exclusion criteria were: presence of other articular prosthetic replacement or bone fixation devices; chronic inflammatory diseases; chronic kidney diseases; history of recurrent infections or malignant neoplasms; previous surgery in the preceding twelve months; diabetes mellitus; immunosuppressive drug or corticosteroid assumption; documented metal allergy and documented cephalosporin allergy.

The patients were divided into two groups, according to the arthritic joint: Group A patients underwent cementless THR, whereas Group B patients underwent cemented TKR, using a bone cement loaded with gentamicin.

All the patients received the same antibiotic prophylaxis: cefazolin 2 grams one hour prior to surgical incision and 1 gram every six hours postoperatively for the first 24 hours. All the arthroplasties were performed by the same surgical and anesthesia team; total operative time (TOT) was defined as the time taken from skin incision to completion of skin closure.

At enrollment anthropometric data, smoking status, osteoarthritis stage according to Kellgren and Lawrence (12), Harris Hip Score (HHS) for Group A patients and Knee Society Score (KSS) for Group B patients, drugs assumption and comorbidities were recorded.

All the patients underwent serial blood tests, including complete blood count, presepsin (PS), C-Reactive Protein (CRP) and Procalcitonin (PCT) 24 hours before arthroplasty (T0) and at 24 (T1), 48 (T2), 72 (T3) and 96 (T4) hours postoperatively. Body temperature (θ) was recorded every six hours in the time lapse T0–T4. The data were gathered and analyzed in blind manner.

Statistical analysis was performed using the SPSS®

11.0 software (SPSS Inc., Chicago, IL, USA). The intergroup variability was evaluated using the unpaired *t*-test after ANOVA. Paired *t*-test was used to assess the postoperative changes of presepsin, PCT, CRP, white blood cells (WBC), neutrophils and lymphocytes. Pearson correlation test was performed. The tests were two-tailed with a confidence level of 5%.

RESULTS

The main data of the study are summarized in Table I. The plasmatic levels of presepsin were assessed in 28 patients undergoing THR (Group A) and in 22 patients undergoing TKR (Group B). Total Operative Time was comparable in both groups.

Fig. 1 A-F shows the plasmatic levels of presepsin, CRP, PCT, WBC, neutrophils and lymphocytes at T0, T1, T2, T3 and T4. The presepsin plasmatic levels were comparable at baseline in both groups at recruitment, while CRP was significantly higher in patients undergoing TKR (Group B), compared with patients undergoing THR. In Group A, a significant

increase of plasmatic presepsin was observed post-operatively, whereas in Group B no significant changes of presepsin were recorded after surgery, compared with baseline. However, no significant changes in presepsin levels between groups were observed after surgery and a comparable trend of this biomarker was found in the two groups, i.e. presepsin increased from T0 to T3, when it reached its maximum value, and its decrease started at T4 (Fig. 1A).

A positive correlation between patients' age and presepsin plasmatic concentration was observed (Group A: $R=0.74$; $p=0.018$. Group B: $R=0.68$; $p=0.027$). In Group A, a positive correlation between presepsin and CRP mean values was found at T0, T1 and T3; a negative correlation between presepsin and mean WBC was also noticed in Group A only.

DISCUSSION

PJI currently represents a big challenge for orthopaedic surgeons. The diagnosis of this

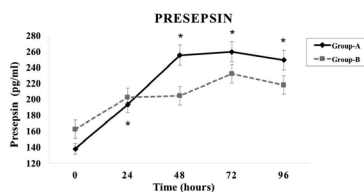


Fig. 1 A

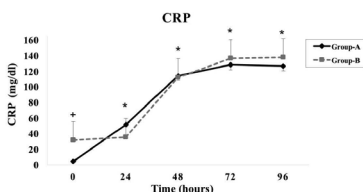


Fig. 1 B

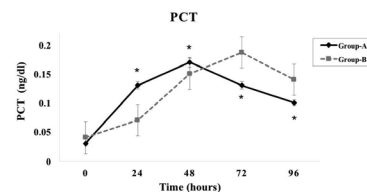


Fig. 1 C

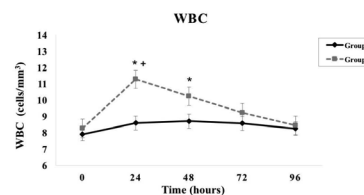


Fig. 1 D

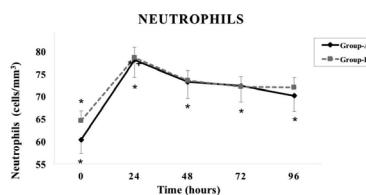


Fig. 1 E

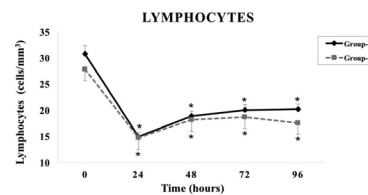


Fig. 1 F

Fig. 1. A) The mean plasmatic presepsin values in both groups at T0-T4. The error bars indicate the standard deviation. **B)** The mean plasmatic CRP values in both groups at T0-T4. The error bars indicate the standard deviation. $^+p<0.05$, p values refer to a comparison between groups (unpaired *t*-test). **C)** The mean plasmatic procalcitonin values in both groups at T0-T4. The error bars indicate the standard deviation. **D)** The mean white blood cell value in both groups at T0-T4. The error bars indicate the standard deviation. **E)** The mean neutrophil count in both groups at T0-T4. The error bars indicate the standard deviation. **F)** The mean lymphocyte count in both groups at T0-T4. The error bars indicate the standard deviation.

* $p<0.05$, p values refer to paired *t*-test. $^+p<0.05$, p values refer to a comparison between groups (unpaired *t*-test).

Table I. Main data of the study.

	GROUP-A (THR)	GROUP-B (TKR)
Number of patients (%)	28 (56%)	22 (44%)
Gender		
Male	9 (32.14%)	10 (45.45%)
Female	19 (67.86%)	12 (54.54%)
Smoking status		
# of smokers	3 (10.7%)	4 (18.2%)
IAI		
#of patients who performed IAI before arthroplasty	4 (14.3%)	18 (81.8%)
Age		
Min-Max	55-76	60-76
Mean±SD	63.46±7.33	65.09±5.86
BMI (kg/m²)		
Mean±SD	26.02±7.19	28.54 ±5.04
KLS		
Mean±SD	3.16±0.54	3.48±0.47
HHS		
Mean±SD	48.85±11.2	N/A
KSS		
Mean±SD	N/A	48.09±5.24
Total Operative Time		
Mean±SD	116.54±28.6	106.37±28.47

N/A: Not Applicable; TKR: Total Knee Replacement; THR: Total Hip Replacement; IAI: Intra-Articular Injection; BMI: Body Mass Index; KLS: Kellgren-Lawrence Score; HHS: Harris Hip Score KSS: Knee Society Score.

infectious disease, indeed, may be difficult, due to its heterogeneous clinical manifestation (13) and to the poor standardized diagnostic criteria. Moreover, in the treatment of PJI there are still lots of unanswered questions (13, 14), including: the diagnostic value of plasmatic biomarkers, synovial fluid analysis, radiological and nuclear medicine imaging; the timing of surgery; the usefulness of prolonged antibiotic assumption, as well as the optimal timing to perform the antibiotic-loaded spacer implant removal and the final reimplantation of a revision total joint prosthesis.

On the bases of the emerging role of presepsin in the diagnosis and prognosis of several infectious diseases, ranging from sepsis to pacemaker and ICD pocket infections, we have hypothesized this biomarker could be helpful in the management of PJI.

In this study, we assessed the normal perioperative changes of plasmatic presepsin, compared with CRP, PCT, WBC, neutrophils and lymphocytes, in patients

undergoing cementless THR or cemented TKR, in order to define a reference interval for this marker in joint replacement surgery.

At baseline, the presepsin levels were comparable in both groups, while a significantly higher CRP was recorded in patients undergoing THR (Group B) (Fig. 1B).

The significant correlation observed at baseline between the patients' age and the presepsin values is an interesting datum, which highlights that the ideal presepsin plasmatic concentration should be corrected according to the patient's age.

Even if prolonged operative time is presently considered a risk factor for PJI (15), surprisingly, in this study we no found a significant correlation between presepsin plasmatic levels and total operative time.

Postoperatively, a significant increase of plasmatic presepsin, compared to baseline, was observed in Group A patients, whereas the values of presepsin recorded in Group B were comparable to baseline.

Nevertheless, there was no significant difference of presepsin values between the two groups: the mean presepsin values were higher in Group B at T0 and T1, while at T2 and T3 presepsin reaches higher values in Group A, compared with Group B (Fig. 1C). Finally, in both groups, a decrease of the mean presepsin value is observed at T4 (Fig. 1C).

These data show that presepsin has a characteristic perioperative trend in patients undergoing THR or TKR: the plasmatic concentration of this biomarker increases until 72 hours postoperatively, when it reaches its maximum, and starts decreasing at 96 hours post-operatively (Fig. 1B).

These data suggest a potential prognostic value of presepsin in patients with PJI: a successful implant of an antibiotic-load spacer should cause a normalization of presepsin levels; conversely, a persistent high presepsin plasmatic concentration after such treatment could predict a poor outcome.

We finally recommend assessing routinely, in patients undergoing total joint replacement, the plasmatic presepsin concentration: before surgery, to excluded the presence of an unknown infection; at 72 hours post-operatively, to evaluate the maximum value of presepsin, which is higher in cementless arthroplasties compared with cemented ones; and at 96 hours post-operatively when a decrease of presepsin should be found. The lack of a presepsin decrease at 96 hours post-operatively should be a predictive factor of PJI.

Our study has some limitations. The sample size is quite small, but it was exceeded by applying several inclusion and exclusion criteria at recruitment. Furthermore, our data refer only to the perioperative period, but all the patients will undergo a long-term follow-up, to better define the role of presepsin in orthopaedic prosthetic surgery.

REFERENCES

1. Yaegashi Y, Sato N, Suzuki Y, et al. Evaluation of a newly identified soluble CD14 subtype as a marker for sepsis. *J Infect Chemother* 2005; 11:234-38.
2. Shozushima T, Takahashi G, Matsumoto N, Kojika M, Okamura Y, Endo S. Usefulness of presepsin (sCD14-ST) measurements as a marker for the diagnosis and severity of sepsis that satisfied diagnostic criteria of systemic inflammatory response syndrome. *J Infect Chemother* 2011; 17:764-69.
3. Zou Q, Wen W, Zhang X-C. Presepsin as a novel sepsis biomarker. *World J Emerg Med* 2014; 5:16-19.
4. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* 1990; 249:1431-33.
5. Wu J, Hu L, Zhang G, Wu F, He T. Accuracy of presepsin in sepsis diagnosis: a systematic review and meta-analysis. *PLoS One* 2015; 10:e0133057.
6. Lin J, Li Z, Zheng Y, Zhang Y, Shao C, Liu G, Li J. Elevated presepsin levels are associated with severity and prognosis of severe acute pancreatitis. *Clin Lab* 2016; 62:1699-708.
7. Ebihara Y, Kobayashi K, Ishida A, et al. Diagnostic performance of procalcitonin, presepsin, and C-reactive protein in patients with hematological malignancies. *J Clin Lab Anal* 2017:e22147.
8. Leoni D, Rello J. Severe community-acquired pneumonia: optimal management. *Curr Opin Infect Dis* 2017; 30:240-47.
9. Bomberg H, Klingele M, Wagenpfeil S, et al. Presepsin (sCD14-ST) is a novel marker for risk stratification in cardiac surgery patients. *Anesthesiology* 2017; 126:631-42.
10. Lennerz C, Vrazic H, Haller B, et al. Biomarker-based diagnosis of pacemaker and implantable cardioverter defibrillator pocket infections: A prospective, multicentre, case-control evaluation. *PLoS One Public Library of Science* 2017; 12:e0172384.
11. Bozic KJ, Kurtz SM, Lau E, et al. The epidemiology of revision total knee arthroplasty in the United States. *Clin Orthop Relat Res* 2010; 468:45-51.
12. Kellgren JH, Lawrence JS. Radiological assessment of osteo-arthritis. *Ann Rheum Dis* 1957; 16:494-502.
13. Henderson RA, Austin MS. Management of periprosthetic joint infection: the more we learn, the less we know. *J Arthroplasty* 2017; 32:2056-59.
14. Deirmengian C, Kardos K, Kilmartin P, Cameron A, Schiller K, Parvizi J. Diagnosing periprosthetic joint infection: has the era of the biomarker arrived? *Clin Orthop Relat Res* 2014; 472:3254-62.

LETTER TO THE EDITOR

**LIPID-LOWERING EFFECT OF BERGAMOT POLYPHENOLIC FRACTION:
ROLE OF PANCREATIC CHOLESTEROL ESTER HYDROLASE**

V. MUSOLINO^{1,2}, M. GLIOZZI^{1,2}, C. CARRESI^{1,2}, J. MAIUOLO^{1,2}, R. MOLLACE^{1,3}, F. BOSCO^{1,2},
F. SCARANO^{1,2}, M. SCICCHITANO^{1,2}, A. MARETTA^{1,2}, E. PALMA^{1,2}, M. IANNONE⁴,
V.M. MORITTU⁵, S. GRATTERI⁶, C. MUSCOLI^{1,2,7}, M. FINI^{2,7} and V. MOLLACE^{1,2,7}

¹Institute of Research for Food Safety and Health (IRC-FSH), Department of Health Science, Magna Graecia University of Catanzaro, Catanzaro, Italy; ²Nutramed S.C.A.R.L, Complesso "Nini Barbieri" Roccelletta di Borgia, Catanzaro, Italy; ³Department of Life, Health & Environmental Sciences, University of L'Aquila, Italy; ⁴CNR, Institute of Neurological Sciences, Section of Pharmacology, Roccelletta di Borgia, Borgia, Italy; ⁵Department of Health Science, Magna Graecia University of Catanzaro, Catanzaro, Italy; ⁶Interdepartmental Services Centre of Veterinary for Human and Animal Health, Magna Graecia University of Catanzaro, Catanzaro, Italy; ⁷Department of Medical and Surgical Sciences, Magna Graecia University, Catanzaro, Italy; ⁷Centre for Clinical and Basic Research IRCCS San Raffaele Pisana Rome Italy

Received May 22, 2017 – Accepted September 7, 2017

Bergamot polyphenolic fraction (BPF) has been shown to positively modulate several mechanisms involved in metabolic syndrome, suggesting its use in therapy. In particular, it is able to induce a significant amelioration of serum lipid profile in hyperlipemic patients at different levels. The purpose of our study was to investigate the effect of BPF on cholesterol absorption physiologically mediated by pancreatic cholesterol ester hydrolase (pCEH). An *in vitro* activity assay was performed to study the effect of BPF on pCEH, whereas the rate of cholesterol absorption was evaluated through *in vivo* studies. In particular, male, Sprague-Dawley rats (200–225 g) were fed either normal chow or chow supplemented with 0.5% cholic acid, 5.5% peanut oil, and varying amounts of cholesterol (0 to 1.5%). BPF (10 mg/Kg) was daily administrated by means of a gastric gavage to animals fed with lipid supplemented diet for 4 weeks and, at the end of the study, plasma lipids and liver cholesteryl esters were measured in all experimental groups. Our results show that BPF was able to inhibit pCEH activity and this effect was confirmed, *in vivo*, via detection of lymphatic cholesteryl ester in rats fed with a cholesterol-rich diet. This evidence clarifies a further mechanism responsible for the hypolipemic properties of BPF previously observed in humans, confirming its beneficial effect in the therapy of hypercholesterolemia and in the treatment of metabolic syndrome.

To the Editor,

Evidence has been accumulated showing that bergamot polyphenolic fraction (BPF), rich

in pectins and flavonoids, causes lowering of cholesterol levels by modulating hepatic HMG-CoA levels and via an enhanced excretion of fecal sterols

Key words: *pancreatic cholesterol ester hydrolase, BPF, anti-lipemic effect*

Mailing address:

Prof. Vincenzo Mollace,
University "Magna Graecia"
Viale Europa, Loc. Germaneto,
88100 Catanzaro, Italy
Tel.: +39 327475800
e-mail: mollace@libero.it

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
**DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF
INTEREST RELEVANT TO THIS ARTICLE.**

in rats, thereby contributing to its hypolipemic and hypoglycemic effect subsequently found in patients on BPF treatment (1-3).

In particular, evidence exists that dietary hesperetin reduces hepatic TG accumulation which is associated with the reduced activity of TG synthetic enzymes, such as phosphatidate phosphohydrolase (4). In addition, *in vitro* studies suggest that naringenin and hesperetin decrease the availability of lipids for assembly of apoB-containing lipoproteins, an effect mediated by reduced activities of acyl CoA: cholesterol acyltransferases (ACAT) (5). On the other hand, BPF has been shown to reduce cholesterol absorption at the gastrointestinal level (6), although the mechanism is still to be better clarified.

Pancreatic, bile acid-dependent cholesteryl ester hydrolase (pCEH) is secreted into the small intestine where it catalyzes the hydrolysis of dietary cholesteryl esters and other acylated lipids. However, several observations suggest that the role of pCEH extends beyond that of simply hydrolyzing dietary lipids. In particular, it has been proposed that pCEH, in addition to its role in lipid hydrolysis, may catalyze cholesteryl ester formation after uptake of the enzyme into the mucosal cells. Data support a role for pCEH in the uptake of both unesterified cholesterol and cholesteryl ester into intestinal cells, the latter independent of ester hydrolysis (7, 8). Thus, pCEH may be involved at several steps or sites during cholesterol absorption (i.e., cholesteryl ester hydrolysis, cholesteryl ester synthesis, uptake of unesterified and esterified cholesterol).

If the above putative functions of pCEH are correct, it suggests that pharmacologic inhibition of this enzyme might lead to inhibition of cholesterol absorption and thereby a reduction in plasma cholesterol concentration. This emerged from evidence suggesting that selective inhibition of pCEH counteracts cholesterol absorption in rats, dogs, and quail (9, 10).

The purpose of the present study is to investigate the activity of BPF on pCEH in animal models in which more plasma cholesterol is transported in low density lipoprotein (LDL).

MATERIALS AND METHODS

Preparation of BPF

Bergamot juice (BJ) was obtained from peeled fruits by industrial pressing and squeezing as previously described (1). The juice was oil fraction-depleted by stripping, clarified by ultra-filtration and loaded on suitable polystyrene resin columns absorbing polyphenol compounds of MW between 300 and 600 Da (Mitsubishi). Polyphenol fractions were eluted by a mild KOH solution. Next, the fitocomplex was neutralized by filtration on cationic resin at acidic pH. Finally, it was vacuum dried and minced to the desired particle size to obtain BPF powder. BPF powder was analysed by HPLC for flavonoid and other polyphenol content. In addition, toxicological analyses were performed including heavy metal, pesticide, phthalate and sinephrine content which revealed the absence of known toxic compounds. Standard microbiological tests detected no mycotoxins and bacteria.

Detection of activity of pCEH in vitro

The *in vitro* inhibitory effects of BPF on pCEH activity were assessed as previously described (11), by using the porcine enzyme (0.0025 U/mL final concentration; Calbiochem, La Jolla, CA, USA). The substrate was a micellized lipid suspension composed of [3 H]cholesteryl oleate (2 μ Ci/mL), 1 mM lysophosphatidylcholine, 40 mM cholate, and 0.01 mM cholesteryl oleate dispersed in a buffer of 150 mM NaCl, 1 mM EDTA and 20 mM 2-(*N*-morpholino) ethanesulfonic acid, pH 6.0. The substrate was prepared by combining aliquots of 100 mM sodium cholate in chloroform/methanol (2:1, vol/vol), 100 mM lysophosphatidylcholine in chloroform/methanol (2:1, vol/vol), 1 mM cholesteryl oleate (in chloroform), and a volume of [3 H]cholesteryl oleate. Solvents were removed by evaporation under a stream of N₂. The dried lipids were resuspended in the appropriate volume of buffer to yield the desired final concentrations of lipids. The pCEH (32.7 IU/mg) was dissolved at a concentration of 0.1 mg/mL in the same buffer as that used for substrate. A more diluted lipase solution (0.15 μ g/mL) was used for the assay. The assay was performed at room temperature. Five microliters of the test compounds (BPF or vehicle), dissolved in ethanol at a concentration 40-fold greater than the final desired test concentration, was added to individual wells

of a 96-well plate. The assay wells without test compound received the same volume of ethanol alone. One hundred microliters of carboxyester lipase (0.15 $\mu\text{g/mL}$) was added to each well. The assay reaction was initiated by the addition of 100 μL of substrate and stopped 10 min later by the addition of 20 μL of 1 N HCl. For quantitation of the substrate and lipolytic products, the contents of each well were transferred to glass high-performance liquid chromatography autosampler vials (12 x 32 mm) and combined with 0.6 mL of ethyl acetate/acetone (2:1, vol/vol). The vials were capped, vortexed twice, and lightly centrifuged for 5 min. The organic phase was transferred to clean autosampler vials and dried under a stream of nitrogen followed by vacuum dessication for at least 1 h. The dried extracts were dissolved in 50 μL of dioxane and then further diluted with 150 μL of isooctane. The [^3H]-labeled lipids were then quantitated by high-performance liquid chromatography as previously described (12).

Studies on cholesterol absorption in vivo

Male, Sprague-Dawley rats (200–225 g, Charles River, Portage, MI, USA) were fed either normal chow or chow supplemented with 0.5% cholic acid, 5.5% peanut oil, and varying amounts of cholesterol (0 to 1.5%).

In all experiments, BPF 1-50 mg/Kg was given daily by means of a gastric gavage. The vehicle for oral gavage was drinking water. BPF and diets were initiated at the same time and continued for 4 weeks. Plasma lipids and liver cholesteryl esters were measured by standard methods.

Although liver cholesteryl ester content correlates well with measures of cholesterol absorption, cholesterol absorption was measured directly in rats by determining the rate of appearance of cholesterol and cholesteryl esters into mesenteric lymph after a single bolus of into a duodenal cannula. A lipid emulsion was then infused, which was prepared by dissolving 200 mg of cholesterol and 250 mg of egg phosphatidylcholine in chloroform. After removal of the chloroform by evaporation under N_2 and vacuum, 5 g safflower oil, 100 mg sodium taurocholate, and 100 mL of saline were added and the entire mixture was emulsified by sonication. The rate of infusion into the duodenum was 2.5 mL/h.

Statistical analysis

Data were expressed as mean $\pm$ standard deviation

(SD) and statistically evaluated for differences using one-way analysis of variance (ANOVA), followed by Tukey-Kramer multiple comparison test (GraphPad software for science). A P value of <0.05 was considered statistically significant.

RESULTS

Effect of BPF on pCEH in vitro

BPF significantly inhibited porcine pCEH with IC_{50} values of 0.62 μM , compared to vehicle (Fig. 1). In addition, BPF has also been shown to lack significant inhibitory activity against ACAT; it does not inhibit other serine hydrolases, including pancreatic triglyceride lipase (not shown), thus suggesting that the action on cholesterol absorption is mainly due to inhibition of pCEH.

Effect of BPF on cholesterol absorption in vivo

In our laboratory the effect of BPF was first evaluated in normal, chow-fed animals using gastric gavage administration. Plasma total cholesterol concentrations and triglycerides were unchanged in rats at doses of 1, 10 and 50 mg/kg. In contrast, liver cholesteryl esters decreased at the highest dose (Table I).

Since pCEH have been implicated as playing a role in the absorption of exogenous (dietary) cholesterol, it was of interest to determine whether BPF would prevent diet-induced hypercholesterolemia, and furthermore, at what level of dietary cholesterol optimal efficacy would be observed. To this end, groups of rats were fed varying amounts of cholesterol and dosed orally with BPF for 4 weeks (10 mg/kg). Plasma cholesterol in controls increased steadily from about 50 mg/dL (chow alone) to almost 300 mg/dL (from 1 to 1.5% dietary cholesterol, Table II). This effect was significantly counteracted by treating rats with BPF 10 mg/Kg, thus confirming previous data from our group.

In order to study the mechanism of action of BPF, rats were fitted with duodenal and mesenteric lymph cannulas to measure lymphatic cholesteryl ester output during active lipid absorption. After a single oral dose of BPF (10 mg/kg), the rise in

Table I. Effect of BPF (10 mg/Kg; $n=3$ for each dose) on serum cholesterol and triglycerides (mg/dL) and liver cholesteryl ester content (mg/g) in chow-fed rats. The lack of changes in serum cholesterol levels, shows that BPF is not involved in the reabsorption of biliary (endogenous) cholesterol, while affecting liver cholesterol ester contents.

* $p<0.05$ BPF 10 mg/Kg vs BPF 1mg/Kg; BPF 50 mg/Kg vs BPF 1 mg/Kg.

Treatment	Doses (mg/Kg)	Serum cholesterol	Serum triglycerides	Liver cholesteryl esters
BPF	1	58 ± 5	142 ± 6	865 ± 30
BPF	10	62 ± 4	146 ± 6	725 ± 27
BPF	50	62 ± 4	140 ± 4	651 ± 24

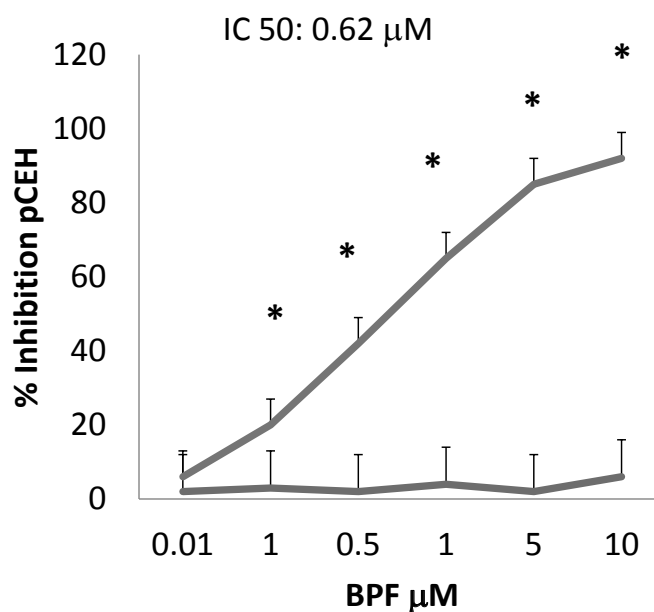


Fig. 1. Inhibition curve for BPF against porcine pancreatic cholesteryl ester hydrolase (pCEH) compared to vehicle. Each point represents the mean $\pm$ SE of triplicate determinations. The concentration required to inhibit enzyme activity 50% (IC₅₀) is indicated. * $p<0.05$ BPF vs vehicle

Table II. Effect of BPF (10 mg/Kg) on plasma cholesterol levels after increasing dietary cholesterol load (0-1.5%; $n=3$ for each supplementation) on plasma cholesterol levels (mg/dL) in rats Data show that only exogenous (dietary) cholesterol is modulated by BPF supplementation. * $p<0.05$ BPF vs CTRL

Dietary cholesterol	CTRL	BPF
0	60 $\pm$ 3	60 $\pm$ 2
0.1	85 $\pm$ 2	71 $\pm$ 4
0.2	112 $\pm$ 4	81 $\pm$ 3
0.5	165 $\pm$ 5	90 $\pm$ 5
1	245 $\pm$ 7	102 $\pm$ 5
1.25	281 $\pm$ 6	125 $\pm$ 5
1.5	315 $\pm$ 6	141 $\pm$ 6

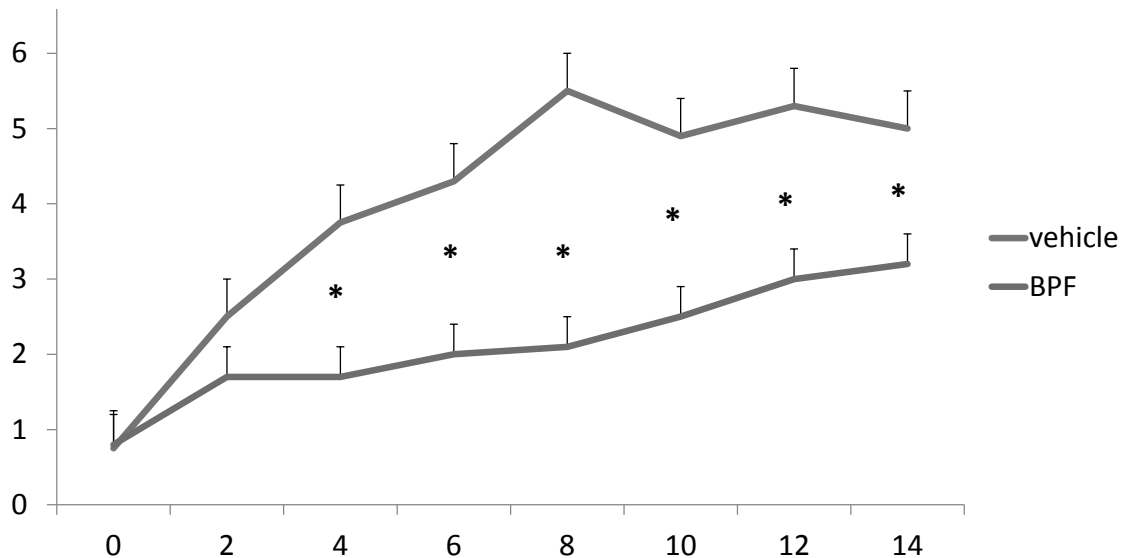


Fig. 2. Effect of a single dose of BPF (10 mg/Kg) on the lymphatic output (transport) of cholesteryl esters in rats with a mesenteric lymph fistula. After collecting basal lymph for 2 h the compound or vehicle alone was injected directly into the duodenum via a cannula, and lymph was collected hourly. Each point represents the mean $\pm$ SE of three animals.

* $p<0.05$ BPF vs vehicle

cholesteryl ester output was blunted significantly, whereas the output of triglycerides was unaffected (Fig. 2). Lymphatic unesterified cholesterol, which was a small percentage of the total, was unaffected by drug treatment (data not shown).

DISCUSSION

The present data show, for the first time, that BPF enhances cholesterol excretion via pCEH inhibition. In particular, BPF has been shown to inhibit pCEH under *in vitro* settings via direct activity on pCEH enzyme. On the other hand, this has been confirmed *in vivo* via detection of lymphatic cholesteryl ester in rats fed with a cholesterol rich diet. This may account for some of the hypolipemic properties shown with BPF both in rats and in humans.

In particular, we have recently shown that fecal output of total bile acids and neutral sterols was found to be enhanced in the BPF-treated group compared to the hyperlipidemic group. The conversion of cholesterol to bile acids is the major pathway of cholesterol elimination, and it accounts for about 50% of daily cholesterol excretion.

Cholesterol 7R-hydroxylase, the rate-determining enzyme in the conversion of cholesterol to bile acids, is mainly regulated by feedback inhibition of bile acids reabsorbed from the intestine (13). Lipid fecal excretion was found to be increased in hyperlipidemic rats, and BPF treatment enhanced, significantly, their excretion. The increase in the excretion of bile acids seems to activate cholesterol 7R-hydroxylase, enhancing the conversion of liver cholesterol to bile acids for excretion. This leads to a decrease in hepatic cholesterol content, which in turn stimulates LDL receptor expression and lowers blood cholesterol levels. In fact, the hypocholesterolemic activity of BPF could be due to the promotion of cholesterol and bile excretion. This is in agreement with previous studies carried out with implementation of diet polyphenols in hyperlipemic patients. Indeed, evidence has been collected showing that grape seed-derived gallic acid, catechin, and epicatechin significantly inhibited pancreatic cholesterol esterase in a concentration-dependent manner (14). Furthermore, similar response was found by using

resveratrol, an active compound of red wine, and of a whole red wine polyphenolic extract (RWE) on the pancreatic bile salt-dependent lipase (BSDL). BSDL is involved in the duodenal hydrolysis of lipid esters and in part of cholesteryl esters, thus favoring the bioavailability of free cholesterol (15).

Thus BPF, alongside with several polyphenols used as supplementation therapy, may inhibit directly the absorption of dietary (exogenous) cholesterol at the gastrointestinal level, being not involved in the reabsorption of biliary (endogenous) cholesterol. This effect occurs via pCEH enzyme inhibition, which explains the beneficial response in lowering serum cholesterol of BPF found in hyperlipemic patients.

ACKNOWLEDGEMENTS

This paper was supported by PON03PE_00078_1 and PON03PE_00078_2.

REFERENCES

1. Mollace V, Sacco I, Janda E, et al. Hypolipemic and hypoglycaemic activity of bergamot polyphenols: from animal models to human studies. *Fitoterapia* 2011; 82:309-16.
2. Gliozzi M, Walker R, Muscoli S, et al. Bergamot polyphenolic fraction enhances rosuvastatin-induced effect on LDL-cholesterol, LOX-1 expression and protein kinase B phosphorylation in patients with hyperlipidemia. *Int J Cardiol* 2013; 170:140-45.
3. Mollace V, Ragusa S, Sacco I, et al. The protective effect of bergamot oil extract on lecithine-like oxyLDL receptor-1 expression in balloon injury-related neointima formation. *J Cardiovasc Pharmacol* 2008; 13:120-29.
4. Cha JY, Cho YS, Kim I, Anno T, Rahman SM, Yanagita T. Effect of hesperetin, a citrus flavonoid, on the liver triacylglycerol content and phosphatidate phosphohydrolase activity in orotic acid-fed rats. *Plant Foods Hum Nutr* 2001; 56:349-58.
5. Wilcox LJ, Borradaile NM, de Dreu LE, Huff MW. Secretion of hepatocyte apoB is inhibited by the flavonoids, naringenin and hesperetin, via reduced activity and expression of ACAT2 and MTP. *J Lipid*

- Res 2001; 42:725-34.
6. Gliozzi M, Carresi C, Musolino V, et al. The effect of bergamot-derived polyphenolic fraction on LDL small dense particles and non alcoholic fatty liver disease in patients with metabolic syndrome. *Adv Biolog Chem* 2014; <http://www.scirp.org/journal/abc>.
 7. Lopez-Candales A, Bosner MS, Spilburg CA, Lange LG. Cholesterol transport function of pancreatic cholesterol esterase: directed sterol uptake and esterification in enterocytes. *Biochemistry* 1993; 32:12085-89.
 8. Gallo LL, Wadsworth JA, Vahouny GV. Normal cholesterol absorption in rats deficient in intestinal acyl coenzyme A:cholesterol acyltransferase activity. *J Lipid Res* 1987; 28:381-87.
 9. McKean ML, Commons TJ, Berens MS, Hsu PL, Ackerman DM, Steiner KE, Adelman SJ. Effects of inhibitors of pancreatic cholesterol ester hydrolase (pCEH) on [¹⁴C]-cholesterol absorption in animal models. *FASEB J* 1992; 6:A1388 (Abstract).
 10. Adelman SJ, Commons TJ, McKean ML, Day CE, Steiner KE. Pancreatic cholesterol ester hydrolase (PCEH) inhibitors inhibit dietary hypercholesterolemia (hyperc) in rats and atherosclerosis (ath) in sea (susceptible to experimental ath) quail. XI Int. Symposium on Drugs Affecting Lipid Metabolism 1992; 87 (Abstract).
 11. Krause BR, Sliskovic DR, Anderson M, Homan R. Lipid-Lowering Effects of WAY 121,898, an inhibitor of pancreatic cholesteryl ester hydrolase. *Lipids* 1998; 33:489-98.
 12. Turley SD, Herndon MW, Dietschy JM. Reevaluation and application of the dual-isotope plasma ratio method for the measurement of intestinal cholesterol absorption in the hamster. *J Lipid Res* 1994; 35:328-39.
 13. Krause BR, Anderson M, Bisgaier CL, et al. In vivo Evidence That the Lipid-Regulating Activity of the ACAT Inhibitor CI-976 in Rats Is Due to Inhibition of Both Intestinal and Liver ACAT. *J Lipid Res* 1993; 34:279-94.
 14. Ngamukote S, Mäkynen K, Thilawech T, Adisakwattana S. Cholesterol-lowering activity of the major polyphenols in grape seed. *Molecules* 2011; 16:5054-61.
 15. Sbarra V, Ristorcelli E, Petit-Thévenin JL, Teissedre PL, Lombardo D, Vérine A. In vitro polyphenol effects on activity, expression and secretion of pancreatic bile salt-dependent lipase. *Biochim Biophys Acta* 2005; 1736:67-76.

LETTER TO THE EDITOR

**ANTI-INFLAMMATORY STEROID USE IN IMPACTED THIRD MOLAR SURGERY:
A SYSTEMATIC REVIEW**

G. VARVARA¹, S. BERNARDI², T. CUTILLI², S. BIANCHI², B. SINJARI¹
and M. PIATTELLI¹

¹*Department of Medical, Oral and Biotechnological Sciences, 'G. d'Annunzio' University of Chieti–Pescara, Chieti, Italy;* ²*Department of Life, Health and Environmental Sciences, University of L'Aquila, L'Aquila, Italy*

Received July 3, 2017 – Accepted September 12, 2017

Oral surgery procedures involve traumatization of mucosal and bony tissues, and lengthy interventions can lead to inflammatory post-operative sequelae. In the bony tissues in particular, the inflammatory processes can affect healing. Modern drug therapies provide valid support for lowering the risk of occurrence of post-operative inflammatory signs. The two main types of agents used are nonsteroidal anti-inflammatory drugs and/or corticosteroids, which act on two different molecular pathways in the inflammatory process. The aim of this systematic review is to examine the different corticosteroids used in oral surgery procedures, their indications for use, and their route of administration, to provide the clinician with a useful scheme for correct pharmacological management of post-operative inflammation. To identify studies eligible for inclusion in this systematic review, we performed a literature search up to April 2017 of the electronic databases, considering published papers from 2007 to 2017. The search terms included steroids, third molar, oral surgery, RCT [randomized controlled trial], human, and clinical trial. Only articles in English language were considered.

To the Editor,

Due to its innate characteristics, third molar surgery can lead to post-operative sequelae, such as edema (with local swelling), pain, and trismus. The jaws have unique features, which include being covered by a mucosal layer, being articulated with teeth, and being in contact with the oral environment. However, these features inevitably increase the risks and difficulties associated with surgical procedures that involve bone exposure. Even though any subsequent inflammatory events are indeed normal and have key roles in the healing processes (1-2),

when these are exacerbated, they can have negative effects on the daily life of the patient. Furthermore, these will sometimes represent signs of unexpected complications.

Drug administration for any oral surgery procedure contributes to the prevention and management of intra-operative accidents and post-operative complications. Pharmacological management is designed to control the microbial load and the inflammatory sequelae (3).

The walls and organs belonging to oral cavity are covered with a microbial biofilm (4), whereby

Key words: steroid therapy, edema, pain, trismus, oral surgery, third molar

Mailing address:

Dr Giuseppe Varvara,
Department of Medical,
Oral and Biotechnological Sciences, University of Chieti–Pescara,
Via Dei Vestini 31, 66100 Chieti, Italy
Tel.: +39 0871 3554125 - Fax: +39 0871 3554148
e-mail: gvarvara@unich.it

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
**DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF
INTEREST RELEVANT TO THIS ARTICLE.**

the associated bacteria might have virulence and thus represent high risk of infectious post-operative complications. This microbial load is generally controlled by professional oral hygiene sessions that are performed before surgery, the use of chlorhexidine 0.2% mouthwash, and the administration of broad-spectrum antibiotics.

As dental surgery procedures themselves represent trauma for the soft and hard tissues involved, the inflammation that follows, often as edema and pain, is inevitable. Among its main functions, the inflammation promotes the reparative processes for the traumatized tissues through the actions of inflammatory mediators. These include the prostaglandins, which are the products of arachidonic acid metabolism, and can activate two different pathways: the cyclooxygenase and lipooxygenase pathways (5-6).

Pharmacologically, inflammation can be controlled by the administration of nonsteroidal anti-inflammatory drugs (NSAIDs) and/or corticosteroids. NSAIDs can act on the cyclooxygenase pathway, while corticosteroids can inhibit the activity of phospholipase A₂, thereby preventing the formation of the end products of both the cyclooxygenase and lipooxygenase pathways (7).

The aim of this systematic review is to examine the different corticosteroids used in oral surgery procedures to define time and route of administration. This information will provide the clinician with a useful scheme for correct pharmacological management of inflammatory sequelae.

MATERIALS AND METHODS

Search strategy

To identify studies eligible for inclusion in this systematic review, we performed a literature search up to April 2017 of the electronic databases PubMed, EMBASE, Google Scholar, EBSCO HOST, Science Direct, Scopus, Web of Science, and the Cochrane Library. We considered published papers from 2007 to 2017. To achieve high sensitivity of the search results and to identify all potential studies for inclusion, the search strategy was individually adjusted for each electronic database. The search

terms included steroids, third molar, oral surgery, RCT [randomized controlled trial], human, and clinical trial. Only articles in English language were considered. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines were strictly followed throughout the search process.

Criteria for study selection

Randomized controlled trials on humans were included in the systematic review. Case reports, abstracts, oral communications, conference proceedings were excluded. When the studies reported the use of other anti-inflammatory agents, they were excluded.

Outcome variables

The primary outcome variables considered were class of steroids used, route and time of administration, post-operative edema, pain, and trismus, and comparisons with the controls.

Data extraction

The data from the studies included were independently extracted by two researchers. These data defined study design, sample size, and the primary outcome variables considered. In the event of any disagreement among the researchers, consultation with all of the authors of this report was carried out, with such problems resolved by consensus. In the event of discrepancies in the data, when possible, the authors of the reference paper were contacted by email, for further explanation.

RESULTS

Search

Fifty records emerged from the database search. Thirty-four did not fully match the investigated topic, and the remaining 16 were screened. The findings are summarized here and detailed in Table I.

Steroids tested

The most used steroid in the RCTs was dexamethasone (n = 8), followed by methylprednisolone (n = 3), prednisolone (n = 2) and betamethasone (n = 1). One of the RCTs compared the two steroids dexamethasone and

Table I. Summary of the results reported in the studies included in this systematic review.

Reference	Steroid tested	Administration		Reported post-operative edema, pain, trismus
		Route	Time	
Grossi et al. 2007 [5]	Dexamethasone	Submucosal	Pre-operation	Significantly less edema for dexamethasone vs control (day 2).
Lauretano Filho et al. 2008 [6]	Dexamethasone	Oral	Pre-operation	Significantly less edema, trismus for dexamethasone vs control (day 2).
Zandi 2008 [7]	Dexamethasone vs rubber drain	Intravenous	Pre-operation	Significantly less edema, pain, trismus for steroid vs control. Use of drain reduces facial pain, trismus; no effect on edema. Significantly greater reduction in post-operative edema, pain for dexamethasone vs surgical drain
Tiigimac-Saar et al. (2010) [8]	Prednisolone	Oral	Post-operation	Significantly less edema, pain, trismus for prednisolone vs control.
Kang et al. (2010) [9]	Prednisolone	Oral	Pre-operation	No significant differences for prednisolone vs control.
Kaur et al. (2010) [10]	Methylprednisolone	Intramuscular (masseter)	Post-operation	Significantly less edema, pain, trismus for methylprednisolone vs control.
Majid and Mahmood (2011) [11]	Dexamethasone	Intramuscular, submucosal	Post-operation	Significantly less edema, pain, trismus for dexamethasone vs control.
Majid (2011) [12]	Dexamethasone	Intramuscular, submucosal	Post-operation	Significantly less edema, pain, trismus for dexamethasone vs control.
Antunes et al. (2011) [13]	Dexamethasone	Intramuscular (masseter), oral	Pre-operation	Significantly less edema, pain (painkiller use), trismus for dexamethasone vs control.
Klongnoi et al. (2012) [14]	Dexamethasone	Intramuscular (deltoid)	Pre-operation	Significantly less edema, pain for dexamethasone vs control.
Acham et al. (2013) [15]	Methylprednisolone	Oral	Post-operation	Significantly less edema, pain, trismus for methylprednisolone vs control.
Majid and Mahmood (2013) [16]	Dexamethasone	Intramuscular (deltoid), oral, submucosal, endoalveolar, intravenous	Post-operation	Significantly less edema, pain, trismus for dexamethasone vs control. No difference for routes of administration.
Warraich et al. (2013) [17]	Dexamethasone	Submucosal	Pre-operation	Significantly less edema, pain, trismus for dexamethasone vs control (day 2).
Marques et al. (2014) [18]	Betamethasone	Submucosal	Post-operation	No significant differences for tests vs control.
Koçer et al. (2014) [19]	Methylprednisolone	Intramuscular	Post-operation	Significantly less edema, trismus in intramuscular/ post-operation administration
		(masseter), intravenous		for methylprednisolone vs control. No differences for routes/ time of administration.
		Oral	Pre-operation	
Alacantha et al. (2014) [20]	Dexamethasone vs methylprednisolone	Oral	Pre operation	Significantly less edema, trismus for dexamethasone vs methylprednisolone.

methylprednisolone. One of the RCTs instead compared dexamethasone therapy *versus* use of a rubber drain.

Route and time of administration

The routes of administration used were intramuscular (masseter, gluteus, deltoid), submucosal, oral, intravenous, and endo-alveolar. The most used route of administration was intramuscular (masseter), followed by submucosal, oral and intravenous. Three studies compared the outcomes of the different routes of administration. For the time of administration, in 10 studies the steroid therapy was administered before the intervention, and in five studies the steroid therapy was administered after the intervention. One study compared different times and groups of administration.

Post-operative edema, pain, and trismus, and comparisons with the controls

Most of the studies reported positive effects of steroid therapy for the reduction of edema (n = 12),

pain (n = 9), and trismus (n = 10). Dexamethasone was the most effective on these three parameters when administered post-operatively, and through the submucosal and intramuscular routes. Betamethasone administered post-operatively and injected submucosally through an intraoral approach did not have any effects here. Prednisolone was reported to be effective if administered orally and post-operatively. For methylprednisolone, one RCT that compared different times and routes of its administration reported that it was more effective on edema if administered intramuscularly and post-operatively. The study that compared dexamethasone and methylprednisolone showed that dexamethasone was more effective on edema and trismus when administered orally and pre-intervention.

For the study with dexamethasone therapy and a rubber drain, perioperative intravenous dexamethasone showed positive results for facial edema, pain, and trismus. The rubber drain reduced facial pain and trismus, but not edema. The comparison between these two therapies revealed

that the steroid therapy was more effective than the surgical drain for edema and pain. The effects for the reduction of trismus were similar.

DISCUSSION

It is evident that surgical interventions of any type will result in traumatization of the target tissues. Thus, there are likely to be signs of post-operative inflammation. Clinical inflammatory signs like facial edema, pain, and trismus can create discomfort for the patients, and thus influence their daily activities as well as the healing process of the wound.

Synthetic corticosteroids are a powerful class of anti-inflammatory drugs that are used in both medicine and dentistry. Their use in oral surgery to reduce or prevent the occurrence of these effects is widespread, well known, and well documented. However, which corticosteroid might be the best to use in oral surgery remains controversial. The findings from the present study show that three different corticosteroids can be identified as useful in the management of post-operative inflammation in oral surgery: dexamethasone, methylprednisolone, and prednisolone, with this last being effective with specific timing and route of administration.

Previous reviews have considered the issue of correct steroid choice in the pharmacological management of patients undergoing oral surgical procedures.

On the basis of these considerations, we can confirm that the use of corticosteroids in oral surgery that involves traumatization of bone tissue is promising for the reduction of post-operative morbidity. Specifically, it can be seen that they have positive effects for the reduction of edema and trismus. Their effects on pain reduction remain a topic for further investigation.

REFERENCES

1. Kritas SK1, Saggini A, Varvara G, et al. Mast cell involvement in rheumatoid arthritis. *J Biol Regul Homeost Agents* 2013; 27:655-60.
2. Conti P, Varvara G, Murmura G, et al. Comparison of beneficial actions of non-steroidal anti-inflammatory drugs to flavonoids. *J Biol Regul Homeost Agents* 2013; 27:1-7.
3. Zeka K, Ruparelia KC, Arroo R, et al. *In-vitro* biocompatibility of a new hydrogel with crocin, powerful antioxidant found in *Crocus sativus* L. flowers. *Ital J Anat Embryol* 2013; 118:200.
4. Bernardi S, Zeka K, Mummolo S, Marzo G, Continenza MA. Development of a new protocol: a macroscopic study of the tongue dorsal surface. *Ital J Anat Embryol* 2013; 118:24.
5. Grossi GB, Maiorana C, Garramone RA, Brogonovo A, Farronato D, Santoro F. Effect of submucosal injection of dexamethasone on postoperative discomfort after third molar surgery: a prospective study. *J Oral Maxillofac Surg* 2007; 65:2218-26.
6. Laureano Filho JR, Maurette PE, Allais M, Cotinho M, Fernandes C. Clinical comparative study of the effectiveness of two dosages of dexamethasone to control postoperative swelling, trismus and pain after the surgical extraction of mandibular impacted third molars. *Med Oral Patol Oral Cir Bucal* 2008; 13:129-32.
7. Zandi M. Comparison of corticosteroids and rubber drain for reduction of sequelae after third molar surgery. *Oral Maxillofac Surg* 2008; 12:29-33.
8. Tiigimae-Saar J, Leibur E, Tamme T. The effect of prednisolone on reduction of complaints after impacted third molar removal. *Stomatologija* 2010; 12:17-22.
9. Kang SH, Choi YS, Byun IY, Kim MK. Effect of preoperative prednisolone on clinical postoperative symptoms after surgical extractions of mandibular third molars. *Aust Dent J* 2010; 55:462-67.
10. Kaur J, Sandhu S, Kaur T, Bhullar RS, Sandhu Y, Singh P. Effect of methylprednisolone on postoperative pain, swelling, and trismus following the surgical removal of bilateral impacted mandibular third molars. *Indian J Compr Dent Care* 2011; 1:36-43.
11. Majid OW, Mahmood WK. Effect of submucosal and intramuscular dexamethasone on postoperative sequelae after third molar surgery: comparative study. *Br J Oral Maxillofac Surg* 2011; 49:647-52.
12. Majid OW. Submucosal dexamethasone injection improves quality of life measures after third molar surgery: a comparative study. *J Oral Maxillofac Surg* 2011; 69:2289-97.

13. Antunes AA, Avelar RL, Neto ECM, Frota R, Dias E. Effect of two routes of administration of dexamethasone on pain, edema, and trismus in impacted lower third molar surgery. *Oral Maxillofac Surg* 2011; 15:217-23.
14. Klongnoi B, Kaewpradub P, Boonsiriseth K, Wongsirichat N. Effect of single dose preoperative intramuscular dexamethasone injection on lower impacted third molar surgery. *Int J Oral Maxillofac Surg* 2012; 41:376-79.
15. Acham S, Klampfl A, Truschnegg A, Kirmeier R, Sandner-Kiesling A, Jakse N. Beneficial effect of methylprednisolone after mandibular third molar surgery: a randomized, double-blind, placebo-controlled split-mouth trial. *Clin Oral Investig* 2013; 17:1693-700.
16. Majid OW, Mahmood WK. Use of dexamethasone to minimise post-operative sequelae after third molar surgery: comparison of five different routes of administration. *Oral Surg* 2013; 6:200-8.
17. Warraich R, Faisal M, Rana M, Shaheen A, Gellrich NC, Rana M. Evaluation of postoperative discomfort following third molar surgery using submucosal dexamethasone - a randomized observer blind prospective study. *Oral Surg Oral Med Oral Pathol Oral Radiol* 2013; 116:16-22.
18. Marques J, Pié-Sánchez J, Figueiredo R, Valmaseda-Castellón E, Gay-Escoda C. Effect of the local administration of betamethasone on pain, swelling and trismus after impacted lower third molar extraction. A randomized, triple blinded, controlled trial. *Med Oral Patol Oral Cir Bucal* 2014; 19: e49-54.
19. Koçer G, Yuce E, Tuzuner Oncul A, Dereci O, Koskan O. Effect of the route of administration of methylprednisolone on oedema and trismus in impacted lower third molar surgery. *Int J Oral Maxillofac Surg* 2014; 43:639-43.
20. Alcântara CEP, Falci SGM, Oliveira-Ferreira F, Santos CRR, Pinheiro MLP. Pre-emptive effect of dexamethasone and methylprednisolone on pain, swelling, and trismus after third molar surgery: a split-mouth randomized triple-blind clinical trial. *Int J Oral Maxillofac Surg* 2014; 43:93-98.

LETTER TO THE EDITOR

COLORECTAL CANCER IN PATIENTS WITH TYPE 2 DIABETES MELLITUS: A SINGLE-CENTER EXPERIENCE

E. SINAGRA^{1,2}, V. GUARNOTTA³, D. RAIMONDO¹, F. MOCCIARO⁴, S. DOLCIMASCOLO³,
C.A. RIZZOLO³, F. PUCCIA³, N. MALTESE³, R. CITARRELLA³, M. MESSINA⁵, M. SPADA⁵,
G. TOMASELLO^{2,6}, F. CAPPELLO^{2,6}, A. LEONE⁷, F. ROSSI¹, R. DI MITRI⁴,
G.C. MORREALE⁸, S. MARASÀ⁹, M. MIDIRI¹⁰, F. MIDIRI¹⁰ and D. SINAGRA³

¹Gastroenterology and Endoscopy Unit, Fondazione Istituto G. Giglio, Cefalù, Italy; ²Euro-Mediterranean Institute of Science and Technology (IEMEST), Palermo, Italy; ³Section of Cardio-Respiratory and Endocrine-Metabolic Diseases, Biomedical Department of Internal and Specialist Medicine (DIBIMIS), University of Palermo, Palermo, Italy; ⁴Gastroenterology and Endoscopy Unit, ARNAS Civico-Di Cristina-Benfratelli Hospital, Palermo, Italy; ⁵Oncology Unit, Fondazione Istituto G. Giglio, Cefalù, Italy; ⁶Department of Experimental Biomedicine and Clinical Neuroscience, Section of Human Anatomy, University of Palermo, Palermo, Italy; ⁷Department of Experimental Biomedicine and Clinical Neuroscience, Section of Histology, University of Palermo, Palermo, Italy; ⁸Unit of Gastroenterology, Ospedali Riuniti Villa Sofia-Vincenzo Cervello, Palermo, Italy; ⁹Centro Diagnostico Marasà, Palermo, Italy; ¹⁰DIBIMED, Section of Radiology, University of Palermo, Palermo, Italy

Received April 26, 2017 – Accepted September 29, 2017

Abstract Type 2 diabetes mellitus (T2DM) is associated with an increased risk of colorectal cancer (CRC). The aim of the study is to evaluate the prevalence of CRC in a cohort of Caucasian patients with T2DM and the association with other variables previously known to be related with increased risk of CRC. We retrospectively evaluated the data of 741 consecutive Caucasian patients with T2DM who underwent colonoscopic screening in our tertiary referral center. A control cohort of 333 patients with thyroid disease was selected to evaluate the difference in the incidence of CRC. At a median follow-up of 132.5 months (range 33.3-175.7), 67 cases of cancer (prevalence 9%) occurred; among these, 14 cases of CRC were reported (prevalence 1.88%) among the diabetic patients, while only two case (one of these was a CRC) (overall prevalence 0.006%, prevalence of CRC 0.003%) occurred in the control group; the difference between the prevalence of CRC was statistically significant (chi-square 4.21, $p=0.04$). The median duration of T2DM to CRC diagnosis was 168 months (range 12-768). At the univariate analysis, older age ($p=0.001$, r 0.138) and diabetes duration ($p=0.001$, r 0.138) were related to higher risk of cancer, while metformin seems to be protective towards cancer ($p=0.07$, r -0.098). In the subset of patients with CRC, the age (RR = 2.25; 95% CI: 0.30 - 17.31; $p < 0.001$), the diabetes duration (RR = 1.93; 95% CI: 0.25 - 14.77; $p = 0.001$) and the sulphonylureas treatment (RR = 2.33; 95% CI: 0.78 - 7.38; $p = 0.007$) were independently correlated with CRC. In our study, the prevalence of CRC in the cohort of patients with T2DM was higher compared to that from the National Tumor Register in 2010 (0.5%). Furthermore, we could speculate that sulphonylureas may play a role in CRC carcinogenesis impairing the physiological insulin secretion.

Key words: type 2 diabetes, colorectal cancer, anti-diabetic drugs

Mailing address:

Dr. Emanuele Sinagra,
Gastroenterology and Endoscopy Unit,
Fondazione Istituto Giuseppe Giglio,
Contrada Pietra Pollastra Pisciotto, 90015 Cefalù, Italy
Tel.: +39 921920712 - Fax: +39 921920406
e-mail: emanuelesinagra83@googlemail.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
**DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF
INTEREST RELEVANT TO THIS ARTICLE.**

To the Editor,

Colorectal cancer (CRC) is the most common malignancy of the gastrointestinal tract (1, 2). The incidence of CRC increased during the last two decades, even though there were many strategies for prevention.

On the other hand, the prevalence of diabetes mellitus (DM) has increased significantly over the past several decades, mostly due to the growing epidemic of obesity. Approximately 8% of the US population has DM, classified as type 2 DM (T2DM) in more than 90% of cases, with insulin resistance as the main underlying pathophysiological cause (3).

CRC and DM, share several risk factors including high caloric diet, obesity, hyperinsulinemia, cigarette smoking and physical inactivity. Hyperinsulinemia may promote colorectal carcinogenesis through a cross-talk with the insulin-like growth factor-1 (IGF-1) receptor, which stimulates proliferation and prolongs cell survival (4). A stimulatory effect on cell growth of intestinal epithelial and colon cancer cells was shown in preclinical studies (5, 6). In addition, hyperinsulinemia was also associated with increased tumor growth in *in vivo* experiments (7).

Many studies have reported a positive association between T2DM and CRC (8, 9), even though others have not reported any association (10, 11).

The aim of the current study is to evaluate the prevalence of CRC in a cohort of Caucasian patients with T2DM and the potential factors associated with the risk of CRC in patients with T2DM.

MATERIALS AND METHODS

We retrospectively studied the data of 741 patients (381 M, 360 F; mean age 67 ± 9.6 years) with T2DM, without any gastrointestinal symptoms, who underwent colonoscopic screening for colorectal cancer, consecutively admitted to the Section of Endocrinology of the University of Palermo from January 2000 to December 2008. Three hundred and thirty-three subjects matched for gender (145 M, 188 F) and age (64 ± 9 years) were enrolled among patients with thyroid disease as controls. Diabetic patients were on treatment with insulin in 310 cases, metformin in 485, sulphonylureas in 68, glinides in 263, while 115 patients were on other hypoglycemic drugs.

The exclusion criteria included a history of colonic disease, such as colitis, polyps, or cancer, prior colonic surgery or colon polypectomy, or medical history of severe hematologic or connective disorders.

The reason why colonoscopy instead of fecal occult blood test and sigmoidoscopy was chosen is that flexible sigmoidoscopy can only identify lesions in the distal 60 cm of the bowel and abnormal findings in the distal bowel require colonoscopy for visualization of the entire colon. On the other hand, colonoscopic examination showed the highest effectiveness for screening colon cancer (12), because the procedure can show the entire colon and lesions can be removed at the same time.

The diagnosis of T2DM was made according to the American Diabetes Association guidelines (13).

Data were collected from baseline until the end of the observation period (2008). Body mass index (BMI), alcohol and non steroidal anti-inflammatory drug (NSAID) consumption, smoking, family history of CRC, HbA1c levels, patient comorbidities (hypertension, hypertriglyceridemia, obesity), age at diabetes onset and duration of diabetes, treatment with insulin or other hypoglycemic drugs at the diagnosis, were from patient charts.

Statistical analysis

Data were collected in Microsoft Excel and imported into the PASW Statistics 18.0 for Windows (SPSS Inc., IL, USA) for statistical analysis. The normality of quantitative variables was tested with the Shapiro-Wilk test. Baseline characteristics were presented as mean $\pm$ SD for continuous variables; rates and proportions were calculated for categorical data. Differences between the two groups were detected using the unpaired Student's t-test for continuous variables (after testing for equality of variance: Levene test) and the χ^2 -test and Fisher's exact test (when appropriate) for categorical variables. Simple univariate correlations among continuous variables with non-normal distribution were determined by Pearson's test. Variables associated with the dependent variable on univariate analysis (probability threshold, $p \leq 0.10$) were included in two multivariate regression models. In both models, the following independent variables were included: age and diabetes duration as continuous variables. In the first model, smoking, metformin and sulphonylureas

treatment were included as categorical variables. In the second model, gender and metformin treatment were included as categorical variables. A two-sided p value < 0.05 was considered statistically significant.

RESULTS

Clinical and demographic data of patients are summarized in Table I. Diabetic patients represented 68.9% of the study population [mean age at DM onset 51.2 ± 11.1 years; median duration of disease 168 months (range 12-768)]. The mean BMI was 29.9 ± 5.4 kg/m² and mean blood glycated hemoglobin level was 7.6 ± 1.4 %. Hypertension was found in 81.2% of patients and hypertriglyceridemia in 40.8%.

The control group represented 31.1% of the study population. The mean age at thyroid disease onset

was 50 ± 11 years, and the median duration of thyroid disease was 98 months (range 12-180).

At a median follow-up of 132.5 months (range 33.3-175.7), 67 cases of cancer (prevalence 9%) occurred in the entire diabetic population, while in the control group only 2 patients had cancer (overall cancer prevalence 0.006%). Fourteen cases of CRC were reported (prevalence 1.88%) among diabetic patients (Table II), while only one was reported in the control group (prevalence 0.003%). The difference between the groups was statistically significant (*chi-squared* 4.21, *p*=0.04) (data not shown). The median duration of DM to CRC diagnosis was 156 months (range 1-768).

In patients with diabetes, at the univariate analysis, age (*p*=0.001, *r* 0.138) and diabetes duration (*p*=0.001, *r* 0.138) were found to be correlated

Table I. Baseline features of diabetic patients and control group.

Variables	Diabetic patients (N=741)	Control group (N=333)
	Mean $\pm$ SD	Mean $\pm$ SD
Age (years)	67 $\pm$ 9.6	64 $\pm$ 9
Age at diabetes onset (years)	51.2 $\pm$ 11.1	NA
Body mass index (Kg/m ²)	29.9 $\pm$ 5.4	27 $\pm$ 4.7
Glycated hemoglobin (%)	7.6 $\pm$ 1.4	NA
	Subjects (%)	Subjects (%)
Gender: Male	381 (51.4%)	145 (43.5%)
Female	360 (48.6%)	188 (56.5%)
Smokers	189 (25.5%)	35 (10.5%)
Alcohol consumption	4 (0.005%)	2 (0.006%)
Non-steroidal antiinflammatory drug consumption	306 (41.2%)	5 (0.01%)
Family history of colorectal cancer	13 (0.02%)	5 (0.01%)
Diabetes treatment		
Sulphonylureas	68 (0.09%)	0 (0)
Glinides	263 (35.4%)	0 (0)
Metformin	485 (65.4%)	0 (0)
Insulin	310 (41.8%)	0 (0)
Others	115 (15.5%)	0 (0)
Comorbidities		
Hypertension	602 (81.2%)	103 (30.9%)
Hypertriglyceridemia	302 (40.7%)	81 (24.3%)

Table II. *Malignant cancer cases and their distribution by type in diabetic patients and controls.*

Type of cancer	Diabetic patients (N= 741)	Controls (N=333)
	Subjects (%)	Subjects (%)
Total	67 (9%)	2 (0.6%)
Colorectal cancer	14 (1.8%)	1 (0.3%)
Breast cancer	15 (4.1%)	1 (0.3%)
Lymphomas	6 (0.8%)	0
Bladder cancer	6 (0.8%)	0
Cancer of uterus	5 (1.38%)	0
Prostatic cancer	5 (1.38%)	0
Papillary thyroid cancer	4 (0.54%)	0
Non-melanoma skin cancer	3 (0.4%)	0
Pancreatic cancer	2 (0.27%)	0
Hepatocellular carcinoma	2 (0.27%)	0
Penile cancer	1 (0.13%)	0
Lung cancer	1 (0.13%)	0
Ovarian cancer	1 (0.13%)	0
Larynx cancer	1 (0.13%)	0
Renal cancer	1 (0.13)	0

with CRC. As expected, metformin appeared to be protective towards cancer ($p=0.07$, $r = -0.98$) (Table III). Data were further analyzed using two binary logistic regression models (Table III). Among all independent variables examined, the age ($RR = 2.25$; 95% CI: 0.30 - 17.31; $p < 0.001$), the diabetes duration ($RR = 1.93$; 95% CI: 0.25 - 14.77; $p = 0.001$) and the sulphonylureas treatment ($RR = 2.33$; 95% CI: 0.78 - 7.38; $p = 0.007$) were independently correlated with CRC (Table III). An inverse independent association was found for metformin treatment ($RR = 0.32$; 95% CI: 0.13 - 0.76; $p = 0.005$) (Table III).

With regard to other tumors (not including CRC), age ($RR = 1.04$; 95% CI: 1.02 - 1.05; $p = 0.007$) and diabetes duration ($RR = 1.07$; 95% CI: 0.24 - 4.69; $p = 0.005$) showed an independent association, while metformin treatment showed an inverse independent association ($RR = 0.44$; 95% CI: 0.19 - 0.98; $p = 0.009$) (Table IV).

DISCUSSION

Epidemiological studies on the association between T2DM and risk of CRC are scarce and inconclusive. The results of the current study demonstrated that overall cancer prevalence, and in detail the CRC prevalence, was higher in diabetic patients than in the control group and this prevalence was also higher than that reported in the Italian tumor registry (15), supporting the results of several epidemiological studies and meta-analyses (8, 16, 17).

In the univariate analysis, we found a correlation between CRC and age or diabetes duration, suggesting that age and diabetes duration are independent factors influencing CRC. Metformin was found to be a protective factor for overall malignant neoplasms, even though this correlation was not found for CRC. The finding of the current study that treatment with sulphonylureas increased the CRC risk ($p=0.001$),

Table III. *Univariate and multivariate analysis (logistic regression model) of risk factors associated with colon cancer in patients with and without diabetes.*

Variable	Patients with diabetes and colon cancer (N=14)	Patients with diabetes without colon cancer (N=727)	Univariate analysis	Multivariate Analysis	
	Mean $\pm$ SD	Mean $\pm$ SD	<i>p</i>	RR (95% CI)	<i>p</i>
Age (years)	74.81 $\pm$ 9.21	66.81 $\pm$ 9.52	<0.001	2.25 (0.303-17.317)	<0.001
Body Mass Index (kg/m ²)	30.27 $\pm$ 3.69	29.9 $\pm$ 5.45	0.501		
Diabetes duration (months)	230.21 $\pm$ 168.6	168.05 $\pm$ 115.5	0.013	1.93 (0.258-14.778)	0.001
Glycated haemoglobin (%)	7.55 $\pm$ 1.79	7.56 $\pm$ 1.42	0.680		
	Subjects (%)	Subjects (%)			
Gender					
Male	8 (57.1%)	369 (50.7%)	0.665		
Female	6 (42.8%)	358 (49.3%)			
Smoking	9 (64.2%)	261 (35.9%)	0.087	1.24 (0.548-3.172)	0.325
Alcohol consumption	0	5 (0.68%)	0.780		
Non-steroidal anti-inflammatory drugs consumption	6 (42.8%)	300 (41.2%)	0.626		
Family history of cancer	1 (7.14%)	11 (16.1%)	0.480		
Diabetes treatment					
Insulin treatment	7 (50%)	299 (41.1%)	0.980		
Metformin treatment	5 (35.7%)	477 (65.6%)	0.007	0.32 (0.128-0.767)	0.005
Sulphonylureas treatment	4 (28.5%)	64 (8.8%)	0.001	2.32 (0.788-7.384)	0.007
Glinides treatment	4 (28.5%)	257 (35.3%)	0.980		
Comorbidities					
Hypertension	10 (71.4%)	587 (80.7%)	0.330		
Hypertriglyceridemia	6 (42.8%)	295 (40.5%)	0.781		
Hypo-HDL	7 (50%)	288 (39.6%)	0.121		

but not the insulin treatment, may exclude an adverse property of the insulin formulation itself. We believe that the strength of the study is the evaluation of many parameters as crucial factors associated with CRC and the finding of increased prevalence of CRC in diabetic patients compared to the Italian reported prevalence. However, some study limitations should be considered. The study has a retrospective design, and the control group is quite small. With regards

to the control group, we included patients with thyroid disease, even though the role of clinical hypothyroidism or hyperthyroidism in cancer growth is under investigation (18).

Therefore, the effect of anti-diabetic drugs in the carcinogenesis and the factors involved in the CRC need to be further investigated. Prospective case-control studies are required to evaluate better the long-term safety of anti-diabetic drugs.

Table IV. Univariate and multivariate analysis (logistic regression model) of risk factors associated with other malignant tumors (excluding colon cancer) in patients with and without diabetes.

Variable	Patients with diabetes and other tumors (N= 53)	Patients with diabetes without other tumors (N= 688)	Univariate analysis	Multivariate Analysis	
	Mean $\pm$ SD	Mean $\pm$ SD	<i>p</i>	RR (95% CI)	<i>p</i>
Age (years)	71.02 $\pm$ 7.39	66.72 $\pm$ 9.69	0.001	1.04 (1.023-1.056)	0.007
Body mass index (kg/m ²)	29.28 $\pm$ 4.81	29.97 $\pm$ 5.46	0.510		
Diabetes duration (months)	203.32 $\pm$ 128.4	168.29 $\pm$ 116.7	0.030	1.07 (0.247-4.690)	0.005
Glycated haemoglobin (%)	7.50 $\pm$ 1.29	7.55 $\pm$ 1.44	0.830		
	Subjects (%)	Subjects (%)			
Gender					
Male	22 (41.5%)	357 (51.9%)	0.043	0.91 (0.484-2.464)	0.440
Female	31 (58.5%)	331 (48.1%)			
Smoking	18 (34%)	250 (36.3%)	0.722		
Alcohol consumption	0	5 (0.72%)	0.560		
Non-steroidal anti-inflammatory drug consumption	18 (34%)	288 (41.8%)	0.081		
Family history of cancer	1 (1.9%)	11 (1.6%)	0.570		
Diabetes treatment					
Insulin treatment	25 (47.2%)	282 (40.9%)	0.129		
Metformin treatment	30 (56.6%)	452 (65.6%)	0.050	0.44 (0.192-0.985)	0.009
Sulphonylureas treatment	5 (9.4%)	63 (9.1%)	0.370		
Glinides treatment	17 (32.1%)	245 (35.6%)	0.881		
Comorbidities					
Hypertension	45 (84.9%)	555 (80.6%)	0.607		
Hypertriglyceridemia	22 (41.5%)	280 (41.9%)	0.880		
Hypo-HDL	20 (37.7%)	278 (41.6%)	0.680		

Nevertheless, despite our study showing the above-mentioned methodological limits, we should take into consideration that, given that high prevalence of diabetes, even a small increase of CRC risk may have important public health implications and a CRC screening of patients with diabetes may be required.

REFERENCES

1. Omran S, Ismail AA. Knowledge and beliefs of Jordanians toward colorectal cancer screening. *Cancer Nurs* 2010; 33:141-48.
2. Sinagra E, Raimondo D, Gallo E, et al. Could JC virus provoke metastasis in colon cancer? *World J Gastroenterol* 2014; 20:15745-49.
3. Centers for Disease Control and Prevention: National diabetes fact sheet: national estimates and general information on diabetes and prediabetes in the United States, 2011. Atlanta, US Department of Health and Human Services, Centers for Disease Control and Prevention, 2011.
4. Pollak M. The insulin and insulin-like growth factor receptor family in neoplasia: an update. *Nat Rev Cancer* 2012; 12:159-69.
5. Koenuma M, Yamori T, Tsuruo T. Insulin and insulin-like growth factor 1 stimulate proliferation of metastatic variants of colon carcinoma 26. *Jpn J Cancer Res* 1989; 80:51-58.
6. Björk J, Nilsson J, Hultcrantz R, Johansson C. Growth-regulatory effects of sensory neuropeptides, epidermal growth factor, insulin, and somatostatin on the non-transformed intestinal epithelial cell line IEC-6 and the colon cancer cell line HT 29. *Scand J Gastroenterol* 1993;28:879-84.
7. Tran TT, Medline A, Bruce WR. Insulin promotion of colon tumors in rats. *Cancer Epidemiol Biomarkers Prev* 1996; 5:1013-15.
8. Larsson SC, Giovannucci E, Wolk A. Diabetes and colorectal cancer incidence in the cohort of Swedish men. *Diabetes Care* 2005; 28:1805-7.
9. Deng L, Gui Z, Zhao L, Wang J, Shen L. Diabetes mellitus and the incidence of colorectal cancer: an updated systematic review and meta-analysis. *Dig Dis Sci* 2012; 57:1576-85.
10. Limburg PJ, Vierkant RA, Fredriksen ZS, et al. Clinically confirmed type 2 diabetes mellitus and colorectal cancer risk: a population-based, retrospective cohort study. *Am J Gastroenterol* 2006; 101:1872-79.
11. Inoue M, Iwasaki M, Otani T, Sasazuki S, Noda M, Tsugane S. Diabetes mellitus and the risk of cancer: results from a large-scale population-based cohort study in Japan. *Arch Intern Med* 2006; 166:1871-77.
12. Steenland K, Nowlin S, Palu S. Cancer incidence in the National Health and Nutritional Study I Follow-up data: diabetes, cholesterol, pulse, and physical activity. *Cancer Epidemiol Biomarkers Prev* 1995; 4:807-11.
13. Levin B, Lieberman DA, McFarland B, et al. Screening and surveillance for the early detection of colorectal cancer and adenomatous polyps, 2008: a joint guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. *CA Cancer J Clin* 2008; 58:130-60.
14. American Diabetes Association. Standards of medical care in diabetes – 2017. *Diabetes Care* 2017; 40 (S 1):1-142.
15. AIRTUM Working Group. I tumori in Italia, rapporto 2010. La prevalenza dei tumori in Italia. *Epidemiol Prev* 2010; 34:1-148.
16. L, Gui Z, Zhao L, Wang J, Shen L. Diabetes mellitus and the incidence of colorectal cancer: an updated systematic review and meta-analysis. *Dig Dis Sci* 2012; 57:1576-85.
17. Sun L, Su Y. Diabetes mellitus is an independent risk factor for colorectal cancer. *Dig Dis Sci* 2012; 57:1586-97.
18. Lin HY, Chin YT, Yang YC, et al. Thyroid Hormone, Cancer, and Apoptosis. *Compr Physiol* 2016; 6:1221-37.

LETTER TO THE EDITOR

CYTOKINE EXPRESSION PROFILE AND BLOOD PARAMETER EVALUATION OF PATIENTS UNDERGOING CARDIAC SURGERY

V. DE IULIIS^{1,2}, V. DADORANTE¹, A. MARINO^{1,2}, I. GRIFFO^{1,2}, A. PENNELLI²,
V. BREDA¹, I. ROBUFFO³, S. URSI², S. MARTINOTTI^{1,2}, S. CAPUTI¹
and E. TONIATO¹

¹Department of Medical, Oral and Biotechnological Sciences, University G. d'Annunzio of Chieti, Italy; ²SS Annunziata University Hospital, Unit of Clinical Molecular Biology and Predictive Medicine, University of Chieti, ASL Lanciano-Vasto-Chieti, Chieti, Italy; ³CNR, Institute of Molecular Genetics, Section of Chieti, Chieti, Italy

Received April 6, 2017 – Accepted October 2, 2017

Cardiac surgery is accompanied by an important immune response that is poorly understood. This inflammatory response is caused by several stimuli: surgical trauma, cardiopulmonary bypass apparatus, aortic-cross clamping, reperfusion injury and hypothermia. The aim of the present study is to investigate the cytokine level profile involved in the inflammatory pathway of patients undergoing cardiac surgery. One hundred and two patients undergoing elective cardiac surgery utilizing cardiopulmonary bypass (CPB) apparatus were enrolled in the study. In the hematological and biochemical profiles investigated, we observed a significant increase of WBC and blood glucose concentration and a strong decrease of RBC, HB, HCT and PLT 24 h post-surgery compared to baseline and immediately after surgery groups. Furthermore, we found a modulation of cytokine levels mostly for IL-10 and an increase of IL-6, detected at 6 h post-surgery, IL-8 at 6 and 24 h, and TNF- α only at 24 h post-surgery. In conclusion, these findings evidence a time course profile on cytokine levels and a balance between pro- and anti-inflammatory cytokine activation during and after cardiac surgery. In fact, IL-6 and IL-10, a pro- and an anti-inflammatory cytokine, respectively, increased immediately after surgery. The plasma level of TNF- α could be inhibited by the high concentration of IL-10 up to 6 h post-surgery. An IL-10 reduction at baseline level, after 24 h post-surgery, could explain a rise of TNF- α plasma concentration. On the other hand, considering the dual role of IL-6 on inflammation acting both as an activator of inflammatory cascade or an anti-inflammatory agent, the increased IL-6 levels 24 h after surgery could be related to the negative feedback action on TNF α activity.

Letter to the Editor

The immune response in patients undergoing cardiac surgery is caused by the following stimuli: surgical trauma, blood exposure to artificial surface of the cardiopulmonary bypass (CPB), reduction in pulmonary blood flow during aortic-cross clamping,

damage of intestinal mucosa, reperfusion injury due to operative procedures and hypothermia (1, 2). This inflammatory activation is modulated by an increasing release of cytokines, such as TNF- α , IL-1, IL-6, IL-8, and expression of adhesion molecules, as well as L-selectin, CD11b and CD18 as markers of

Key words: cytokine, cardiac surgery, cardiopulmonary bypass

Mailing address:

Prof. Elena Toniato,
Department of Medical, Oral and Biotechnological Sciences,
University of Chieti,
Via dei Vestini 31, Chieti, Italy
Tel.: +39 0871 3554161 - Fax: +39 0871 357340
e-mail: e.toniato@unich.it

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

neutrophil potential adhesion (3, 4). Furthermore, a correlation between these cytokines, involved in the inflammatory modulation patterns, and post-operative fever was demonstrated in patients undergoing coronary artery bypass graft surgery (CABG) using CPB (5). Several studies demonstrated that TNF- α , IL-1 β and IL-6 play a pivotal role in febrile response in animal models (6) and in patients after cardiac surgery (7). In addition, various data suggest that myocardium play an important role as a source of cytokines during CPB surgery and reperfusion (8). Furthermore, the pro-inflammatory response and the cytokine production and release were more prominent in surgical procedures performed with CPB compared to those without CPB, known as off-pump surgery (9). The myocardium produces and releases important concentrations of cytokines only in the case of surgery with CPB (10).

The aim of this study was to evaluate the blood parameters, specifically the hematological, coagulation and biochemical profiles, and the cytokine secretion in patients undergoing cardiac surgery performed with CPB, since the artificial surface of the cardiopulmonary bypass apparatus may be responsible for the immunological changes (11).

MATERIALS AND METHODS

Study subjects

One hundred and two patients undergoing elective cardiac surgery, utilizing cardiopulmonary bypass (CPB) apparatus, were enrolled in the study. Exclusion criteria were: (a) presence of immunologic disease, (b) recent myocardial infarction (< 3 months), (c) previous stroke, (d) acute or repeat surgery, (e) recent infections, (f) coagulopathy, (g) tumor, (h) acute or chronic renal failure, and (i) respiratory impairment. The Medical Research Ethics Committee of the "SS. Annunziata University Hospital" of Chieti, Italy, approved the study and patients gave their written informed consent. Investigations were performed in accordance with the Declaration of Helsinki.

Blood sampling

Blood samples were collected from the peripheral vein at baseline (1 Day before the operation), immediately after surgery (t0), and 6 and 24 h post-surgery. Blood

samples were collected in sterile vacuum tubes containing EDTA, clot activator or sodium citrate, for the analysis of hematological parameters [RBC ($10^{12}/L$), WBC ($10^9/L$), PLT ($\times 10^9/L$)] and cytokine concentrations (TNF- α , IL-10, IL-6, IL-8, IL-1 β , IL-12), the biochemical profile with the serum determination of blood glucose (mg/dL), BUN (mg/dL), creatinine (mg/dL), Na⁺ (mEq/L), K⁺ (mEq/L), and the coagulation parameters AT III and Fibrinogen (mg/dL), respectively.

Cytokine determination

Plasma concentration of several cytokines released during and after cardiac surgery was determined using flow cytometry (Becton Dickinson, NJ, USA) as previously described (12). A series of FITC-mouse anti-human antibodies were used (Santa Cruz Biotechnology, TX, USA). A panel of 6 different cytokine antibodies (TNF- α , IL-10, IL-6, IL-8, IL-1 β , IL-12) was used and different levels of induction were observed between them.

Statistical analysis

Data are expressed as means $\pm$ SEM. The significance of differences regarding cytokine plasma concentrations and hematological, coagulation and biochemical parameters was analyzed by an ANOVA test followed by the Bonferroni post-hoc test (Stat View 4.0 software, Abacus Concepts, Berkeley, CA, USA). P values < 0.05 were considered statistically significant, with a confidence interval of 95%.

RESULTS

Characteristics of the study participants

The demographic and clinical characteristics of the 102 study participants are shown in Table I.

Modulation of blood parameters after cardiac surgery

The hematological and biochemical profiles of all patients were analyzed at baseline and immediately after surgery and 24 h later. A significant increase of WBC and blood glucose was evident 24 h post-surgery compared to baseline and immediately after surgery groups (Fig. 1, $p < 0.001$). Furthermore, a significant decrease between 24 h post-surgery and the baseline and immediately after surgery groups

Table I. *Characteristics of the study participants.*

Characteristics	Cases (n = 102)
Age, mean (SD), yrs	67.41 (8.44)
Male/female	69 (67.6) / 33 (32.4)
Hypercholesterolemia	61 (59.8)
Hypertension	81 (79.4)
Diabetes	34 (33.3)
Body mass index*, mean (SD)	27.8 (4.9)
Stable angina	25 (24.5)
Unstable angina	24 (23.5)
Myocardial infarction°	36 (35.3)
Chronic kidney disease	8 (7.8)
Cardiac arrhythmia	27 (26.5)
NYHA class	
I	79 (69.6)
II	15 (14.7)
III	16 (15.7)

*Data are expressed as n° (%) unless otherwise indicated. Abbreviations: SD, Standard deviation; ECC, extracorporeal circulation. * Body mass index was calculated as weight in kilograms divided by the square of height in meters. ° More than 3 months ago.*

was highlighted for the following parameters: RBC, HB, HCT and PLT (Fig. 1, $p < 0.001$). Conversely, a significant modulation was not shown of the coagulation parameters (AT III and Fibrinogen).

Modulation of cytokine levels after cardiac surgery

A modulation was noted in the expression of several cytokines analyzed at baseline and immediately after surgery, as well as 6 h and 24 h later. Specifically, the following were observed: a strong increase of IL-10 plasma concentration immediately after surgery and a slight boost 6 h post-surgery compared to baseline ($p < 0.001$; Fig. 2A); a rise of IL-6 plasma concentration at the end of the surgery and 6 h later and a slight increase 24 h post-surgery compared to baseline ($p < 0.001$; Fig. 2A); a strong increase of

IL-8 plasma concentration respectively at 6 and 24 h post-surgery compared to baseline ($p < 0.001$; Fig. 2A); a sharp rise of TNF- α plasma concentration at 24 h post-surgery compared to baseline ($p < 0.001$; Fig. 2A). There was no significant difference with regard to IL-1 β and IL-12 plasma concentrations before or after cardiac surgery (IL-1 β , $p = 0.181$; IL-12, $p = 0.566$; Fig. 2A).

DISCUSSION

The inflammatory response seems to be a key role during and after cardiac surgery. In fact, several studies demonstrated a prominent pro-inflammatory response and a myocardium production of TNF- α , IL-1, IL-6 and IL-8 (8-11), counterbalanced by an increased expression of anti-inflammatory cytokines, such as IL-10 (1). In this study, one hundred and two patients undergoing elective cardiac surgery, utilizing CPB apparatus, were enrolled. First of all, a significant increase was observed of WBC and blood glucose concentration and a meaningful decrease of RBC, HB, HCT and PLT at 24 h post-surgery compared to baseline and immediately after surgery, as previously described (13). In addition, the following cytokine modulation was observed: (i) a significant increase of IL-10 plasma concentration immediately after surgery and a slight increase at 6 h post-surgery compared to baseline; (ii) a strong rise in IL-6 plasma concentration at the end of surgery and 6 h later and a slight boost at 24 h post-surgery compared to baseline; (iii) a strong increase of IL-8 plasma concentration 6 and 24 h post-surgery compared to baseline; (iv) a sharp rise in TNF- α plasma concentration 24 h post-surgery compared to baseline. These findings showed a time course of cytokine levels. In fact, IL-6 and IL-10, a pro- and an anti-inflammatory cytokine, respectively, increased immediately after surgery, and high levels of these two cytokines were also maintained 6 h post-surgery. The plasma level of TNF- α could be inhibited by the high concentration of IL-10 up to 6 h post-surgery. After 24 h post-surgery, we observed an IL-10 reduction up to baseline level that could explain a rise in TNF- α plasma concentration. On the other hand, considering the dual role of IL-6

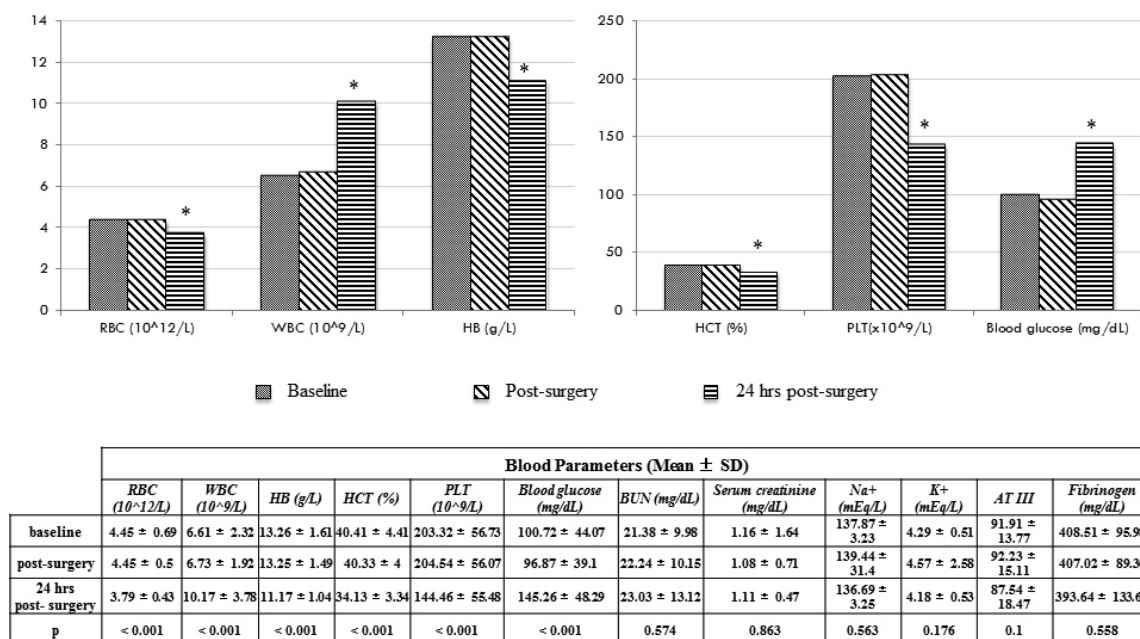
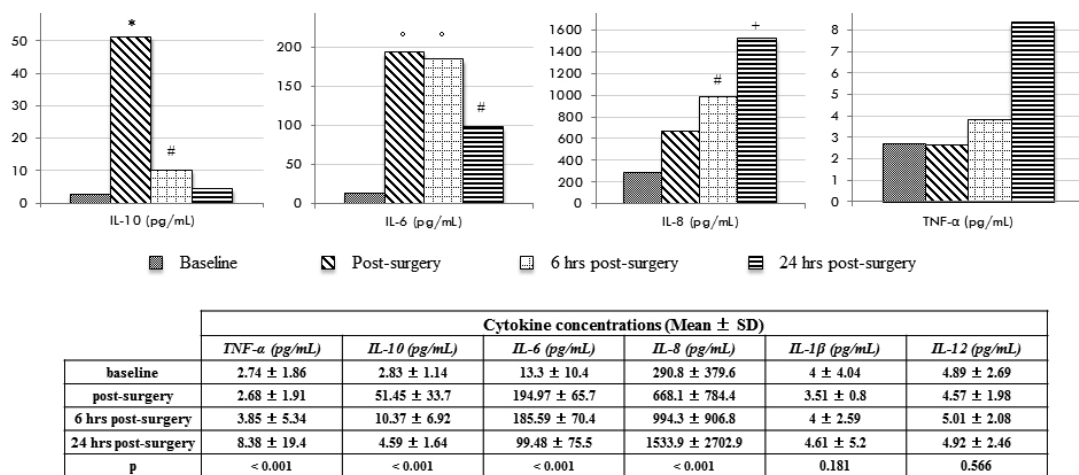
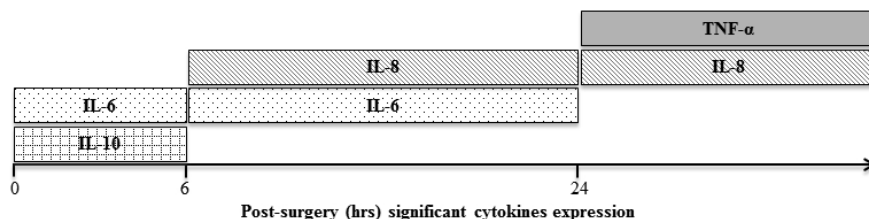


Fig. 1. Blood parameter modification after cardiac surgery. * $p < 0.001$ compared to baseline and immediately after surgery. Abbreviations: RBC, red blood cell count; WBC, white blood cell count; HB, hemoglobin; HCT, hematocrit; PLT, platelet count; BUN, blood urea nitrogen; AT III, antithrombin III. Abbreviations: SD, Standard deviation.



A



B

Fig. 2. A) Modulation of cytokine plasma concentrations during and after cardiac surgery. * $p < 0.001$ compared to baseline, 6 and 24 h post-surgery. # $p < 0.001$ compared to baseline. ° $p < 0.001$ compared to baseline and 24 h post surgery. + $p < 0.001$ compared to baseline and post-surgery. ^ $p < 0.001$ compared to baseline, post-surgery, 6 and 24 h post-surgery. Abbreviations: SD, standard deviation. B) Time course of cytokine level after cardiac surgery.

on inflammation acting both as an activator of inflammatory cascade or an anti-inflammatory agent, the increased IL-6 levels, 24 h after surgery, could be related to the negative feedback action on TNF α activity (14, 15). On the basis of these findings, in subsequent studies, we will evaluate the correlations of clinical manifestations with blood parameters and cytokine modulation. We will also evaluate the comparison of clinical outcomes of patients treated with corticosteroids after 6 h post-surgery, when the anti-inflammatory cytokine IL-10 begins to decrease, in respect to patients treated before and after 24 h post-surgery.

ACKNOWLEDGEMENTS

This project was partially funded by 60% Ateneo grants from Ministero dell'Università, Italy to S.M. and E.T. We also received partial contribution to research expenses by the Department of Medical, Oral and Biotechnologic Sciences, University of Chieti "G. d'Annunzio".

REFERENCES

1. Brix-Christensen V. The systemic inflammatory response after cardiac surgery with cardiopulmonary bypass in children. *Acta Anaesthesiol Scand* 2001; 45(6):671-79.
2. Schwartz JD, Shamamian P, Schwartz DS, et al. Cardiopulmonary bypass primes polymorphonuclear leukocytes. *J Surg Res* 1998; 75(2):177-82.
3. McBride WT, Armstrong MA, Crockard AD, McMurray TJ, Rea JM. Cytokine balance and immunosuppressive changes at cardiac surgery: contrasting response between patients and isolated CPB circuits. *Br J Anaesth* 1995; 75(6):724-33.
4. Wan S, Yim AP. Cytokines in myocardial injury. Impact on cardiac surgical approach. *Eur J Cardiothorac Surg* 2014; 46(1):107-11.
5. Mitchell JD, Grocott HP, Phillips-Bute B, Mathew JP, Newman MF, Bar-Yosef S. Cytokine secretion after cardiac surgery and its relationship to postoperative fever. *Cytokine* 2007; 38(1):37-42.
6. Leon LR. Invited review: cytokine regulation of fever: studies using gene knockout mice. *J Appl Physiol* 2002; 92(6):2648-55.
7. Sharma M, Ganguly NK, Chaturvedi G, Thingnam SK, Majumdar S, Suri RK. Release of pro-inflammatory mediators during myocardial ischemia/reperfusion in coronary artery bypass graft surgery. *Mol Cell Biochem* 2003; 247(1-2):23-30.
8. Wan S, DeSmet JM, Barvais L, Goldstein M, Vincent JL, LeClerc JL. Myocardium is a major source of proinflammatory cytokines in patients undergoing cardiopulmonary bypass. *J Thorac Cardiovasc Surg* 1996; 112(3):806-11.
9. Chuai JB, Shi L2, Ma XY, Wu D, Kang K, Jiang SL. Curative effect of mechanical heart valve replacement and anticoagulant therapy after surgery. *J Biol Regul Homeost Agents* 2016; 30(1):141-46.
10. Buffalo de Andrade JCS, Branco JNR, Teles CA, Anguiar LF, Gomes WJ. Coronary artery bypass grafting without cardiopulmonary bypass. *Ann Thorac Surg* 1996; 61(1):63-66.
11. Mazzei V1, Nasso G, Salamone G, Castorino F, Tommasini A, Anselmi A. Prospective randomized comparison of coronary bypass grafting with minimal extracorporeal circulation system (MECC) versus off-pump coronary surgery. *Circulation* 2007; 116(16):1761-67.
12. Marinari S, De Iuliis V, Dadorante V, et al. Cytokine modulation in patients with idiopathic pulmonary fibrosis undergoing treatment with steroids, immunosuppressants and IFN- γ -1b. *J Biol Regul Homeost Agents* 2017; 31(1):59-69.
13. Westenbrink BD, Kleijn L, de Boer RA, et al., IMAGINE Investigators. Sustained postoperative anaemia is associated with an impaired outcome after coronary artery bypass graft surgery: insights from the IMAGINE trial. *Heart* 2011; 97(19):1590-96.
14. Menghini L, Leporini L, Scanu N, Pintore G, Ferrante C, Recinella L, Orlando G, Vacca M, Brunetti L. A multiherbal formulation influencing immune response in vitro. *Minerva Med* 2012; 103(1):13-21.
15. Menghini L, Leporini L, Pintore G, Ferrante C, Recinella L, Orlando G, Vacca M, Brunetti L. A natural formulation (imoviralTM) increases macrophage resistance to LPS-induced oxidative and inflammatory stress in vitro. *J Biol Regul Homeost Agents* 2014; 28(4):775-82.

LETTER TO THE EDITOR

ANTERIORLY PLACED TUMORS TO THE CONUS: REMOVAL BY INTERRADICULAR WINDOWP. CIAPPETTA¹, S. TAURONE², M. SPOLETINI² and M. ARTICO²¹*Department of Basic Medical Sciences, Neuroscience and Sensory Organs, University of Bari;*²*Department of Sensory Organs, University of Rome "Sapienza", Italy**Received August 21, 2017 – Accepted October 19, 2017*

Tumors anteriorly situated to the medullary conus are rarely encountered and represent a true surgical challenge. We examined the literature on this topic, concluding that there are no previous reports on alternative surgical techniques different to the traditional one. We report two cases of intradural extramedullary tumor operated on by a technique performed through a window opened between the spinal roots, which allows an easy, effective and useful resection. We describe a new operative technique which ensures a complete removal of these tumors and discuss clinical implications in the light of the available literature on this topic.

To the Editor,

This report focuses on the technical difficulties that may be encountered during the surgical removal of intradural extramedullary tumors located anteriorly to the medullary conus. The usual approach for the surgical resection of these tumors is the classic posterior technique (1-3). However, this approach may lead to unsatisfactory results and may even be dangerous in anteriorly placed tumors of the region of the medullary conus, due to the possibility of intraoperative damage of the medullary conus and of the neighbouring spinal roots.

To be defined as anteriorly placed to the conus, tumors must strictly fulfill some fundamental requirements: i) location of the tumor anterior to the equator of the spinal canal, medially to the denticulate ligament; ii) a tumor completely covered by the conus and the spinal roots, and therefore not visible after laminectomy (unless a small part of it protrudes between the spinal roots). A spinal meningioma presenting the above-reported features

may be defined as "anterior" to the medullary conus only when its attachment is strictly confined to the anterior dura within the spinal canal. Similar tumors are very unusual and present technical difficulties that highlight the problems involved in their removal, which imply a higher risk of intraoperative and postoperative morbidity. The authors describe a new operative technique (a window performed between the spinal roots of the cauda equina) which may ensure an easy and effective resection of these rare tumors and discuss the relevant clinical implications in the light of available literature.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. The study was approved by the Local Ethics Committee of Policlinico Umberto I, Sapienza University of Rome, (Prot. 726/10, approved on 16.09.2010). Informed consent was obtained from all individual participants included in the study.

Key words: complete resection, intradural extramedullary tumors, medullary conus, spinal roots, interradicular window

Mailing address:

Prof. Marco Artico,
Department of Sensory Organs,
University of Rome "Sapienza",
V.le del Policlinico 155, 00161 Rome, Italy
Tel./Fax: +39 0649918054
e-mail: marco.artico@uniroma1.it

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

Clinical presentation

Case 1

A 60-year-old woman with a three-month history of flaccid paraparesis came to our attention and was rapidly hospitalized. On admission, she presented a flaccid paraparesis associated with modest sphincteric disturbances, a level of hypoesthesia at L1 and a saddle anesthesia. MRI showed a tumor located in the anterior portion of the spinal canal which extended from T12 to L1 (Fig. 1a). A relevant posterior dislocation of the conus, that was compressed by the lesion, was also visible. The lesion was hyperintense on T2 weighted sequences, showing significant enhancement after administration of paramagnetic contrast medium. Surgery was performed by a midline skin incision made from T11 to the L2 spinous process tip. The paravertebral muscles were detached on both sides. A limited laminectomy was performed on the laminae of T12, L1. Intraoperative neurophysiological monitoring of conus medullaris and cauda equina was performed as previously reported by Kothbauer and Deletis (4). On opening the dura mater, the conus appeared posteriorly displaced by the tumor. The surface of the neoplasm showed enlarged and varicose vessels. The tumor was not visible (Fig. 1b) because it was covered by the spinal roots and the filum terminale. Only after section of the arachnoidal adhesions between the filum and caudal roots was it possible to observe the inferior tumor pole. The latter was incised, but this surgical maneuver was not sufficient to reduce the compression on the conus. Since it was impossible to obtain a good visualization of the lesion, a particular intraoperative approach was performed. In fact, the lesion was covered by the anterior-inferior part of the conus and its removal would easier through a window between the spinal roots (Fig. 1c). This procedure allowed the visualization of the anteriorly placed portion of the tumor and an optimal debulking of the mass. Dissection was then conducted towards the upper pole of the tumor and, after the debulking of the mass by Cavitron Ultra Sonic Aspirator (CUSA), the total resection was achieved by rotating and sliding the anteriorly placed tumor through the obtained window (Fig. 1, d and e). After resection, the conus tended to resume its original

position. Intraoperative findings suggested a typical ependymoma. Subsequent histological examination confirmed a myxopapillary ependymoma.

The postoperative course was uneventful and a prompt recovery of the lower limb movements was observed. A complete improvement of the sphincter functions occurred within 15 days after the surgical operation. Postoperative MRI showed the complete removal of the tumor (Fig. 1f).



Fig. 1. Case 1: ependymoma D12-L1. **a)** Preoperative MRI: an intradural extramedullary lesion situated anteriorly to the medullary conus at D12-L1 level and displacing posteriorly the lower portion of the spinal cord is visible. **b)** Intraoperative view: the tumor is covered by the medullary conus, anchored by a stretched filum terminale, and spinal roots. **c)** Intraoperative view: the filum terminale has been sectioned (a clip is visible near the section) in order to mobilize the lower pole of the tumor. **d)** Intraoperative view: the window performed between the spinal roots ensures the visualization of the tumor in the depth of the operative field. **e)** The tumor (on the left) is removed through a surgical window performed between the spinal roots. **f)** Postoperative MRI showing the complete resection of the tumor.

Case 2

The second case was a 42-year-old woman with a five-month history of flaccid paraparesis associated with urinary retention (treated by a permanent urinary catheter). Neurological examination showed a flaccid paraparesis, saddle hypoesthesia and sphincter disturbances. MRI (Fig. 2a) documented a subarachnoidal T12-L1 ovoidal mass isointense in T1-weighted sequences, mildly hyperintense in T2-weighted sequences, and a mild homogeneous contrast medium enhancement. Surgery was performed as follows: a midline skin incision was made from T11 to the L2 spinous process tip. The paravertebral muscles were detached on both sides. A limited laminectomy was performed on the laminae of T12, L1. Intraoperative neurophysiological monitoring of conus medullaris and cauda equina was performed (4). On midline opening of the dura mater, the conus appeared as displaced posteriorly by a firm not-visible mass (Fig. 2b). An arachnoidal dissection was therefore performed in order to obtain an operating window (Fig. 2c) similar to that described in Case 1. The dural attachment of the mass (anteriorly placed in the spinal canal), placed at the level of the central portion of T12 (Fig. 2d), was carefully resected by also coagulating the dura mater. The tough-elastic consistency of the tumor produced a kind of “anchoring” of the conus in its preoperative location, with no changes after the removal (Fig. 2e), even after cutting the arachnoidal adhesions. In this case, the resection was easier in comparison to Case 1. The postoperative histological diagnosis was endothelial meningioma.

The postoperative course was complicated by a mild CSF leakage treated by a CSF drainage for 7 days. The patient showed a progressive neurological improvement and was discharged after two weeks. Two months after surgery a clinical evaluation showed a complete recovery of the previous neurological deficits and the MRI confirmed complete removal of the tumor (Fig. 2f).

DISCUSSION

Spinal meningiomas located in the lumbar segment or in the dorso-lumbar segment are extremely rare. A

statistical analysis reported in the available literature (5) reports a percentage varying between 2% and 14% of all intradural extramedullary tumors. On the other hand, anteriorly located tumors, which are very frequent in the cervical spine, are extremely rare in the dorso-lumbar spine (6). Myxopapillary extramedullary ependymomas are rare, slow-growing lesions, originating from the filum terminale and may reach enormous size, sometimes filling out

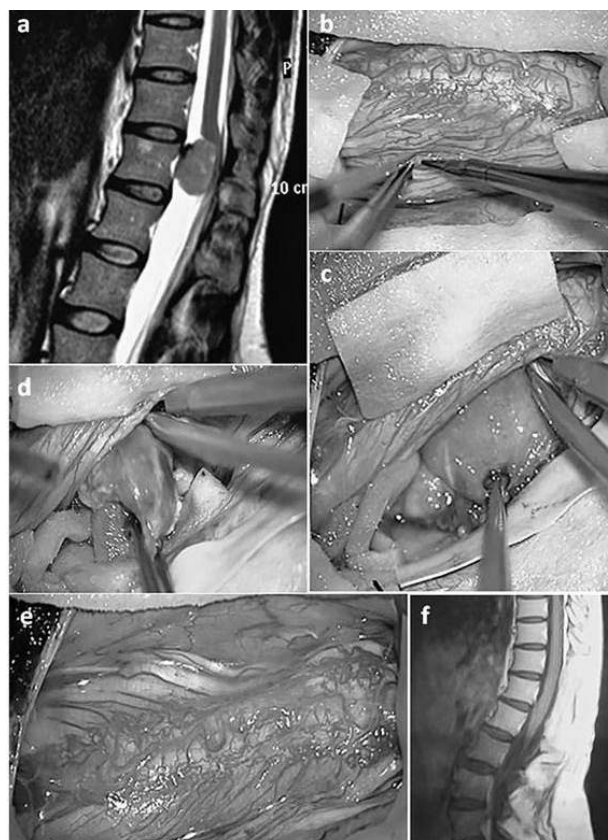


Fig. 2. Case 2: meningioma D12-L1. **a)** Preoperative MRI: an intradural extramedullary ovoidal mass arising anteriorly to the medullary conus at D12-L1 level and strongly displacing the lower portion of the spinal cord is visible. **b)** Intraoperative view. The lesion is hidden by the medullary conus and the spinal roots; the window between the spinal roots was thus performed by an accurate dissection. **c)** Intraoperative view: the window opened between the spinal roots allowed an easy removal of the meningioma and the coagulation of its dural attachment. **d)** Intraoperative view: the meningioma is easily removed through the window performed between the spinal roots. **e)** Intraoperative view: repositioning of the medullary conus after the debulking and complete resection. **f)** Postoperative control MRI which demonstrates the complete resection of the tumor.

the entire lumbosacral canal. Klekamp (7) reports an incidence rate of 4.5% among all intradural tumors, which is similar to those reported by other authors (8, 9) who found an incidence of 5.1%. In the available literature, however, no cases of tumors with an exclusive anterior location removed by the surgical technique described in this report (“window between the spinal roots”) were found.

The lateral approach to the lesion as well as the visual perspective tangential to the anterior dural plane of the spinal canal represent fundamental aspects. This result was achieved by performing a surgical corridor between the roots, laterally to the conus, selecting the side on the basis of the eccentricity of the lesion. In cases in which the tumor grows in the central portion of the spinal canal, the option is related to the preferences of the surgeon. The surgical corridor consists of a window obtained by a sharp dissection of the arachnoidal adhesions. This “window” may also be achieved in different areas, on the basis of the length of the mass, with the aim of performing a correct debulking when the lesion is partially or completely hidden by the spinal roots. In fact, in the first above-reported case, the inferior pole of the tumor was scarcely visible. This condition forced us to extend the corridor superiorly between the spinal roots, thus obtaining a complete debulking and an easier removal of the tumor.

REFERENCES

1. Tessitore E, Cabrilo I, Boex C, Schaller K. Cauda equina tumor surgery: how I do it. *Acta Neurochir (Wien)* 2012; 154(10):1815-20.
2. Lee JP1, Wang AD, Wai YY, Ho YS. Spinal extradural cavernous hemangioma. *Surg Neurol* 1990; 34(5):345-51.
3. Rovira A, Rovira A, Capellades J, Zauner M, Bella R, Rovira M. Lumbar extradural hemangiomas: report of three cases. *AJNR Am J Neuroradiol* 1999; 20(1):27-31.
4. Kothbauer KF, Deletis V. Intraoperative neurophysiology of the conus medullaris and cauda equina. *Childs Nerv Syst* 2010; 26(2):247-53.
5. Gottfried ON, Gluf W, Quinones-Hinojosa A, Kan P, Schmidt MH. Spinal meningiomas: surgical management and outcome. *Neurosurg Focus* 2003; 15:14(6):e2.
6. Galgano MA, Beutler T, Brooking A, Deshaies EM. Spinal Meningiomas: A Review. *J Spine* 2014; 3(1):1-5.
7. Klekamp J1. Spinal ependymomas. Part 2: Ependymomas of the filum terminale. *Neurosurg Focus* 2015; 39(2):E7.
8. Hirano K1, Imagama S, Sato K, et al. Primary spinal cord tumors: review of 678 surgically treated patients in Japan. A multicenter study. *Eur Spine J* 2012; 21(10):2019-26.
9. BhatAR, KirmaniAR, Wani MA, Bhat MH. Incidence, histopathology, and surgical outcome of tumors of spinal cord, nerve roots, meninges, and vertebral column-Data based on single institutional (Sher-i-Kashmir Institute of Medical Sciences) experience. *J Neurosci Rural Pract* 2016; 7(3):381-91.

LETTER TO THE EDITOR

FOCAL ACTIVE COLITIS AS A PREDICTOR OF INFLAMMATORY BOWEL DISEASE: RESULTS FROM A SINGLE-CENTER EXPERIENCE

E. SINAGRA^{1,2}, D. RAIMONDO¹, G. POMPEI³, G. FUSCO⁴, F. ROSSI¹, G. TOMASELLO^{2,5}, A. LEONE⁶, F. CAPPELLO^{2,5}, G.C. MORREALE⁷, F. MIDIRI⁸, M. MIDIRI⁸ and A.G. RIZZO³

¹Gastroenterology and Endoscopy Unit, Fondazione Istituto San Raffaele Giglio, Cefalù, Italy;

²Section of Nutrition, Euro-Mediterranean Institute of Science and Technology (IEMEST), Palermo, Italy; ³Pathology Unit, Fondazione Istituto San Raffaele Giglio, Cefalù, Italy; ⁴Division of Internal Medicine, Ospedali Riuniti Villa Sofia – Vincenzo Cervello, Palermo, Italy; ⁵Section of Human Anatomy, BIONEC, University of Palermo, Italy; ⁶Section of Histology, BIONEC, University of Palermo, Italy; ⁷Division of Gastroenterology, Ospedali Riuniti Villa Sofia – Vincenzo Cervello, Palermo, Italy; ⁸Section of Radiology, DIBIMED, University of Palermo, Italy

Received April 26, 2017 – Accepted October 13, 2017

The term focal active colitis (FAC) is conventionally used to describe the presence of isolated cryptitis, characterized by an inflammatory infiltrate consisting of intraepithelial neutrophils and/or neutrophils invading the lumen of the cryptae, with no other microscopic alteration of the colonic mucosa and, in particular, without the presence of signs of chronic inflammation. To date, only four studies, including one conducted in a pediatric population, have been performed to evaluate the clinical significance of this disease. The aim of this retrospective study on prospectively-collected data is to evaluate the clinical implications of the focal active colitis, since there still remains a marked uncertainty regarding this topic and about how often such a diagnosis will presage a diagnosis of inflammatory bowel disease (IBD). Clinical, endoscopic, and pathological data were retrospectively reviewed from 30 patients with focal active colitis, who had no other diagnostic findings on colorectal biopsy and no history of chronic inflammatory bowel disease. The histological findings were correlated with clinical diagnoses. Thirty patients (11 males, 19 females; age 24-80 years, median 56 years) (0.5%) out of 5,600 undergoing colonoscopy between January 2012 and December 2016 presented a definitive diagnosis of FAC. Follow-up ranged from 6 to 60 months (median 24 months). At endoscopy, 19 patients (63%) had mild and non-specific changes, such as mild mucosal erythema, while 11 (37%) had normal findings. Eight patients were documented as having irritable bowel syndrome, while nine cases could be attributed to the effects of drugs, five presented FAC as incidental finding, one a diagnosis of infectious colitis, and seven a diagnosis of IBD (4 with Crohn's disease). FAC was confirmed to be a more significant predictor of IBD than the previous literature would indicate, even if larger prospective studies, targeted to study this relationship, are needed to understand more clearly its clinical significance.

To the Editor,

The term focal active colitis (FAC) is conventionally used to describe the presence of

isolated cryptitis, characterized by an inflammatory infiltrate consisting of intraepithelial neutrophils and/or neutrophils invading the lumen of the cryptae,

Key words: focal active colitis; Crohn's disease; ulcerative colitis; irritable bowel syndrome

Mailing address:

Dr Emanuele Sinagra,
Fondazione Istituto G. Giglio,
Contrada Pietrapollastro Pisciotto, 90015 Cefalù, Palermo, Italy
Tel.: +393270193383 - Fax: +39921920406
e-mail: emanuelesinagra83@googlemail.com

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

with no other microscopic alteration of the colonic mucosa and, in particular, without the presence of signs of chronic inflammation (Fig. 1).

To date, only four studies, including one conducted on a pediatric population, have been carried out to evaluate the clinical significance of this disease (1-4). Initially, it was thought that the main causes of focal active colitis were (a) polypharmacy [with particular regard to the concomitant assumption of anti-inflammatory drugs (NSAIDs)], and (b) intestinal bacterial infections. However, thereafter, it was found that active focal colitis can evolve towards a chronic inflammatory bowel disease, particularly to a colonic Crohn's disease and, in a smaller percentage of cases, be associated with irritable bowel syndrome, or represent an incidental finding (1).

The aim of this retrospective study on prospectively-collected data is to evaluate the clinical implications of focal active colitis, since there still remains a marked uncertainty on this topic and about how often such a diagnosis will presage a diagnosis of inflammatory bowel disease (IBD).

MATERIALS AND METHODS

Patients

A computer search of the pathology files at the Fondazione Istituto Giuseppe Giglio was used to generate a list of all colorectal biopsies signed out between January 2012 and December 2016 with the terms 'focal' and 'colitis' mentioned anywhere in the text of the reports. Cases from this list were reviewed and entered into the study provided they did not have an antecedent history of IBD and had the histological changes of FAC.

Cases in which other diagnostic features were present in the same set of biopsies, such that FAC was the only histological abnormality identified, were excluded from the study. FAC was defined, according to the first definition performed by Greenson et al. (2), as the presence of at least a single crypt infiltrated by neutrophils with associated epithelial damage; activity was no longer considered focal when most crypts in a single biopsy were infiltrated by neutrophils. Crypt abscesses were also included as part of the spectrum of FAC.

Thirty patients with no antecedent history of IBD were

selected for the study. Prior and subsequent colorectal biopsies were also reviewed when available. Patients ranged in age from 24 to 80, with a median age of 56, and consisted of 11 men and 19 women.

Clinical features

Clinical and endoscopic information for FAC patients was obviously reviewed. The presenting symptoms/indications for endoscopy, medications at initial presentation, past medical history, endoscopic findings, and instituted therapy were noted. Length of follow-up (which ranged from less than 6 to 60 months, with a median of 24 months), final clinical diagnosis, and symptoms and treatment at the time of follow-up were also determined from the patients' charts.

We used the criteria hereunder to establish the final clinical diagnosis of the patients enrolled.

Infectious-type colitis

The patients were defined as affected by self-limiting colitis-like diarrhoeal illness with or without positive stool culture. Positive stool culture was not mandatory in this subset of patients, as highlighted in previous studies, since it was recognized that only about 40% of self-limiting colitis, with histological features suggestive of an infective pathology, presents culture-positive stools (1, 5-7).

Drug-induced colitis

The patients were defined as affected by "drug-induced colitis" when there was a relevant drug history that was likely to cause the change in bowel habit, which resolved after the discontinuation of the drug.

Irritable bowel syndrome

According to Rome IV criteria (8), IBS is defined on the basis of the presence of recurrent abdominal pain, on average, at least 1 day per week in the last 3 months, associated with 2 or more of the following criteria:

- Related to defecation,
- Associated with a change in frequency of stool,
- Associated with a change in form (appearance) of stool.

These criteria should be fulfilled for the last 3 months with symptom onset at least 6 months before diagnosis. In this subset of patients there was no relevant drug history or clinical, radiological, endoscopic or histopathological

signs of IBD. Most of these patients showed an IBS with predominant diarrhoea (IBS-D), and no alarming features had developed during the period of follow-up.

Incidental focal active colitis

The patients were affected by incidental FAC when they underwent colonoscopic assessment for reasons other than a change in bowel habit, and FAC was an incidental finding on biopsy. They had no relevant drug history or evidence of IBD.

Inflammatory bowel disease

The patient was categorized as affected by IBD if he subsequently represented with clinical, endoscopic and histopathological features of IBD. Histological assessment presented a pivotal role in this categorization.

Only the patients with a confident final histopathological diagnosis of IBD were included in this category. Patients were subclassified further into ulcerative colitis or Crohn's disease groups when this was possible or, when there were features equivocal for the two types of IBD, into the IBD unclassified category (IBDU), according to the European Crohn's and Colitis Organization (ECCO) guidelines (9).

Evaluation of histological features

FAC was conventionally defined as neutrophil-mediated crypt epithelial injury involving at least one crypt and <50% of the biopsy specimen. Biopsies diagnosed as FAC were reviewed independently by two experienced pathologists.

All the cases with chronic inflammation, granulomas or crypt architectural abnormalities, or if there was a previous diagnosis of IBD or any other specific diagnosis were excluded from the study.

For the purpose of this study, we followed the protocol also experienced by Shetty et al. (1), thus evaluating the following histological features: site(s) of involvement in the colorectum and possible ileal involvement, amount of polymorphonuclear neutrophil (PMN) crypt activity based on the number of crypts involved in the most affected biopsy, position of PMNs in the crypt(s), number of crypt abscesses, PMNs in the lamina propria, association of inflamed crypts to lymphoglandular complexes (LGC), basal FAC, crypt apoptosis, subepithelial apoptosis and melanosis coli. All cases were

consigned by one experienced gastroenterologist to one of the aforementioned clinical diagnostic categories, according to the findings of follow-up. When there was doubt, the categorization was corroborated by a further gastroenterologist.

RESULTS

Thirty patients (0.5%) out of 5,600 who underwent colonoscopy between January 2012 and December 2016, presented a definitive diagnosis of FAC. Of the 30 patients, 11 were males (37%) and 19 females (63%). Patients ranged in age from 24 to 80, with a median age of 56. Length of follow-up ranged from 6 to 60 months, with a median of 24 months. The predominant symptoms were diarrhoea alone in 15 (50%), diarrhoea and pain in 10 (34%) and altered bowel habit in 4 (13%). One patient (3%) was asymptomatic. In all the patients the colonic preparation was recorded in the endoscopy report: 21 patients (70%) had oral Selg-Esse (PEG-3350, sodium sulfate, sodium chloride, potassium chloride) colonic preparation, 9 (30%) had oral Moviprep (PEG-3350, sodium sulfate, sodium chloride, potassium chloride, ascorbic acid and sodium ascorbate). Table I gives the clinical categorization of the 30 FAC cases. At endoscopy, 19 patients (63%) had mild and non-specific changes, such as mild mucosal erythema, while 11 (37%) had normal findings.

Eight patients (27%) were documented as having IBS, while nine cases (30%) could be attributed confidently to the effects of drugs. At the time of the diagnosis of FAC, 18 (60%) of the 30 patients were on drugs which had been described previously as potentially causing FAC: five patients were on NSAIDs (cardioaspirin), five on thyroxine, eight on PPIs and the others were on multiple drugs (bisoprolol, carvedilol or bisoprolol; furosemide; ticlopidin; rivaroxaban; simvastatin; telmisartan). Of the 9 patients (30%) with evidence of drug-induced FAC, 4 (13%) were on thyroxine, 4 (13%) on PPIs, three (10%) on NSAIDs (cardioaspirin), and two (7%) on metformin. In only one patient (3%), a self-limiting colitis was diagnosed, due to an *Entamoeba histolytica* infection, shown on

stool cultures. For 7 patients (23%) an ultimate diagnosis of IBD was made: four patients (13%) ultimately developed Crohn's disease and three (10%) developed ulcerative colitis with evidence of characteristic endoscopic and histological findings affecting at least the rectum. The period of time that elapsed between the initial diagnosis of FAC and the subsequent diagnosis of IBD varied between 2 and 5 years. In four patients with Crohn's disease, there was restriction of the disease to the right colon or to the whole colon but, in one, there was involvement of the ileum and colon; in the three patients with ulcerative colitis, two presented with proctitis, while only one presented with left-side colitis.

Finally, five patients (17%) presented a definitive diagnosis with incidental FAC. All the patients presenting such diagnosis were not taking any drug with the exception of one patient who was taking omeprazole; however, the discontinuation of this drug did not affect the clinical symptoms of the patient nor modify the subsequent endoscopic and histological evaluation.

With regard to the endoscopic findings, 11 (37%) out of 30 colonoscopies appeared endoscopically normal: five (45%) presented a final diagnosis of IBS-D, five (45%) presented a final diagnosis of drug-induced colitis, while only one (10%) represented an incidental FAC.

The proximal colon was involved by FAC in biopsies from 6 patients (20%), distal colon in 6 patients (20%) and rectum in 3 patients (10%). In 2 patients (7%) both proximal and distal colon showed involvement by FAC in mucosal biopsies, while in two further patients there was an ileal non-specific inflammatory involvement.

Interestingly, in the four patients (13%) with a definitive diagnosis of Crohn's disease, two presented with a FAC in the proximal colon, one with a left-sided FAC, and one with a FAC extended to both proximal and distal colon. On the other hand, in the three patients (10%) with a definitive diagnosis of ulcerative colitis, the rectum was involved in all the cases, and only in one case there was an involvement of the left colon.

According to the interesting report performed by Shetty and coworkers, we also evaluated the "basal

FAC", defined as a florid FAC, orientated basally in the crypts and relatively diffusely in a single biopsy. Interestingly, this finding was shown to be present in all the patients with a definitive diagnosis of IBD (1).

Data on the amount of PMN crypt activity in the most affected biopsy, position of PMNs, number of crypt abscesses, PMNs in the lamina propria, association of inflamed crypts to lympho-glandular complexes, basal FAC, crypt apoptosis, subepithelial apoptosis, eosinophils in lamina propria and melanosis coli are shown in Table II.

Due to the small number of patients enrolled in the study, there were no statistically significant differences between the five clinical categories of FAC in regard to the age at diagnosis, the number and sites of affected biopsies, the number of crypts involved, the percentage of crypt involvement in the most affected biopsy, the percentage of crypt involvement in all biopsies, the number of crypt abscesses, the presence of polymorphs in the lamina propria, the association of inflamed crypts to LGCs, subepithelial apoptosis and melanosis coli.

The main, although not statistically significant, findings concerned :

- the association of cryptitis to LGC in 3 out of 4 patients with a definitive diagnosis of Crohn's disease;
- the presence of basal FAC in all the patients with a definitive diagnosis of IBD (both Crohn's disease and ulcerative colitis);
- the simultaneous presence of a basal FAC with a cryptitis associated to LGC, when the latter was reported;
- the presence of crypt apoptosis in two patients with Crohn's disease and in one patient with ulcerative colitis, while all the other patients did not show this pathological finding.

DISCUSSION

Few data are available on the clinical implications of FAC. However, with regard to previous studies carried out to better understand the clinical course of FAC, the present study showed some interesting findings. Firstly, only in one patient (3%) a definitive diagnosis of infective colitis was made and,

Table I. *Clinical definitive diagnosis for all the cases of focal active colitis.*

Diagnosis	Male	Female	Total
Drug-induced colitis	3	6	9
Irritable bowel syndrome	4	4	8
Crohn's disease	1	3	4
Ulcerative colitis	1	2	3
Infectious colitis	0	1	1
Incidental focal active colitis	2	3	5
Total	11	19	30

Table II. *Pathological findings of focal active colitis.*

Polimorph neutrophils crypt activity	None 6 (20%)	Mild 15 (50%)	Moderate 9 (30%)	Severe 0 (0%)
Position of polymorph neutrophils	Basal 10 (33%)	Basal and superficial 20 (64%)	Superficial 0 (0%)	Complete 0 (0%)
Number of crypt abscesses	None 29 (97%)	Less than two 0 (0%)	More than 5 1 (3%)	—
Polimorph neutrophils in lamina propria	None 1 (3%)	Mild 22 (73%)	Moderate 7 (24%)	Severe 0 (0%)
Association of inflamed crypts to linfoglandular complex	Absent 24 (80%)	Present 6 (20%)	—	—
Basal focal active colitis	Absent 7 (24%)	Present 23 (76%)	—	—
Crypt apoptosis	Absent 27 (90%)	Present 3 (10%)	—	—
Subepithelial apoptosis	None 28 (93%)	Mild 2 (7%)	Moderate 0 (0%)	Severe 0 (0%)
Melanosis coli	None 29 (97%)	Mild 0 (3%)	Moderate 1 (0%)	Severe 0 (0%)

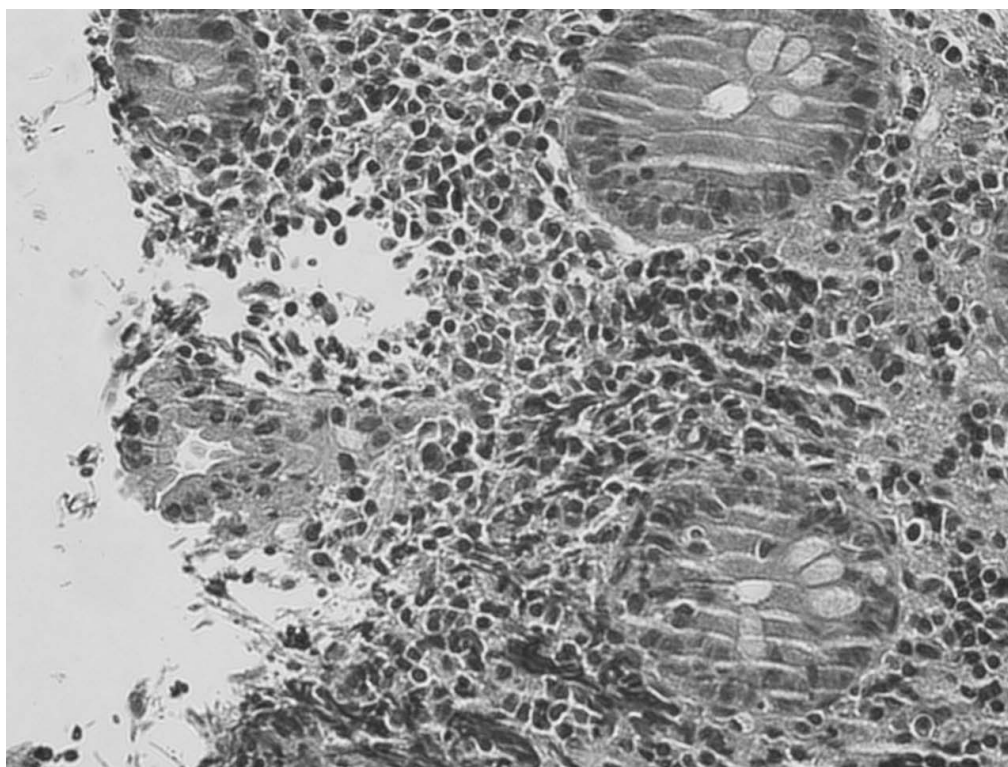


Fig. 1. *Example of isolated cryptitis.*

interestingly, this was secondary to an infection due to *Entamoeba histolytica*. This unexpected finding may be due to several reasons, such as the progressively widespread use of antibiotics. Secondly, in nine patients (30%) a definitive diagnosis of drug-induced FAC was made. Thirdly, IBS was the second most reported diagnosis in the present study. Recently, the scientific community focused its attention on the pivotal role of low grade mucosal inflammation in IBS (10, 11). To date, however, it results unclear whether this event occurs only in selected subsets of IBS patients, where probably other factors (12) may be involved in the manifestation of the definitive phenotype of IBS patients. Finally, 7 out of 30 (23%) patients reported a definitive diagnosis of IBD, the third most represented diagnosis in the present study. In fact, in previous studies, the diagnosis of ulcerative colitis was under-represented, while the present study reports the highest prevalence of ulcerative colitis between the studies regarding FAC. The patients with a definitive diagnosis of ulcerative colitis presented a mild pattern of the disease, extended to

the rectum (in two cases) or to the sigmoid colon (in one case), without extra intestinal manifestations which are, in contrast, more frequently associated to pancolitis (13). There is a growing interest in the role that inflammatory cytokines, which sustain the pathogenesis of IBD (14, 15), could play in shaping the definitive inflammatory phenotype of IBD. In conclusion, despite the major pitfall of the present study, represented by the small number of patients enrolled, our experience shows that FAC was confirmed to be a more significant predictor of IBD than the previous literature would indicate, even if larger prospective studies, targeted to study this relationship, are needed to understand more clearly its clinical significance.

REFERENCES

1. Shetty S, Anjarwalla SM, Gupta J, Foy CJ, Shaw IS, Valori RM, Shepherd NA. Focal active colitis: a prospective study of clinicopathological correlations in 90 patients. *Histopathology* 2011; 59(5):850-56.

2. Greenson JK, Stern RA, Carpenter SL, Barnett JL. The clinical significance of focal active colitis. *Hum. Pathol* 1997; 28:729-33.
3. Volk E, Shapiro BD, Easley KA, Goldblum JR. The clinical significance of a biopsy-based diagnosis of focal active colitis: a clinicopathologic study of 31 cases. *Mod. Pathol* 1998; 11:789-94.
4. Xin W, Brown PI, Greenson JK. The clinical significance of focal active colitis in paediatric patients. *Am J Surg Pathol* 2003; 27:1134-38.
5. Surawicz CM, Belic L. Rectal biopsy helps to distinguish acute self-limited colitis from idiopathic inflammatory bowel disease. *Gastroenterology* 1984; 86:104-13.
6. Nostrant TT, Kumar NB, Appelman HD. Histopathology differentiates acute self-limited colitis from ulcerative colitis. *Gastroenterology* 1987; 92:318-28.
7. Shepherd NA. Pathological mimics of chronic inflammatory bowel disease. *J Clin Pathol* 1991; 44:726-33.
8. Mearin F, Lacy BE, Chang L, et al. Bowel Disorders. *Gastroenterology* 2016 18. doi: 10.1053/j.gastro.2016.02.031. [Epub ahead of print]
9. Magro F, Langner C, Driessen A, et al; European Society of Pathology (ESP).; European Crohn's and Colitis Organisation (ECCO).. European consensus on the histopathology of inflammatory bowel disease. *J Crohns Colitis*. 2013; 7(10):827-51.
10. Barbara G, De Giorgio R, Stanghellini V, Cremon C, Corinaldesi R. A role for inflammation in irritable bowel syndrome? *Gut* 2002; 51 Suppl 1:i41-4.
11. Sinagra E, Pompei G, Tomasello G, et al. Inflammation in irritable bowel syndrome: Myth or new treatment target? *World J Gastroenterol* 2016; 22(7):2242-55.
12. Sinagra E, Romano C, Cottone M. Psychopharmacological treatment and psychological interventions in irritable bowel syndrome. *Gastroenterol Res Pract* 2012; 2012:486067.
13. Sinagra E, Aragona E, Romano C, et al. The role of portal vein thrombosis in the clinical course of inflammatory bowel diseases: report on three cases and review of the literature. *Gastroenterol Res Pract* 2012; 2012:916428.
14. Sinagra E, Perricone G, Romano C, Cottone M. Heart failure and anti tumor necrosis factor-alpha in systemic chronic inflammatory diseases. *Eur J Intern Med* 2013; 24(5):385-92.
15. Sinagra E, Tomasello G, Cappello F, et al. Probiotics, prebiotics and symbiotics in inflammatory bowel diseases: state-of-the-art and new insights. *J Biol Regul Homeost Agents* 2013; 27(4):919-33.

LETTER TO THE EDITOR

**INSTANT LOADING WITH INTRAORAL WELDING TECHNIQUE
AND PRAMA IMPLANTS: A NEW PROSTHETIC APPROACH**R. CELLETTI¹, S. FANALI¹, C.U. LAICI², C. SANTORI³, P. PIGNATELLI¹ and B. SINJARI¹¹Department of Medical, Oral and Biotechnological Sciences, University G. d'Annunzio Chieti-Pescara, Chieti, Italy; ²Private Practice Ripe San Ginesio, Italy; ³Private Practice, Giulianova, Italy*Received May 18, 2017 – Accepted October 27, 2017*

When splinting multiple implants passive fit of the framework should be achieved to avoid excessive force distribution on the implants. Recently, a protocol was suggested for immediate loading of multiple implants by welding a titanium bar to implant abutments directly in the oral cavity so as to create a customized, precise and passive metal-reinforced provisional restoration. The intraoral welding technique subsequently proves to be a successful option in the full-arch immediate restorations of the mandible and maxilla. The aim of this article is to present a case report in which a new prosthetic approach, using trans-mucosal implants, is described. Dental implants are instantly loaded with a provisional prosthesis supported by an intraoral welded titanium framework to obtain a precise passive fit of the immediate loaded prosthesis.

To the Editor,

The evolution of implant dentistry brought an increasing interest in immediate loading and esthetic replacement of missing teeth. In Branemark's traditional protocol the healing time suggested for mandibular implant osseointegration was at least 3 months and 6 months for the maxilla (1). Recent protocols reduce this healing period and immediately expose the implants to functional occlusion forces (2-8). The exposition to these mechanical stresses before biological osseointegration may lead to implant micromovements causing an early implant failure (8-9). New studies describe the intraoral welded technique that seems to offer a practical solution for the stabilization of the implants with high and low torque values (10-13). A new implant system (Prima, Sweden and Martina, Due Carrare Italy) with an innovative trans-mucosal surface morphology (BOPT) was recently developed (14-15). Biologically Oriented

Preparation Technique (BOPT) is a prosthetic approach designed originally for periodontal treated teeth (14); these concepts were transferred to Prima Implants who offer all the characteristics needed for an implant-prostheses rehabilitation for the benefit of both hard and soft tissues (15). BOPT is designed to stabilize the clot that forms in the gingival sulcus during the preparation. The healing process determines the reinsertion and thickening of gingival tissue which adapts to the new emergence profile obtained by the preparation technique without a finishing line. Interactions between preparation-restoration-gingiva facilitate soft tissues to adapt to the new prosthetic shape.

The aim of this study is to evaluate the clinical use of biologically oriented preparation technique (BOPT) with transmucosal implants (PRIMA, Sweden and Martina, Due Carrare, Italy) in addition to the intraoral welding technique. Prima implants

Key words: immediate loading, intraoral welding, biologic width, trans-mucosal implants, marginal bone loss

Mailing address:

Prof. Renato Celletti,
Periodontology I,
Department of Medical, Oral and Biotechnological Sciences,
University G d'Annunzio Chieti-Pescara, Via dei Vestini 31,
66100 Chieti, Italy
Tel.: +39 08713554073- Fax: +39 08713554062
e-mail: celletti@unich.it

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
**DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF
INTEREST RELEVANT TO THIS ARTICLE.**

are instantly loaded with a provisional prosthesis supported by an intraoral welded titanium framework to obtain a precise passive fit of the immediate loaded prosthesis.

MATERIALS AND METHODS

An adult patient received a fixed titanium reinforced temporary durable prosthesis attached to six 3.8 mm trans-mucosal conical zirconium sand-blasted acid etched titanium implants (PRAMA, Sweden and Martina, Due Carrare, Italy). All implants were placed immediately after extractions. The patient was treated at the University G. D'Annunzio in Chieti; he was fully informed regarding the intent of the procedure and signed a written consent form. This case report was designed in accordance to the October revision of the World Medical Association Declaration of Helsinki.

Exclusion criteria: i) presence of active periodontal disease; ii) systemic disease that could compromise osseointegration; iii) treatment with radiation therapy within the previous 12 months; iv) smoking habits (more than 5 cigarettes per day); v) pregnancy or lactating; and vi) previous treatment with bisphosphonates.

Preoperative analyses of anatomical features were performed using a panoramic radiograph and a CT scan. The treatment plan included oral prophylaxis, extractions of all hopeless maxillary teeth and immediate placement of implants instantly loaded by means of a temporary durable prosthesis.

Alginate impressions and laboratory casts for both maxillary and mandibular arches were taken. The shade and mold of prosthetic teeth were selected and appropriate wear-resistant commercial composite resin teeth (Enamel Plus Temp, Micerium, GE) was chosen. All teeth were arranged on a cast, mounted on a semi-adjustable articulator and joined with composite resin to create a prosthetic template.

Antimicrobial prophylaxis was administered with the oral use of 2 g Augmentin 1 h before surgery and twice daily for 5 days post-operatively. After administration of local anesthesia (2% articaine/adrenaline 1:100.000) all maxillary hopeless teeth were extracted; a full thickness muco-periosteal flap was elevated and maxillary bone exposed. Six implants (PRAMA, Sweden and Martina, Due Carrare, Italy) were placed in a sub-crestal position leaving the gold portion of the implants above the bone. In

this way periodontal soft tissues heals along the collar of Prama implants. Implant were placed as following (Pos 15 3.8x 13 mm, pos 12 3.8x 13 mm, pos 11 3.8x 10 mm, pos 21 3.8x 10 mm, pos 23 3.8x 11.5 mm, pos 25 3.8x10 mm). All implants had an insertion torque of 50-70Ncm.

Appropriate 1 piece titanium straight 0° abutment (Sweden and Martina, Due Carrare, Italy) with no exagon was connected to all implants and tightened using a calibrated torque wrench applying a force of 25N. A titanium provisional cap (Sweden and Martina, Due Carrare, Italy) was placed over the abutments and tightened.

Then a 1.8 mm diameter titanium bar wire Grade 2, (Sweden and Martina, Due Carrare, Italy) was placed intra-orally next to the abutments and shaped in order to be in contact with all caps and welded using a welding unit (Dent Weld, Sweden and Martina, Due Carrare Italy). The titanium framework was then removed and all abutments were reduced in length. Additional 1.2 mm titanium wire was added to the framework and this was eventually placed over the implants to check the passivity; the framework was removed, retrieved extra orally, sandblasted and a layer of opaque resin (Ivoclar opaquer, Ivoclar-Vivadent, GE) was applied. At this point, the titanium framework was tightened over the implants in the oral cavity. The pre-operative prepared shell was relined intraorally over the framework with a small quantity of dual cure composite resin. After setting, the completed restoration (framework and composite shell) was removed from the oral cavity, relined with more composite resin, trimmed, polished, reinserted and tightened again using a calibrated torque wrench applying a force of 20N. Proper occlusal contacts were checked with an 8 microns aluminum foil (Hanel Shimstock, Coltene/Whaledent). Nylon 4-0 sutures (Atramat, Int Farmaceutica, Mexico) were used to enhance periodontal soft tissue healing (Fig 1-3).

Appropriate analgesics were prescribed and postoperative oral hygiene instructions (16) were given to the patient. An X-ray panorex was taken at day 0.

The patient was checked two weeks after dental implant placement and sutures removed where possible. At this time soft tissues were evaluated for bleeding, suppuration, inflammation, and probing depths; there was no attempt to check screw tightness. The patient was reassessed three months post-placement for a maintenance appointment and a panorex X-ray. Patient occlusion was measured and adjusted where necessary. In addition,

during the examination, the soft-tissue texture, color, and amount of keratinized tissue was checked. This type of exam and maintenance interval was repeated at six and twelve months.

Clinical and radiographic evaluation:

The patient was recorded for the following :

- biologic or prosthodontic complications;
- pocket probing depth measured at the mesial, buccal, distal and lingual/palatal sites using a periodontal pressure-calibrated (0,25N) plastic probe (TPS probe, Ivoclar Vivadent);
- each panorex at 0, 3, 6 and 12 months was taken for evaluation.

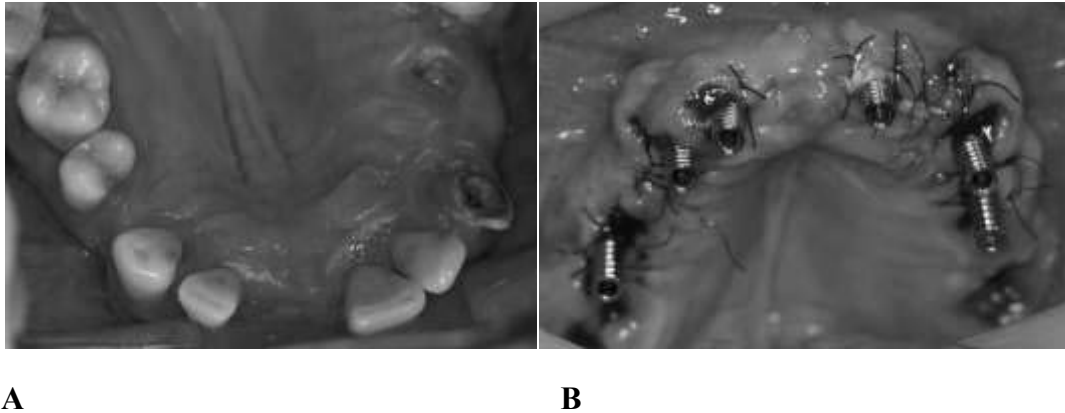


Fig. 1. *A) Pre-operative clinical view. B) Titanium abutments tightened over implants.*

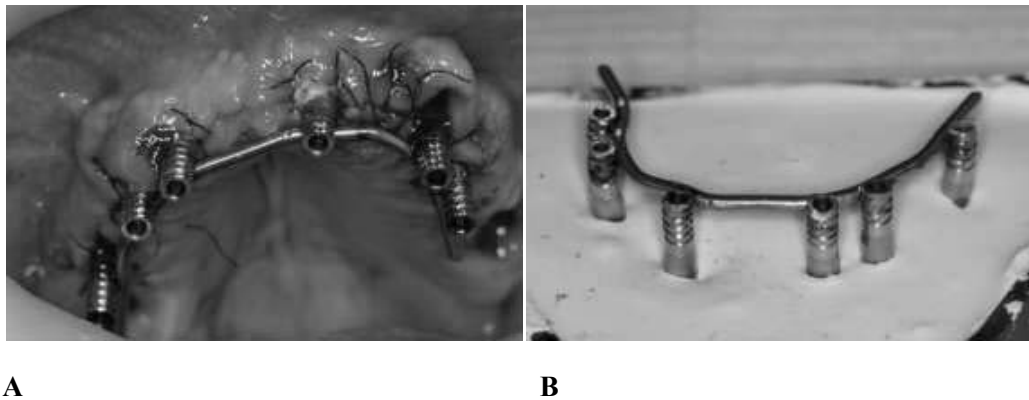


Fig. 2. *A) Titanium bar welded to the abutments. B) Titanium framework with abutments reduced in height.*

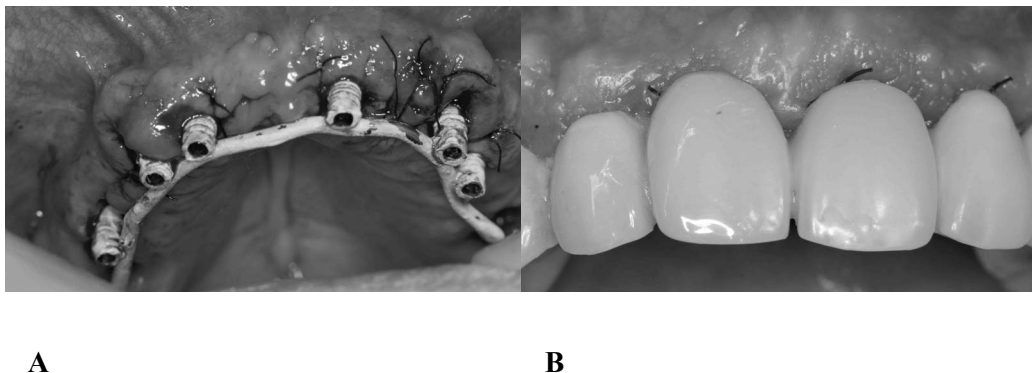


Fig. 3. *A) Titanium framework passivity checked over the implants. B) Completed restoration tightened over implants.*

DISCUSSION

Marginal soft tissue adaptation plays a key role in establishing a physical seal between the oral environment and the bone-surrounded implant which, in turn, is expected to prevent microbial organisms and contaminated products from the oral cavity reaching the underlying bone. Limiting migration of bacteria and bacterial products along the implant surface reduces the risk for peri-implantitis and/or implant failure. Additionally, a stable peri-implant soft tissue attachment, in the presence of bone support, has a critical impact on the long-term esthetic outcomes of implant therapy. It was suggested that the biologic width plays an important role in both achieving and maintaining desired soft tissue contour around dental implants.

To evaluate the influence of the configuration of the marginal aspect of implants placed immediately into extraction sockets on peri-implant hard tissue adaptation, a “platform switching” concept was introduced (17). This concept includes the use of an abutment having a diameter smaller than that of the implant platform in order to preserve the marginal bony crest in a more coronal level compared to the situation in which identical diameter is applied for the implant and the abutment (18-19). However, the distance of the implant surface from the outer contour of the bony ridge may be an important aspect that affects buccal bony crest resorption. To support this statement, we compared the healing of implants with a diameter similar to the dimension of the alveolus, installed immediately after tooth extraction, to smaller diameter implants (15). After 4 months of healing, less supra-crestal exposure of the buccal implant surface was observed with smaller implants compared to the larger implants. The PRAMA design follows the “platform switching” concept. The use of implants with a reduced diameter in coronal aspect may contribute to preservation of the buccal bony crest in a more coronal level compared with conventional implants (7).

In conclusion, the use of trans-mucosal implants, in addition to the rigid immobilization splinting necessary to increase the stability of the implants after surgery, creates a thick gingival tissue around

implants, and a shift of the inflammatory cell infiltrate to the central axis implant and away from the crestal bone level. The creation of this mechanical barrier serving as a defense mechanism prevents bacteria penetration from the oral environment and is a viable technique to reduce bone resorption. Within the limits of this clinical case report, the clinical use of biologically oriented preparation technique (BOPT) using Prama implants in addition to the intraoral welding technique reduces marginal crestal bone loss (MBL) and gingival/periodontal status of trans-mucosal implants.

REFERENCES

1. Brånemark P-I, Zarb GA, Albrektsson T. Tissue integrated prosthesis: osseointegration in clinical dentistry. Chicago, IL: Quintessence 1985; 11-61.
2. Brånemark PI, Hansson BO, Adell R, Ohman A. Osseointegrated implants in the treatment of the edentulous jaw. Experience from a 10-year period. *Scand J Plast Rec Surg* 1977; 16:1-32.
3. Lindhe J, Berglundh T. The interface between the mucosa and the implant. *Periodontol* 2000 1998; 17:47-54.
4. Berglundh T, Abrahamsson I, Welander M, Lang NP, Lindhe J. Morphogenesis of the peri-implant mucosa: an experimental study in dogs. *Clin Oral Implants Res* 2007; 18:1-8.
5. Cochran DL, Hermann JS, Schenk RK, Buser D. Biologic width around titanium implants. A histometric analysis of the implanto-gingival junction around unloaded and loaded non submerged implants in the canine mandible. *J Periodontol* 1997; 68:186-98.
6. Hermann JS, Buser D, Schenk RK, Higginbottom FL, Cochran DL. Biologic width around titanium implants. A physiologically stable dimension. *Clin Oral Impl Res* 2000; 11:1-11.
7. Bressan E, Lang NP, Corazza B, Ricci S, Botticelli D. The platform switching concept revisited. An experimental study in dogs. *Clin Oral Implants Res* 2014; 25:1200-6.
8. Degidi M, Nardi D, Piattelli A. A six-year follow-up of full-arch immediate restorations fabricated with an intraoral welding technique. *Implant Dentistry* 2013; 22:3.

9. Degidi M, Nardi D, Piattelli A. Immediate loading of the edentulous maxilla with a final restoration supported by an intraoral welded titanium bar: a case series of 20 consecutive cases. *J Periodontol* 2008; 79:2207-13.
10. Loi I, Di Felice A. Tecnica di preparazione biologicamente orientata (BOPT). Un nuovo approccio per il restauro protesico di denti parodontalmente sani. *Eur J of Esthetic Dent* 2013; 8:8-21.
11. Todescan S, Lavigne S, Kelekis-Cholakias A. Guidance for the maintenance care of dental implants: clinical review. *J Can Dent Assoc* 2012; 78:107-11.
12. Lazzara R, Porter SS. Platform switching: a new concept in implant dentistry for controlling post restorative crestal bone levels. *Int J Perio Rest Dent* 2006; 26:9-17.
13. Canullo L, fedele GR Jepsen S. Platform switching and marginal bone level alterations: the results of a randomized clinical trial. *Clin Oral Impl Res* 2010; 21:115-21.
14. Bressan E, Lang N, Botticelli D. The platform switching concept revisited. An experimental study on dogs. *Clin Oral Impl Res* 2014; 25:1200-06.
15. Caneva M, Salata LA, Botticelli D, Lang N. Hard tissue formation adjacent to implants of various size and configurations immediately placed into extraction sockets. An experimental study in dogs. *Clin Oral Impl Res* 2010; 21:885-90.
16. Caneva M, Botticelli D, Lang N. Influence of implants with different sizes and configurations immediately placed into extraction sockets on peri-implant hard and soft tissues. An experimental study in dogs. *Clin Oral Impl Res* 2012; 23:396-01.
17. Baffone GM, Botticelli D, Canullo L, Lang N. Effect of mismatching abutments on implants wider platforms. An experimental study in dogs. *Clin Oral Impl Res* 2012; 23:334-39.
18. Ledermann PD, Schenk RK, Buser D. Long-lasting osseointegration of immediately loaded, bar-connected TPS screws after 12 years of function: a histologic case report of a 95-year-old patient. *Int J Perio Rest Dent* 1998; 18:552-63.
19. Weng D, Nagata MJH, Bell M, Bosco AF, de Melo LGN, Richter EJ. Influence of microgap location and configuration on the peri-implant bone morphology in submerged implants. An experimental study in dogs. *Clin Oral Impl Res* 2008; 19:1141-47.

LETTER TO THE EDITOR

ALTERED DIURNAL CARDIAC SYMPATHOVAGAL BALANCE CORRELATES WITH NUTRITIONAL STATUS INDEXES IN SYSTEMIC SCLEROSIS PATIENTS

E. ROSATO¹, A. GIGANTE¹, M. LIBERATORI², ML. GASPERINI¹, B. BARBANO¹,
A. AMOROSO¹, L. TUBANI² and A. LAVIANO²

¹*Department of Clinical Medicine, Scleroderma Unit, Sapienza University of Rome, Italy;*

²*Department of Clinical Medicine, Sapienza University of Rome, Rome, Italy*

Received July 18, 2017 – Accepted November 2, 2017

Autonomic nervous system is involved in body weight regulation. Gastrointestinal manifestations of systemic sclerosis (SSc) can influence patients' nutritional status and facilitate the development of protein–energy malnutrition. The aim of the study is to assess the nutritional status of SSc patients and to explore its possible correlation with autonomic dysfunction using heart rate variability (HRV). We enrolled 19 SSc subjects and 19 healthy subjects as controls. Body mass index (BMI) and body surface area (BSA) were collected and recorded in all patients. HRV was measured and the domains of low frequencies (LF, index of the sympathetic modulation) and high frequencies (HF, index of the parasympathetic modulation) were recorded. As assessed by the LF/HF RATIO, sympathovagal balance is altered in SSc patients because of increased sympathetic modulation and reduced parasympathetic activity. BMI positively correlates with LF ($r=0.57$; $p < 0.01$) and LF/HF RATIO during daytime ($r=0.46$; $p < 0.05$). Similarly, BSA positively correlates with LF ($r=0.51$; $p < 0.05$), LF day time ($r=0.53$; $p < 0.05$) and LF/HF RATIO night time ($r=-0.51$; $p < 0.05$). In SSc patients the autonomic dysfunction is characterized by increased sympathetic modulation. We observed a correlation between autonomic dysfunction and nutritional status in SSc patients.

To the Editor,

Systemic sclerosis (SSc) is a connective tissue disease affecting the skin and internal organs and characterized by fibrosis (1). Some features and complications of SSc, such as microcirculatory impairment, cardiovascular abnormalities, impaired esophageal motility and gastrointestinal dysfunction, might be partly explained by autonomic dysfunction (AD) (2). AD is common in SSc and it is characterized by parasympathetic impairment and sympathetic hyperactivity, starting early in the disease process and possibly preceding the development of fibrosis (3, 4). The autonomic nervous system, and particularly its

sympathetic component, is involved in body weight regulation by modulating energy expenditure (5). Thus, impaired body weight regulation may be triggered and maintained, at least in part, by the AD, as well as in the clinical course of chronic diseases when it may lead to obesity or undernutrition (6). Protein-energy malnutrition is frequently observed in SSc patients, with prevalence ranging from 15% to 30% (7). In healthy individuals nutritional status as measured by body mass index (BMI) correlates with autonomic nervous system activity (6). Moreover, in SSc patients we recently demonstrated that BMI correlates with left ventricular mass, as well as markers of fibrosis

Key words: systemic sclerosis, heart rate variability, autonomic dysfunction, nutrition, malnutrition

Mailing address:

Antonietta Gigante, MD
Sapienza University of Rome,
Department of Clinical Medicine, Scleroderma Unit,
Viale dell'Università 37, 00185 Rome, Italy
Tel./Fax: +39 06 49970308
e-mail: antonietta_gigante@yahoo.it

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

(8). Interestingly, the heart is largely innervated by sympathetic and parasympathetic fibers, influencing cardiac metabolism and function (9).

The aim of the study is to evaluate the impact of AD, assessed by heart rate variability (HRV), in nutritional status of SSc patients in respect to healthy controls. We also evaluated the difference of nutritional status and autonomic dysfunction between SSc and healthy controls.

MATERIALS AND METHODS

Nineteen SSc patients (14 females and 5 males) were enrolled in the study. All patients met the American College of Rheumatology/European League Against Rheumatism Collaborative Initiative criteria for the classification of SSc (10). Median age was 45 years (range 23–58) and the median SSc duration was 6.5 years (range 2–25 years). Six patients had limited cutaneous SSc and 13 had diffuse cutaneous SSc, as defined by LeRoy et al. (1). Nineteen clinically healthy subjects, 12 female and 7 male, served as controls. Their median age was 48 years (range 42–65). Also 19 healthy controls matched for sex and age were enrolled in the study.

SSc patients with cardiac and pulmonary abnormalities were excluded. Patients with involuntary weight loss in the previous months, diabetes mellitus, renal failure, hepatic or thyroid dysfunction and anemia or administered with beta-blockers, anti-arrhythmic drugs, ACE-inhibitors, or angiotensin receptor antagonists were excluded.

Patients enrolled in the study underwent 24-h continuous ECG monitoring according to Holter in order to measure autonomic nervous system activity. Transthoracic echocardiography was also performed for all patients.

The protocol was designed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of our Institution, and the patients gave written informed consent.

Anthropometry

Body weight to the nearest 0.1 Kg and height to the nearest cm were collected using calibrated scale and stadiometer. For each patient we calculated body mass index (kg/m^2). Also, body surface area (BSA; m^2) was calculated according to the formula $0.20247 \times \text{height (m)}^{0.725} \times \text{weight (kg)}^{0.425}$ (11).

Heart rate variability

Autonomic nervous system activity was evaluated by HRV analysis during 24-h ECG recording. Autonomic nervous activity was analyzed following the recommendations of the Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology (12). The standard deviation of normal-to-normal RR intervals (SDNN) (ms) and the square root of the mean of the sum of the squares of differences between adjacent NN intervals (RMSSD) represent markers of global sympathetic and parasympathetic system, respectively. In the frequency domain, Fast Fourier Transform was used to obtain power spectral estimates of HRV. Total power in the frequency range (0–0.40 Hz) was divided into low frequencies (LF: 0.04–0.15 Hz, modulated mainly by sympathetic system) and high frequencies (HF: 0.15–0.40 Hz, modulated by parasympathetic system). The rate LF/HF RATIO represents sympathovagal balance. Subjects were analyzed for 24 h, at 10 min intervals. Artificial data and arrhythmic periods were excluded. The 24-h period was divided into two periods: night (0 am–6 am) and day (7 am–9 pm). Data analyses were performed with software Del Mar Avionics Accuplus 363 (Irvine, California, USA).

Statistical analysis

Commercial software (SPSS version 20.0, Bologna, Italy) was used for statistical analysis. Data are expressed as median and range. All data were tested to see if assumptions of normality held true. The assumptions of normality could not be assumed within the data set mainly because of the small sample size. Therefore non-parametric tests were used. Multivariate analysis was applied for the estimation of relationship of nutritional variables with HRV or disease variables. The Mann–Whitney U-test or Kruskal–Wallis were used to test differences between the two study groups. Spearman's rank order correlation coefficient (r) was used to test for an association between numerical variables. P-values <0.05 were considered significant.

RESULTS

Anthropometry

Median BMI of SSc patients was 22 (range: 19–24) and was not significantly different from that of

healthy controls (23; range: 20-25). No significant difference ($p > 0.05$) of BSA was observed between SSc and healthy controls [1.59 m^2 (range: 1.30-2.16) vs 1.68 (1.37-2.25)].

Heart rate variability

No arrhythmia was observed during ECG recordings either in SSc patients or in healthy controls. HRV results of SSc patients and controls are summarized in Table I. The median value of SDNN was significantly lower ($p < 0.05$) in SSc patients than healthy controls [136.5 (103.3-232.5) vs 120.2 (86.8-161.6)]. No significant difference ($p > 0.05$) of RMSSD was observed between SSc patients and healthy controls.

During daytime, sympathetic activity of SSc patients was significantly reduced when compared to controls, as revealed by lower levels of LF normalized units (nu) (57.12 vs 65.21 , respectively; $p = 0.01$). However, the sympathovagal balance, as expressed by LF/HF RATIO, during daytime was not different from that of controls. In contrast, LF/HF RATIO during nighttime was significantly increased in SSc patients vs controls (2.26 vs 0.96 , respectively; $p < 0.0001$), due to a marked increase of LF nu (55.39 vs 44.58 , respectively; $p < 0.0001$) and a marked decrease of HF nu (31.31 vs 52.44 , respectively; $p < 0.0001$). Therefore, the physiological circadian rhythm of sympathovagal balance was disrupted in SSc patients. In fact, parasympathetic activity prevails in controls during nighttime, whereas sympathetic activity prevails in SSc patients.

HRV and nutritional variables in SSc patients

In SSc patients, 24 hours sympathetic activity, as expressed by LF nu, and the sympathovagal balance during day time, as expressed by LF/HF RATIO day, were significantly and positively correlated with patients' BMI ($r = 0.57$, $p < 0.01$; and $r = 0.46$, $p < 0.05$; respectively) (Fig. 1). Similarly, 24-hr and daytime sympathetic activity, as expressed by LF nu and LF nu day, and the sympathovagal balance during nighttime, as expressed by LF/HF RATIO night, positively correlated with patients' BSA ($r = 0.51$, $p < 0.05$; $r = 0.53$, $p < 0.05$; and $r = 0.51$, $p < 0.05$; respectively) (Fig. 2). We did not observe any

correlation between BMI and HRV parameters of healthy controls.

DISCUSSION

Our study confirms that the autonomic nervous system is pathologically perturbed in SSc patients.

In the literature there are few reports regarding the autonomic nervous system and BMI. The studies were performed on healthy population (6, 13), Finnish employees (14), Parkinson's disease (15) and in metabolic syndrome (16). Most of the authors conclude that specially sympathetic component of the autonomic nervous system is involved in body weight regulation by modulating energy expenditure both in normal and in obese patients (6).

In particular, we observed reduced global sympathetic activity in SSc patients, as revealed by lower values of SDNN, when compared to healthy controls. In contrast, overall parasympathetic activity in SSc patients, as assessed by RMSSD, was not significantly different from controls. Overall sympathovagal balance was significantly increased in SSc patients when compared to controls due to the reduction of 24 hours parasympathetic activity. The further analysis of the daytime and nighttime behavior of HRV time domains revealed the complexity of the AD in SSc patients. In fact, LF resulted significantly increased during nighttime and reduced during daytime. In contrast, HF was significantly reduced only during nighttime. Therefore, in SSc patients we observed the loss of the autonomic circadian rhythm, which is characterized by the pathological dominance of sympathetic activity during nighttime due to the lack of the physiological increase of parasympathetic activity.

The nutritional status of SSc patients and healthy controls, as assessed by BMI, was not different. However, their sympathovagal balance was significantly different. Although this evidence may suggest that autonomic function has a minimal role in influencing nutritional status, it should be acknowledged that we did not assess the patients' and controls' body composition, which could have revealed significant differences. Recently, Monteze et al. showed that visceral fat area, as derived

Table I. Autonomic function variables of SSc patients and controls.

	Control group (n=19)		SSc patients (n=19)		p value
	Median	Range	Median	Range	
LF/HF ratio	1.91	1.12-3.79	2.71	2.01-4.65	0.041
LF (nu)	59.56	45.01-73.53	56.91	51.03-69.83	0.90
HF (nu)	34.72	24.21-43.9	28.53	17.49-35.79	0.03
LF/HF day	2.68	1.36-5.25	2.71	1.95-4.9	0.77
LF day (nu)	65.21	47.04-79.13	57.12	50.52-70.28	0.01
HF day (nu)	28.12	14.78-40.06	30.12	16.45-34.42	0.64
LF/HF night	0.96	0.5-1.54	2.26	1.32-4.54	<0.0001
LF night (nu)	44.58	31.06-55.94	55.39	46.75-74.3	<0.0001
HF night (nu)	52.44	37.25-65.06	31.31	19.3-39.81	<0.0001
SDNN (ms)	136.5	103.3-232.5	120.2	86.8-161.6	0.04
RMSSD (ms)	38.00	19.9-50.6	40.0	15.2-73.7	0.15
HR mean (bpm)	79.48	64.3-88.71	81.90	63-90.6	0.33

$P < 0.05$ was chosen to be the threshold of statistical significance.

LF: low frequency, HF: high frequency, LF/HF ratio: low frequency/high frequency, SDNN: standard deviation of all sinus rhythm RR intervals, RMSSD: square root of the mean of the sum of the squares of differences between adjacent NN intervals, HR: heart rate, night time= 0–6am, day= 7 am–9 pm

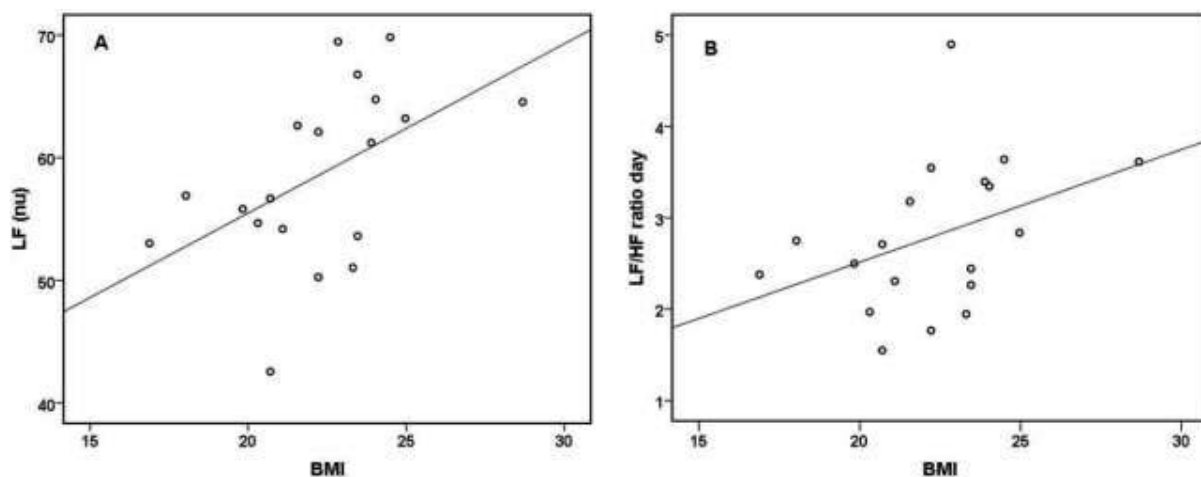


Fig. 1. *A)* Correlation between body mass index (BMI) and low frequencies (LF) in normalized units (nu); *B)* Low frequencies/high frequencies (LF/HF ratio) daytime in SSc patients.

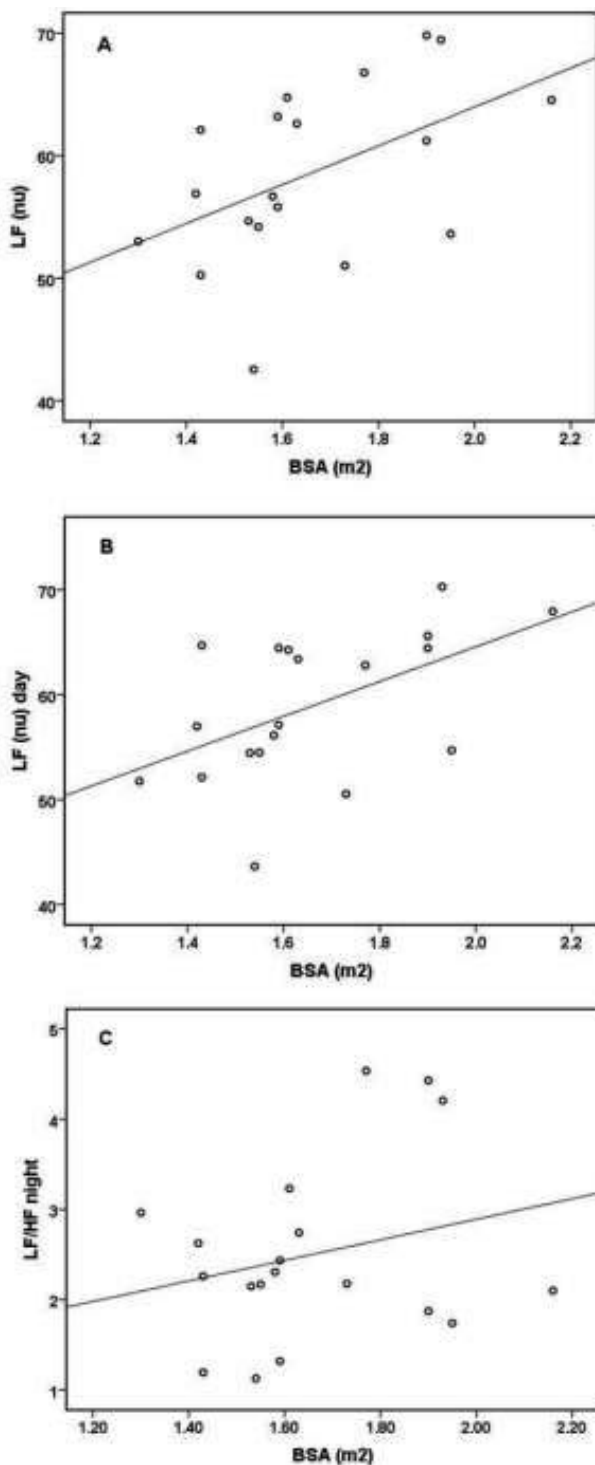


Fig. 2. *A) Correlation between body surface area (BSA) and low frequencies (LF) in normalized units (nu); B) Low frequencies (LF) in normalized units (nu) day time; C) low frequencies/high frequencies (LF/HF ratio) night time.*

from anthropometric measurements, is negatively associated to HRV components in shift workers (17). Also, Koenig et al. studied 8,538 healthy individuals and showed that HRV is related to anthropometry-derived measures of adiposity (18). In contrast, Izumi et al. could not find any association between HRV and DXA-measured changes in body composition in post-partum women (19). Therefore, the issue of whether HRV could reflect changes in fat mass and/or muscle mass remains to be ascertained by studies involving imaging tools. We previously demonstrated that under healthy conditions increased BMI is characterized by increased sympathetic and lower parasympathetic activities (6). We here report in SSc patients a marginal reduction of overall sympathetic modulation, which does not appear to influence the correlation between the size of SSc patients, as measured by BMI or BSA, and LF. However, we observed a substantial increase of sympathetic modulation during nighttime that was paralleled by a similar robust reduction of parasympathetic modulation. It could be speculated that the AD of SSc patients is characterized by preserved increased sympathetic modulation and energy expenditure as BMI increases, which is not counteracted by enhanced parasympathetic modulation, which actually significantly and pathologically decreases during nighttime. Considering that increased parasympathetic activity is associated with enhanced fat deposition (20), it appears that SSc patients are more prone to malnutrition, since they cannot resist weight loss due to limited ability to deposit fat. The clinical relevance of the present study is also revealed by the evidence that the loss of the circadian rhythm with the non-physiological reduction of the parasympathetic component HF at night promotes a constant sympathetic hyperactivity, resulting in increased cardiovascular risk (21). The main limitation of this study is the small sample size. Future larger studies are needed to evaluate the relationship between nutritional status and autonomic dysfunction.

In SSc patients, the autonomic dysfunction is characterized by increased sympathetic modulation. We observed a correlation between autonomic dysfunction and nutritional status in SSc patients.

REFERENCES

1. LeRoy EC, Black C, Fleischmajer R, et al F. Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. *J Rheumatol* 1988; 15:202-5.
2. Di Franco M, Paradiso M, Riccieri V, Basili S, Mammarella A, Valesini G. Autonomic dysfunction and microvascular damage in systemic sclerosis. *Clin Rheumatol* 2007; 26:1278-83.
3. Dessein PH, Joffe BI, Metz RM, Millar DL, Lawson M, Stanwix AE. Autonomic dysfunction in systemic sclerosis: sympathetic overactivity and instability. *Am J Med* 1992; 93:143-50.
4. Gigante A, Rosato E, Liberatori M, et al. Autonomic dysfunction in patients with systemic sclerosis: correlation with intrarenal arterial stiffness. *Int J Cardiol* 2014; 177:578-80.
5. Berthoud HR. The vagus nerve, food intake and obesity. *Regul Pept* 2008; 149:15-25.
6. Molino A, Fiorentini A, Tubani L, Martuscelli M, Rossi Fanelli F, Laviano A. Body mass index is related to autonomic nervous system activity as measured by heart rate variability. *Eur J Clin Nutr* 2009; 63:1263-65.
7. Murtaugh MA, Frech TM. Nutritional status and gastrointestinal symptoms in systemic sclerosis patients. *Clin Nutr* 2013; 32:130-35.
8. Rosato E, Gigante A, Gasperini ML, et al. Nutritional status measured by BMI is impaired and correlates with left ventricular mass in patients with systemic sclerosis. *Nutrition* 2014; 30:204-9.
9. Schwartz PJ, De Ferrari GM. Sympathetic-parasympathetic interaction in health and disease: abnormalities and relevance in heart failure. *Heart Fail Rev* 2011; 16:101-7.
10. van den Hoogen F, Khanna D, Fransen J, et al. 2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European League against Rheumatism collaborative initiative. *Arthritis Rheum* 2013; 65:2737-47.
11. Du Bois D, Du Bois EF. A formula to estimate the approximate surface area if height and weight be known. *Arch Intern Med* 1916; 17:863-71.
12. Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. Heart rate variability: standards of measurement, physiological interpretation and clinical use. *Circulation* 1996; 93:1043-65.
13. Koenig J, Jarczok MN, Warth M, Ellis RJ, Bach C, Hillecke TK, Thayer JF. Body mass index is related to autonomic nervous system activity as measured by heart rate variability--a replication using short term measurements. *J Nutr Health Aging* 2014; 18:300-2.
14. Föhr T, Pietilä J, Helander E, Myllymäki T, Lindholm H, Rusko H, Kujala UM. Physical activity, body mass index and heart rate variability-based stress and recovery in 16,275 Finnish employees: a cross-sectional study. *BMC Public Health* 2016; 16:701.
15. Mochizuki H, Taniguchi A, Nakazato Y, et al. Increased body mass index associated with autonomic dysfunction in Parkinson's disease. *Parkinsonism Relat Disord* 2016; 24:129-31.
16. Boudet G, Walther G, Courteix D, et al. Paradoxical dissociation between heart rate and heart rate variability following different modalities of exercise in individuals with metabolic syndrome: The RESOLVE study. *Eur J Prev Cardiol* 2017; 24:281-96.
17. Monteze NM, Souza BB, Alves HJ, et al. Heart rate variability in shift workers: responses to orthostatism and relationships with anthropometry, body composition, and blood pressure. *Biomed Res Int* 2015; 2015:329057.
18. Koenig J, Windham BG, Ferrucci L, et al. Association strength of three adiposity measures with autonomic nervous system function in apparently healthy employees. *J Nutr Health Aging* 2015; 19:879-82.
19. Izumi M, Manabe E, Uematsu S, Watanabe A, Moritani T. Changes in autonomic nervous system activity, body weight, and percentage fat mass in the first year postpartum and factors regulating the return to pre-pregnancy weight. *J Physiol Anthropol* 2016; 35:26.
20. Balbo SL, Grassioli S, Ribeiro RA, et al. Fat storage is partially dependent on vagal activity and insulin secretion of hypothalamic obese rat. *Endocrine* 2007; 31:142-48.
21. Grassi G, Seravalle G. Autonomic imbalance and metabolic syndrome: unravelling interactions, mechanisms and outcomes. *J Hypertens* 2006; 24:47-49.

LETTER TO THE EDITOR

SAFETY AND TOLERABILITY OF INTRA-ARTICULAR HYALURONIC ACID (SINOVIAL®/GELSYN-3™) INJECTIONS IN THE TREATMENT OF KNEE OSTEOARTHRITIS

M. ABATE and V. SALINI

*Department of Medicine and Science of Aging, University G. d'Annunzio, Chieti-Pescara, Chieti, Italy**Received June 12, 2017 – Accepted November 15, 2017*

Osteoarthritis (OA) is a progressively degenerative joint disease, with a very high prevalence rate that is expected to increase worldwide with the ageing of the population. Considering that OA requires long-term treatment, therapies with minimal side effects and which can be repeated as needed are warranted. Hyaluronic acid (HA), a natural glycosaminoglycan with viscoelastic properties, is a major component of synovial fluid and the extracellular matrix of the joint cartilage, and plays key roles in maintaining synovial fluid viscosity and the bio-mechanical integrity of healthy cartilage. Intra-articular administration of exogenous HA has therefore been used to successfully improve the viscoelastic properties of the joint to improve lubrication, modulate inflammation and modify the catabolic micro-environment. Sinovial®/GELSYN-3™ is a sterile, non-pyrogenic formulation of highly purified, chemically unmodified HA of bio-fermentative origin, which has been introduced in several different concentrations in clinical use within the European market. This expert opinion reports on the published data regarding the efficacy and tolerability of first and multiple injection series of Sinovial®-based product formulations. The data regarding the tolerability of Sinovial® in patients with knee osteoarthritis were analyzed, showing that this formulation, beside favourable therapeutic effects, has a very good tolerability profile, with only mild, transient, and easily managed, local injection-site reactions and absence of systemic reactions. In particular, repetitive cycles of HA have been shown to yield positive results in terms of both efficacy and safety and therefore should be offered to patients who had undergone a successful first course of therapy when their symptoms reoccur.

To the Editor,

Osteoarthritis (OA) is a progressively degenerative joint disease. The prevailing paradigm suggests that OA results from an impaired regenerative ability of the damaged cartilage due to bio-mechanical and biochemical changes. The clinical signs associated with these changes include pain, stiffness, and decreased functionality, which may compromise overall health and quality of life, leading to disability (1).

The prevalence of OA is very high, expected

to affect more than 50 million subjects in the US by 2020, and will increase with the ageing of the population. According to the American College of Rheumatology, nearly 70% of people aged over 70 years have X-ray evidence of OA, although only half ever develop symptoms (2).

While several pharmaceutical approaches to managing the symptoms of OA exist (i.e. analgesic treatment with non-steroidal anti-inflammatory drugs, COX-2 inhibitors and steroids), many of

*Key words: hyaluronic acid, knee, osteoarthritis, Sinovial**Mailing address:*

Dr. Michele Abate,
Department of Medicine and Science of Aging,
University G. d'Annunzio, Chieti-Pescara,
Via dei Vestini 31, 66013 Chieti Scalo (CH), Italy
Tel.: +39 0871 35 82 63 - Fax: +39 0871 35 89 69
e-mail: m.abate@unich.it

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.
Unauthorized reproduction may result in financial and other penalties

these agents carry known systemic risks, with the incidence of adverse reactions increasing with age (3). Furthermore, intra-articular steroids must be used sparingly, with dosing every 3-4 months due to the potential for soft tissue damage (4).

Considering the limits of these treatments, therapies with minimal side effects and the possibility to be repeated as needed are therefore warranted. One such therapy is viscosupplementation, wherein exogenous hyaluronic acid (HA) is injected into the affected joint. During the progression of OA, synthetic levels and concentrations of HA in synovial fluid are reduced. Administration of exogenous HA has therefore been used to restore the viscoelastic properties of the joint, which aids in improving lubrication, modulating inflammation and modifying the catabolic micro-environment (5). Moreover, HA is recommended in patients with intolerance or contraindications to other pharmacological treatments and in patients who are unable to elect surgery.

Recent data have suggested that repeated use of HAs may help to delay the need for total knee replacement (TKR) for up to 3.6 years (6). Several authors found that the time to TKR is proportionate to the number of HA injection series, with ensuing economic benefits and improvement in the quality of life (7).

This review reports on published data regarding the safety and tolerability of first and repeated intra-articular injections with HA in knee OA, focusing on Sinovial®-based product formulations (IBSA, Institut Biochimique SA, Lugano, Switzerland). Sinovial®, branded GELSYN-3™ (Bioventus LLC, Durham, North Carolina, USA) for the US market, is FDA approved based on a single course of therapy.

The pathogenesis of OA and the mechanisms of action of HA, which are widely addressed in the literature (5), are beyond the scope of the present paper.

MATERIALS AND METHODS

Data sources, search strategy and inclusion criteria

We performed a systematic electronic literature search in PubMed, Web of Knowledge and EMBASE using the terms Sinovial or hyaluronic acid or hyaluronan, combined with intra-articular or osteoarthritis or knee. Trials, selected manually and supplemented by searches of the bibliographies of articles, were deemed relevant if they contained original research providing relevant knowledge related to this topic. All searches were limited to human studies; all randomized controlled trials and observational

Table I. Sinovial product family dosing regimen.

Product	Initial (n of injections)	Repeat interval	Repeat Product	Repeat Course (n of injections)	Concentration/volume		
					First course	Repeat course	Total
Sinovial®	Once weekly (3 injections)	6 months	Sinovial®	Once weekly (3 injections)	48 mg/6 mL	48 mg/6 mL	96 mg/12 mL
Sinovial®	Once weekly (3 injections)	6 months	Sinovial® Forte	Once weekly (3 injections)	48 mg/6 mL	96 mg/6 mL	144 mg/12 mL
Sinovial® Forte	Once weekly (3 injections)	6 months	Sinovial® Forte	Once weekly (3 injections)	96 mg/6 mL	96 mg/6 mL	192 mg/12 mL
Sinovial® Forte	Once weekly (3 injections)	4 months	Sinovial® One	Once every 4 months (3 injections)	96 mg/6 mL	150 mg/7.5 mL	246 mg/13.5 mL
Sinovial® One	1 injection	4 months	Sinovial® One	Once every 4 months (1 injection)	50 mg/2.5 mL	50 mg/2.5 mL	100 mg/5 mL
Sinovial® HL	Once weekly (3 injections)	6 months	Sinovial® HL	Once weekly (3 injections)	192 mg/6 mL	192 mg/6 mL	384 mg/12 mL

studies which reported the efficacy and tolerability for intra-articular injection of Sinovial[®] in knee OA patients were included.

Data extraction and outcome measures

From each publication, relevant study details (design, inclusion and exclusion criteria, number of injections, treatment duration, follow-up times), patient characteristics (number, demographic and clinical data), and efficacy outcomes were extracted. The overall incidence of adverse events (AEs) (reported separately by type and severity), after the first and multiple courses of Sinovial[®] injections, were examined and collated.

Product overview

Sinovial[®] is a sterile, non-pyrogenic, sodium chloride (phosphate buffered) solution of highly purified, chemically un-modified HA of bio-fermentative origin (*Streptococci* of Lancefield groups A and C). It is a linear polymer composed of the disaccharide units N-acetyl-D-glucosamine and sodium-D-glucuronate, linked by glycosidic bonds, with a mean molecular weight (MW) of 800-1200 kDa. Sinovial[®] is available in pre-filled, ready to use syringes for injection into large or small joints.

There are several different concentrations of the product in clinical use within the European market, including Sinovial[®] Mini (8 mg/1 mL), Sinovial[®] (16 mg/2 mL; branded GELSYN-3[®] for US market), Sinovial[®] Forte (32 mg/2 mL), Sinovial[®] One (50 mg/2.5 mL). Moreover, a new formulation, Sinovial[®] HL (64 mg/2 mL), composed of both low molecular weight (80-100 kDa) and high molecular weight (1100-1400 kDa)

HA, has recently (2015) become available on the market.

Sinovial[®] Mini is a specific formulation mostly indicated in the treatment of OA of small joints (wrist, hand, foot, temporo-mandibular joint, etc.) and synovial sheaths (e.g. trigger finger), while the others are mainly used in the treatments of OA of large joints (hip, knee, ankle, shoulder, etc.).

The clinical dosing regimen may vary from a single dose (Sinovial[®] One), to two/three (Sinovial[®] Mini, Sinovial[®] HL, Sinovial[®] Forte), and three/five (Sinovial[®]/GELSYN-3TM) (once weekly) for each cycle of treatment. The interval between cycles is usually 4-6 months, but it can be modified by the treating physician on the basis of the specific clinical situation and individual experience (Table I).

RESULTS

Six clinical trials (two retrospective (8, 9), two open double-blind (10, 11) and two open observational (12, 13) studies) were performed on the efficacy and safety of Sinovial[®] product family in the management of knee OA. The design of the studies is reported in Table II.

These trials demonstrated that first and repetitive cycles of Sinovial[®] product family have been shown to yield positive results in terms of clinically relevant pain relief (reduced pain scores at VAS and WOMAC scales), improved joint mobility (Lequesne Index, WOMAC sub-scores and KOOS scale) and reduced use of rescue medication in patients affected by mild to moderate knee OA (Table III).

Table II. Sinovial treatment in knee OA (design of the studies).

Author (year) [ref]	Study type	N. of patients	K-L	HA	N. of injections	Total HA concentration and volume injected/patient	Study Duration
Castellacci (2004) [8]	Retrospective	40 28 M; 12 F	Primary and secondary OA I-IV	Sinovial [®]	5 once weekly	80 mg/10 mL	7 weeks
Depont (2006) [9]	Retrospective observational open label	408 146 M; 262 F	Primary and secondary OA	Sinovial [®]	1	16 mg/2 mL	4-12 months
Pavelka (2011) [10]	Multicenter, phase III, double blind, controlled, randomized, parallel-group, non-inferiority study	192* 188**	Primary OA II-III	Sinovial [®] Synvisc [®]	3 once weekly	48 mg/6 mL 48 mg/6 mL	26 weeks
Polacco (2013) [11]	Open label, double blind, single center study	21 8 M; 13 F	II-IV 16 II; 6 III; 2 IV	Sinovial [®] One (mepivacaine 2%)	1	50 mg/2.5 mL	4 months
Theiler (2005) [12]	Longitudinal, prospective, single group, open label observational	63 26 M; 37 F	Primary and secondary OA II-IV	Sinovial [®]	5 once weekly	80 mg/10 mL	24 weeks
Abate (2015) [13]	Open design, observational trial	15 6 M; 9 F	II-III	Sinovial [®] Forte + Sinovial [®] One	3 once weekly + 3 (one every 4 months)	246 mg/13.5 mL	14 months

* Patients treated with Sinovial[®] (53 M; 139F) ** Patients treated with Synvisc[®] (50 M; 138 F)

Table III. *Sinovial treatment in knee OA (efficacy and safety).*

Author (year) [ref]	VAS scores	WOMAC	WOMAC sub-scores	KOOS	Lequesne Index	Adverse events (AEs)
Castellacci (2004) [8]					From 7.9 to 3.2	40% mild
Depont (2006) [9]	64.5% patients reported subjectively an improved knee function, less pain and less need for pain medication. Successful injections were associated with recent OA onset (<5 years) and lower BMI.					4% mild
Pavelka (2011) [10]	From 64.5 to 26.9	From 55.7 to 22.7			From 11.5 to 7.6	0.5% mild 0.5% severe 0.5% serious
Polacco (2013) [11]	Reduced pain at rest and during activities	From 14.7 to 7.9 in 77.6% of the 24 treated knees				No
Theiler (2005) [12]		From 4 ± 1.9 to 2.4 ± 1.8	Reduced pain Improved stiffness and physical function			5.8%
Abate (2015) [13]	Reduced pain at rest and during activities			From 51.9 to 70.2	From 10 to 5.4	13% mild

In Pavelka's study [10] positive results were observed also in patients treated with Synvisc® (VAS: from 66.7 to 30.5; WOMAC: from 55.5 to 23.1; Lequesne: from 11.6 to 8). Mild, severe and serious AEs were observed in 2.1%, 3.2% and 2.1%, respectively (The authors did not declare in the paper the characteristics of these AEs, however stated that they were not treatment-related).

In Abate's study [13], NSAIDs consumption was reduced, and 73.3% (11/15) of patients reported a rating of "extremely/very satisfied" associated with their treatment.

Moreover, intra-articular injections of Sinovial® have demonstrated a remarkable tolerability profile, with a low incidence of AEs (Table III). Indeed, in all the trials performed, after the first and repeated course of injection, early mild AEs (pain, burning sensation, irritation or erythema at the injection site) were observed in a very low percentage of patients, which, when reported, ranged from 0.5% to 40% of cases. These AEs are common and known reactions to any intra-articular injections. Furthermore, those reported in these studies were short lasting and resolved spontaneously in a few hours post administration or in the following 2-3 days without any concomitant therapy (only RICE treatment). The overall tolerability was judged as "good" or "very good" by nearly all the patients (95%) and investigators (97.5%). None of the patients treated with Sinovial® injections discontinued the treatment due to an AE. All authors considered Sinovial® as safe and well tolerated with a low incidence of AEs

and that treatment did not interfere with the product's clinical performance.

Anecdotal Italian and European researchers' experiences

In our daily clinical practice, we have been using different formulations of Sinovial® for about six years in the management of hip, knee and ankle degenerative/overuse pathologies, performing roughly 150-200 injections of Sinovial®-based formulations per year. The type of Sinovial® used varies according to the subject's characteristics (age, BMI, activity) and severity of OA. Sinovial® or Sinovial® Forte is usually preferred in young patients with low OA degree (K-L I-II) and BMI <25. Sinovial® One or Sinovial® HL are mostly used in elderly and/or obese patients with K-L III-IV score or in subjects who are submitted to higher joint demands (heavy work, professional athletes) in order to exploit the shock absorption activity of HA.

The treatment regimen varies according to the joint to be injected. Specific to the knee, the first course of therapy for Sinovial[®], Sinovial[®] Forte, and Sinovial[®] HL is typically 3 injections spaced once a week; Sinovial[®] One is delivered as a single injection.

After 3 and 6 months, clinical (pain evaluation, swelling, use of painkillers, patients' satisfaction, etc.) and functional (ROM, muscular strength, function, etc.) parameters are evaluated and, on this basis, we judge whether the patient should be re-treated. Typically, in case of positive clinical results re-treatment is carried out approximately six months following the initial course of therapy. The interval between courses can be variable according to the patient's need (on demand).

Among the Sinovial[®] product family, Sinovial[®] Forte (at twice the HA concentration as compared to Sinovial) is mainly used (70% of patients) by our group, while Sinovial[®] One and Sinovial[®] HL are injected in a limited number of cases (25% and 5%, respectively).

Similar to other authors, we have observed mild AEs (pain, burning sensation, irritation or erythema at the injection site) after the first course of injections, but only in very few patients (2-3%). These events were short lasting and resolved spontaneously in a few days without any concomitant therapy. It must be underlined that the percentage of AEs was reduced (below 1%) after repeated courses.

During these years many patients have been treated with multiple repeated courses of the product, with up to 20 total injections of Sinovial[®]-based products with positive results in absence of AEs which may compromise the continuation of the treatment.

These anecdotal data (not supported by statistical analysis) are in line with those reported by several other European clinicians (Table IV) (collated based upon medical records review). In total, 2,475 patients (25-84 years) suffering from moderate-severe knee OA or chondropathy were treated with different products (Sinovial[®], Sinovial[®] One, Sinovial[®] Forte,

Table IV. Sinovial[®] treatment in knee OA (Italian and European experiences).

Name (Facility)	N. of patients	Initial Treatment Course		Subsequent Treatment Course		Total HA concentration and volume injected/patient	Observational period	AEs *
		HA	n. of injections	HA	n. of injections			
AP (Italy)	60 (25-80 ys)	Sinovial [®]	5 (one weekly)	Sinovial [®]	5 (one weekly) after 6 months	160 mg / 20 mL	9 months	No
ADV (Italy)	500 (> 60 ys)	Sinovial [®] Forte	3 (one weekly)	Sinovial [®] One	2 injections 2 weeks apart after 15 days from 3rd injection of Sinovial [®] Forte	196 mg / 11 mL	12 months	4% *
ADV (Italy)	50 (< 50 ys)	Sinovial [®] HL	2 (2 weeks apart)	Sinovial [®] HL	2 (2 weeks apart) after 4-6 months	256 mg / 8 mL	12 months	4% *
FM (Italy)	70 (40-80 ys)	Sinovial [®]	5 (one weekly)	Sinovial [®]	5 (one weekly) after 6 months	160 mg / 20 mL	10 months	No
LF (Italy)	734	Sinovial [®]	5 (one weekly)	Sinovial [®]	3 (spaced 1 month apart) after 5th injection of first course	128 mg / 16 mL	From January 2015 to May 2016	No
LT (Italy)	81	Sinovial [®] Forte	3 (one weekly)	Sinovial [®] Forte	3 (one weekly) after 6 months	192 mg / 12 mL	From April 2014 to April 2016	No
LT (Italy)	45	Sinovial [®] Forte	3 (one weekly)	Sinovial [®] Forte	3 (one weekly) after 6 months	192 mg / 12 mL	From April 2014 to April 2016	No
AP (Italy)	62 (44-84 ys)	Sinovial [®]	3 (one weekly)	Sinovial [®]	One injection/month for 12 months	240 mg / 30 mL	12 months	No
AR (Italy)	195	Sinovial [®] Forte	3 (one weekly)	Sinovial [®] One	One injection after 4 months from 3rd injection of Sinovial [®] Forte	146 mg / 8.5 mL	From June 2015 to June 2016	No
KP (Czech Rep)	208	Sinovial [®] or Sinovial [®] One	3 (one weekly) or 1	Sinovial [®] or Sinovial [®] One	3 (one weekly) or 1	96 mg / 12 ml or 100 mg / 5 mL	From January 2014 to December 2015	10-20% §
ES (Czech Rep)	314	Sinovial [®] or Sinovial [®] One	3 (one weekly) / 1	Sinovial [®] / Sinovial [®] One	3 (one weekly) / 1	96 mg / 12 ml or 100 mg / 5 mL	From May 2013 to April 2015	0.6% *

* Local pain in the area of the injection

§ slight pain or hematoma in the injection site

Sinovial[®] HL) and dose regimens (1 injection, 2-5 injections; single, one every 1 or 2 weeks). In all the reports available, subsequent injections were administered, ranging from one month up to thirteen months after the initial course of therapy. Total HA exposure per patient ranged from 96 mg (Table IV: PK and ES) to up to 256 mg (Table IV: ADV).

No AEs were observed after the first cycle of treatment or subsequent cycles, except for some mild post-injection pain for 3-4 days [which disappeared spontaneously in a few hours or in the following 2-3 days without any concomitant therapies (only RICE treatment)]. These AEs were experienced in subjects treated with Sinovial[®] One and Sinovial[®] HL, both of which are at higher total concentration of HA as compared to Sinovial/GELSYN-3[®].

Post-marketing surveillance information on Sinovial[®] product family

The Sinovial[®] (GELSYN-3[®] for US market) formulation was initially introduced onto the European market in November 2002. A recent analysis of post-marketing surveillance data (through December 2015) shows that there have been a total of 64 AEs (8 serious and 56 non-serious) reported to the manufacturer (14). Of the serious cases, only five were considered possibly related to the Sinovial[®] product; three events were associated with injection site reactions (swelling, inflammation or pain) and the other two involved myositis and septic arthritis (confirmed with a positive bacterial culture). Of the 56 non-serious events, approximately 50% (i.e., 29) were associated with injection site reactions and occurred either immediately or within 24 hours of the injection; in most cases, RICE therapy without a need for drugs or surgical intervention was required. The remaining reactions were mainly considered to be associated with the underlying disorder (e.g., arthritis) or an anticipated reaction due to the injection procedure (e.g., tachycardia triggered by anxiety arising from the injection).

Considering that approximately 5 million syringes of the Sinovial[®] 16mg concentration has been sold to date (15), the incidence of adverse reactions to the product is remarkably low (0.001%). Although within this data the proportion of patients receiving

repeated courses of therapy is not known, it is not unreasonable to assume that it is common practice across Europe to do so as outlined in the preceding sections. Thus, these data spanning 13 years supports a long-standing, safe clinical history of repeated use of Sinovial[®]/GELSYN-3[®].

DISCUSSION

According to published clinical trial data, Sinovial[®] product family is considered safe and has a remarkable tolerability profile with a low incidence of mild local AEs at the injection site. Such events are not clinically relevant, and are more likely associated with the procedure of any intra-articular injection itself rather than the product, with most AEs observed during the first course of treatment. Indeed, a similar incidence of this type of AEs has been observed in most studies with intra-articular injections, including the placebo groups (5).

Repeat treatment with a course of Sinovial[®]/GEL-SYN-3[®] would equate to 96 mg of sodium hyaluronate. As summarized in this report, repeated exposure up to 4-times this amount (i.e., 384 mg) of sodium hyaluronate has not given rise to any safety signals for Sinovial[®]-based formulations. Moreover, repeated administration of Sinovial[®]-based products is currently standard clinical use within Europe. It must be underlined that two clinical consortiums consisting of several experts in OA and viscosupplementation from Europe and US [European Society for Clinical and Economic Aspects of Osteoporosis, Osteoarthritis and Musculoskeletal Diseases (ESCEO) and European Viscosupplementation Consensus (EUROVISC) group] recommend, in a systemic literature review, that repeated treatments of HAs should be offered to patients who had undergone a successful first course of therapy when their symptoms reoccur.

Adverse events to Sinovial[®] are very low in patients who received both a single or subsequent courses of injections. The risk associated with a high frequency of Sinovial[®] use is not increased and therefore there is sufficiently valid scientific evidence to offer reasonable assurance of the safety of this product.

Declaration of conflict of interest

The preparation of this manuscript was conducted independently from the influence of the funding source. Both authors were involved in the preparation of this manuscript from its inception and approved the final version for submission. MA has received consulting fees or honorarium and support for travel to meetings from IBSA; VS has received consulting fees or honorarium and support for travel to meetings from IBSA.

REFERENCES

1. Abate M, Pulcini D, Di Iorio A, Schiavone C. Viscosupplementation with intra-articular hyaluronic acid for treatment of osteoarthritis in the elderly. *Curr Pharm Des* 2010; 16(6):631-40.
2. Altman R, Alarcón G, Appelrouth D, et al. The American College of Rheumatology criteria for the classification and reporting of osteoarthritis of the hip. *Arthritis Rheum* 1991; 34(5):505-14.
3. Griffin MR, Yared A, Ray WA. Nonsteroidal antiinflammatory drugs and acute renal failure in elderly persons. *Am J Epidemiol* 2000; 151(5):488-96.
4. Jüni P, Hari R, Rutjes AW, Fischer R, Silleta MG, Reichenbach S, da Costa BR. Intra-articular corticosteroid for knee osteoarthritis. *Cochrane Database Syst Rev* 2015; (10):CD005328.
5. Migliore A, Procopio S. Effectiveness and utility of hyaluronic acid in osteoarthritis. *Clin Cases Miner Bone Metab* 2015; 12(1):31-3.
6. Altman R, Lim S, Steen RG, Dasa V. Hyaluronic acid injections are associated with delay of total knee replacement surgery in patients with knee osteoarthritis: evidence from a large U.S. health claims database. *PLoS One* 2015; 10(12):e0145776.
7. Hatoum HT, Fierlinger AL, Lin SJ, Altman RD. Cost-effectiveness analysis of intra-articular injections of a high molecular weight bioengineered hyaluronic acid for the treatment of osteoarthritis knee pain. *J Med Econ* 2014; 17(5):326-37.
8. Castellacci E, Polieri T. Antalgic effect and clinical tolerability of hyaluronic acid in patients with degenerative diseases of knee cartilage: an outpatient treatment survey. *Drugs Exp Clin Res* 2004; 30(2):67-73.
9. Depont F. Sinovial dans la gonarthrose. Une étude en situation réelle d'utilisation chez 408 patients. *Rhumatos* 2006; (3).
10. Pavelka K, Uebelhart D. Efficacy evaluation of highly purified intra-articular hyaluronic acid (Sinovial[®]) vs hylan G-F20 (Synvisc[®]) in the treatment of symptomatic knee osteoarthritis. A double-blind, controlled, randomized, parallel-group non-inferiority study. *Osteoarthritis Cartilage* 2011; 19(11):1294-300.
11. Polacco A, Beomonte Zobel B, Polacco M, Scarlata S, Gasparro F, Del Vescovo R, Scarciolla L. The effect of intra-articular hyaluronic acid (SINOVIAL[®] One) on knee osteoarthritis: a preliminary study. *Euro J Inflamm* 2013; 11(3).
12. Theiler R, Brühlmann P. Overall tolerability and analgesic activity of intra-articular sodium hyaluronate in the treatment of knee osteoarthritis. *Curr Med Res Opin* 2005; 21(11):1727-33.
13. Abate M, Vanni D, Pantalone A, Salini V. Hyaluronic acid in knee osteoarthritis: preliminary results using a four months administration schedule. *Int J Rheum Dis* 2015; May 5.
14. IBSA Post-Marketing Data (Internal Data)
15. RPT-000529, Bioventus data on file

LETTER TO THE EDITOR

ROLE OF BIOMARKERS IN ADULT SEPSIS AND THEIR APPLICATION FOR A GOOD LABORATORY PRACTICE: A PILOT STUDY

V. SARGENTINI¹, D. COLLEPARDO², M. D'ALESSANDRO², G. PETRALITO²,
G. CECCARELLI³, F. ALESSANDRI⁴, A. PICIOCCHI⁵, A. ANGELONI⁶, M. VENDITTI³
and A. BACHETONI¹

¹Clinical Pathology, Department of Experimental Medicine, Sapienza University, Rome, Italy; ²Clinical Pathology, Department of Surgery, Sapienza University, Rome, Italy; ³Department of Public Health and Infectious Diseases, Sapienza University, Rome, Italy; ⁴Department of Anaesthesiology and Intensive Care, Sapienza University, Rome, Italy; ⁵Department of Statistical Sciences, Sapienza University, Rome, Italy; ⁶Clinical Pathology, Department of Molecular Medicine, Sapienza University, Rome, Italy

Received January 31, 2017 – Accepted March 24, 2017

This study measured Procalcitonin (PCT), Presepsin (PRE-S) and pro-Adrenomedullin (pro-ADM) in intensive care unit (ICU) patient's blood to assess their contribution to accurate diagnosis of sepsis and potential predictive impact on prognosis. The final aim was to improve the use of infection biomarkers for optimizing the impact of laboratory medicine on clinical outcomes, focusing on the good management of resources designed to produce maximum effectiveness and efficiency. Sixty-four adult patients were studied during their hospitalization in ICU; blood samples were collected and categorized according to their clinical diagnosis and illness severity, and sepsis marker levels were measured on automated immunoassay platforms. PCT, PRE-S and pro-ADM infection markers were significantly lower in controls than in sepsis or septic shock groups. The area under the curve, by ROC curve analysis, was 0.945 for PCT, 0.756 for PRE-S and 0.741 for pro-ADM. Sepsis diagnostic accuracy was not improved by combining PCT, PRE-S and pro-ADM measures. Preliminary data demonstrated that, despite PRE-S and pro-ADM being able to differentiate between septic and non-septic patients with accuracy, PCT remains the most reliable marker available. The results obtained still do not allow us to consider a combination of markers, because it would merely increase laboratory costs without improving diagnostic performance. Furthermore, the results confirm a possible prognostic role of pro-ADM in septic states, but no correlation between biomarker levels and survival at 48 h was detected. Hence PCT, PRE-S, nor pro-ADM can be used to predict short-term prognosis.

To the Editor,

Sepsis is a leading cause of critical illness and mortality worldwide (1). In order to treat patients successfully, sepsis needs to be diagnosed accurately and early, and applicable diagnostic criteria are

therefore a fundamental research target. To this end, the third edition of international guidelines regarding sepsis and septic shock (Sepsis-3) has been recently published (2). Since the scoring systems have proven insufficient to define sepsis accurately, the focus is now

Key words; : procalcitonin, presepsin, pro-adrenomedullin, sepsis, laboratory practice

Mailing address:

Valeria Sargentini, PhD
Clinical Pathology,
Department of Experimental Medicine,
Sapienza University
V.le del Policlinico 155, 00161 Rome, Italy
Tel.: +39 06 49970132 - Fax: +39 06 49970443
e-mail: valeria.sargentini@uniroma1.it

0393-974X (2017)

Copyright © by BIOLIFE, s.a.s.

This publication and/or article is for individual use only and may not be further reproduced without written permission from the copyright holder.

Unauthorized reproduction may result in financial and other penalties
DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

on finding new diagnostic and prognostic biomarkers as promising alternatives (3). Microbiological culture is considered the reference standard for diagnosing infectious conditions, but results require several days (4). Hence, many biomarkers have been examined to improve the early diagnosis of sepsis and predicting prognosis. C-reactive protein (CRP) and Procalcitonin (PCT) are acute phase biomarkers of a systemic reaction, nevertheless PCT is supposed to be more specific for bacterial systemic infection (5). Despite its undoubted helpfulness in diagnosing sepsis, increased PCT levels have been also described in many noninfectious conditions (6, 7). The search for biomarkers with better specificity for the systemic response against infectious microorganisms is still ongoing, particularly among the molecules involved in innate immunity. In this context, Presepsin (PRE-S), a soluble subtype of CD14 (sCD14-ST) has been recently proposed as a potentially useful biomarker for sepsis in clinical settings (8, 9). Another molecule recently studied in febrile and septic patients is Adrenomedullin (ADM) (10); in particular, the mid-regional fragment of pro-ADM (MR-proADM) was detected in the plasma of patients with septic shock (11). Although these promising biomarkers have been assessed clinically, none alone has been definitively tested for its diagnostic accuracy for sepsis (12). Moreover, future research efforts are being directed toward the use of combinations of different biomarkers with clinical signs (13). This study assesses PCT, PRE-S and pro-ADM measurements in sepsis patients to determine which, if any, marker is more prevalent and may contribute to an accurate diagnosis of sepsis, alone or in combination, and evaluates the possible predictive impact of pro-ADM. The goal is to improve clinical outcomes by utilizing validated infection biomarkers. This approach will also lead to effective deployment of laboratory resources.

MATERIALS AND METHODS

The study was performed as a single-centre, observational study including adult patients hospitalized in the Policlinico Umberto I Intensive Care Unit (ICU) (Rome, Italy) between September 2015 and March 2016.

Patients provided informed consent, and the local Ethics Committee approved the study.

Study population and control group

Specimens were collected during hospitalization of 64 patients with: 14 cases of sepsis and 16 of septic shock at the time of clinical diagnosis (Sepsis and Septic Shock groups, respectively), and 34 clinical cases of acute medical or post-traumatic conditions without signs of bacterial infection (No Sepsis group). Clinical information of the patients are summarized in Table I.

The exclusion criteria were patients <18 years, pregnancy and major surgery in the previous 72 hours. Sepsis and septic shock were defined according to the new guidelines provided in the Third International Consensus Definition for Sepsis and Septic Shock (Sepsis-3) (6), patients were categorized according to their clinical diagnosis and the severity of their illness. Thirty healthy subjects, who did not meet the diagnostic criteria for bacterial infection or inflammation, were assessed as controls (Ctr).

Sampling and laboratory analysis

Blood samples were collected and immediately centrifuged at 2000 g for 10 min at 20°C, and plasma was stored at -20°C until use. PRE-S levels were measured by means of a PATHFAST® Presepsin analyser (Mitsubishi Chemical Medience Corporation, Japan), which is based on a chemiluminescent enzyme immunoassay. Method precision was tested on three different plasma pools with mean PRE-S concentrations of 460 pg/ml, 750 pg/ml, 7200 pg/ml, respectively. Between-series coefficients of variance (CoV) of 4.1%, 3.5% and 6.3%, respectively, were calculated for the above. Quantitative analysis of serum PCT and pro-ADM was performed using BRAHMS TM PCT sensitive KRYPTOR® immunoassay (ThermoFisher, Hennigsdorf, Germany). Method precision was tested on three different plasma pools with mean PCT concentrations of 0.07 ng/ml, 1.4 ng/ml and 71.3 ng/ml (obtaining between-series CoVs of 8.5%, 5.3% and 1.5%) respectively, and mean pro-ADM concentrations of 0.3 nmol/L, 1.5 nmol/L and 4 nmol/L (between-series CoVs of 8.1%, 3.5% and 1.4%), respectively.

Statistical analysis

Patient characteristics were summarized by means of cross-tabulations for categorical variables, or by means

of quantiles for continuous variables, as appropriate. In univariate analyses, non-parametric tests were applied for comparisons among groups; Kruskal-Wallis tests were performed to compare sepsis marker concentrations among groups (Ctr group, No Sepsis group, Sepsis group and Septic Shock group). Box plots were used to compare the differential distribution of PCT, PRE-S and pro-ADM among groups. The correlation between pro-ADM and SOFA score was calculated using Pearson test. The diagnostic values of biomarkers were assessed by a receiver operating characteristic (ROC) and area under the curve (AUC), performed by comparing the infection (Sepsis + Septic Shock) group with the non-infection (Ctr + No Sepsis) group. The optimal cut-off point was determined, i.e. that with the best combination of sensitivity and specificity, and logistic regression models were applied to estimate the effect of sepsis markers on the infection group. All tests were two sided, with $p < 0.05$ as statistically significant level; Confidence Intervals were calculated at 95% confidence level. All analyses were performed using SAS software (release 9.4 - SAS Institute, Cary, NC, USA).

RESULTS

Diagnostic roles of PCT, PRE-S and pro-ADM

The median with interquartile range (IQR) concentrations of PCT in serum were 0.1 (0.0–0.1) ng/ml in the Ctr group, 0.2 (0.1–0.3) ng/ml in the No Sepsis group, and 1.4 (0.8–3.5) ng/ml and 1.6 (0.1–2.7) ng/ml in Sepsis and Septic Shock groups, respectively. PCT values were significantly lower in the Ctr group with respect to the No Sepsis ($p < 0.001$), Sepsis ($p < 0.0001$) and Septic Shock ($p < 0.0001$) groups. Differences between No Sepsis and Sepsis groups ($p < 0.0001$) and between No Sepsis and Septic Shock groups ($p < 0.0001$) were also significant (Fig. 1A).

The median PRE-S concentrations in plasma were 497 (400–584) pg/ml in the Ctr group, 622 (322–934) pg/ml in the No Sepsis group, and 1046 (429–2344) pg/ml and 1739 (937–3235) pg/ml in the Sepsis and Septic Shock groups, respectively. PRE-S values were significantly lower in Ctr subjects in comparison to the Sepsis ($p < 0.05$) and Septic Shock ($p < 0.0001$) groups. There was also a significant difference between levels in the No Sepsis and Septic

Shock groups ($p = 0.001$) (Fig. 1B). Pro-ADM concentrations were also evaluated in a smaller number ($n=42$) of Ctr, No Sepsis, Sepsis and Septic Shock samples, respectively, and preliminary data on this new molecule were analyzed. The median values obtained were 0.5 (0.4–0.6) nmol/L in the Ctr group, 0.4 (0.3–1.2) nmol/L in the No Sepsis group, and 2.4 (0.5–4.2) nmol/L and 1.2 (0.5–2.1) in the Sepsis and Septic Shock groups, respectively. Pro-ADM values were significantly lower in Ctr in respect to Sepsis ($p < 0.05$) and Septic Shock ($p < 0.05$) groups. Differences between No Sepsis and Sepsis groups ($p < 0.05$) and between No Sepsis and Septic Shock groups ($p < 0.05$) were also significant (Fig. 1C), but differences in PCT, PRE-S and pro-ADM levels between cases of Sepsis and Septic Shock were not significant.

Median values with IQR and p-values of the markers described above measured in the four populations are shown in Table II.

Comparison of diagnostic accuracy of PCT, PRE-S and pro-ADM for sepsis

In order to compare the accuracy of PCT, PRE-S and pro-ADM measurements in the diagnosis of sepsis, we performed ROC curve analysis for all diagnostic markers, comparing the infection (Sepsis + Septic Shock) group with the non-infection (Ctr + No Sepsis) group. The AUCs calculated from the ROC curve were 0.945 for PCT, 0.756 for PRE-S, and 0.741 for pro-ADM. The best diagnostic cut-off value, to discriminate between infection and no infection, was 0.5 ng/ml for PCT. This was associated with a sensitivity of 86.6% (95% CI 69.2–96.2), and a specificity of 92.1% (95% CI 82.7–97.4). The best diagnostic cut-off value for PRE-S was 650 pg/ml, corresponding to 80.0% (95% CI 67.4–92.2) sensitivity and 66.6% (95% CI 52.7–78.1) specificity. Finally, the best diagnostic cut-off value for pro-ADM was 0.65 pg/ml, corresponding to 68.2% (95% CI 45.1–86.1) sensitivity and 79.4% (95% CI 63.5–90.7) specificity (Fig. 2).

Combined PCT, PRE-S and pro-ADM tests for sepsis diagnosis

The sensitivity and specificity achieved by the

Table I. Clinical characteristic of patients, divided in the four groups, used as study population.

No Sepsis		Sepsis		Septic Shock		Healthy Controls	
Number of Patients	34	Number of Patients	14	Number of Patients	16	Number of Patients	30
Mean age	65	Mean age	58	Mean age	62	Mean age	50
Male	13	Male	6	Male	6	Male	13
Female	21	Female	8	Female	10	Female	17
Disorders at baseline (n)		Disorders at baseline (n)		Disorders at baseline (n)			
Multi trauma	4	Multi trauma	1	Multi trauma	2		
Pulmonar disorders	9	Pulmonar disorders	6	Pulmonar disorders	5		
Neurological diseases	18	Neurological diseases	5	Neurological diseases	7		
Abdominal diseases	3	Abdominal diseases	2	Abdominal diseases	2		
Patients survived at 48h (%)	91	Patients survived at 48h (%)	64	Patients survived at 48h (%)	25		
SOFA score (median)	9	SOFA score (median)	11	SOFA score (median)	15		

Table II. Median values (IQR) and p-values of PCT, PRE-S and pro-ADM.

	Ctr	No Sepsis		Sepsis		Septic Shock	
	Median (IQR)	Median (IQR)	p-value	Median (IQR)	p-value	Median (IQR)	p-value
PCT ng/ml	0.1 (0.0–0.1)	0.2 (0.1–0.3)	vs CTR ** vs Sepsis *** vs S. Shock ***	1.4 (0.8–3.5)	vs CTR *** vs No Sepsis *** vs S. Shock ns	1.6 (0.1–2.7)	vs CTR *** vs No Sepsis *** vs Sepsis ns
PRE-S pg/ml	497 (400–584)	622 (322–934)	vs CTR ns vs Sepsis ns vs S. Shock **	1046 (429–2344)	vs CTR * vs No Sepsis ns vs S. Shock ns	1739 (937–3235)	vs CTR ** vs No Sepsis ** vs Sepsis ns
pro-ADM nmol/L	0.5 (0.4–0.6)	0.4 (0.3–1.2)	vs CTR ns vs Sepsis ns vs S. Shock *	2.4 (0.5–4.2)	vs CTR * vs No Sepsis ns vs S. Shock ns	1.2 (0.5–2.1)	vs CTR * vs No Sepsis ns vs Sepsis ns

ns = not significant: $p > 0.05$; * = $p < 0.05$; ** = $p < 0.001$; *** = $p < 0.0001$

Table III. Univariate logistic regression model.

Parameter	Odds Ratio	95% CI	Limits	p-value
PCT	1.060	0.922	1.219	0.4156
PRE-S	1.000	1.000	1.000	0.9151
pro-ADM	1.169	0.774	1.765	0.4576
SOFA	1.198	0.976	1.471	0.0842

p value = not significant if > 0.05

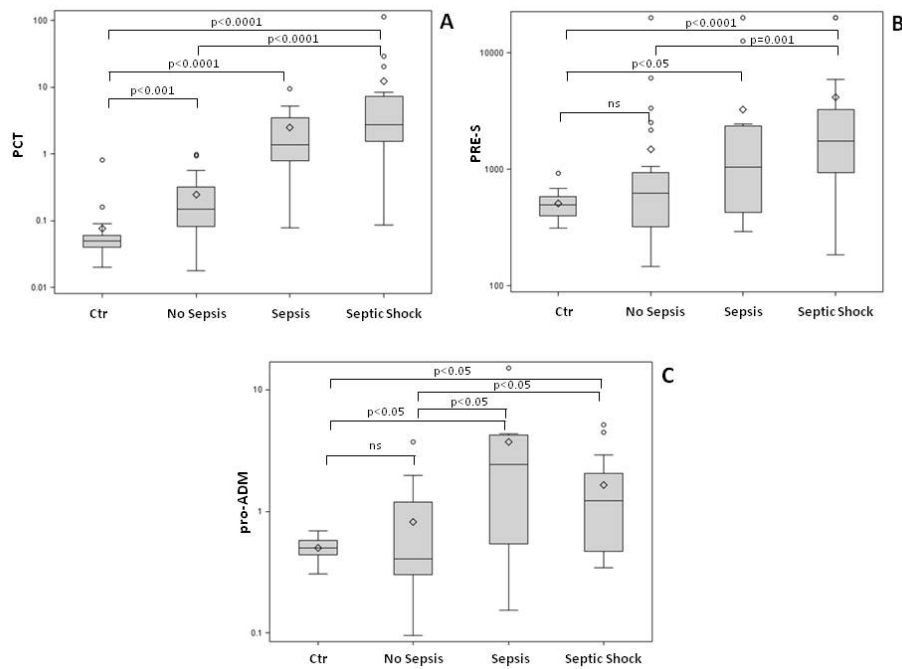


Fig. 1. Serum PCT (A), PRE-S (B) and pro-ADM (C) levels in control subjects and patients without infection (No Sepsis), sepsis and septic shock. Box plots show median values with IQR (Q1–Q3). Differences were considered significant at p -values of <0.05 . ns = not significant

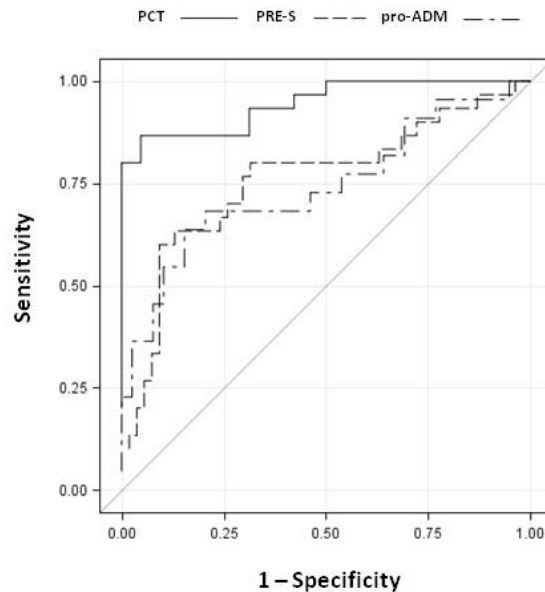


Fig. 2. ROC curves of PCT, PRE-S and pro-ADM for diagnosis of sepsis. Areas under the ROC curve: PCT 0.945 (95% CI 0.89–0.99), $p < 0.0001$ (solid line); PRE-S 0.756 (95% CI 0.64–0.87), $p = 0.0001$ (dashed line); and pro-ADM 0.741 (95% CI 0.61–0.88), $p = 0.001$ (dash dot line). Arrows indicate the optimum cut-offs (0.5 ng/ml for PCT, 650 pg/ml for PRE-S and 0.65 pg/mL for pro-ADM), i.e. those with the best combination of sensitivity and specificity.

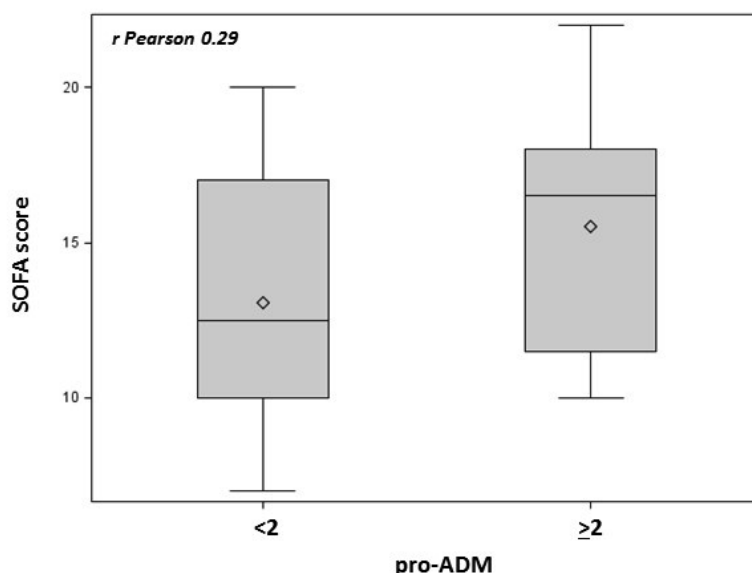


Fig. 3. Association between pro-ADM and SOFA score. Box plots show median values with IQR of SOFA scores between the two groups of patients affected by sepsis and septic shock, respectively, in particular patients with pro-ADM values <2 and ≥ 2 ($p = 0.29$).

combined use of PCT, PRE-S and pro-ADM were also evaluated. Specifically, we considered those samples that were positive for both PCT and PRE-S, with the two cut-offs >0.5 ng/ml and >650 pg/ml, respectively. This combined approach yielded a sensitivity of 73.3% (95% CI 54.1–87.7) and a specificity of 98.2% (95% CI 91.6–99.9). We also combined PCT and PRE-S with pro-ADM (cut-off 0.65 pg/ml) obtaining a sensitivity of 68.8% (95% CI 45.2 - 86.4) and a specificity of 97.4% (95% CI 87.1 – 100).

Association between pro-ADM and SOFA score

As pro-ADM has recently been described as a prognostic marker in sepsis patients (14), we correlated the pro-ADM values measured with the patients' SOFA scores – a scoring system designed to determine the extent of organ function or rate of failure in ICU patients. We indeed, found high pro-ADM values associated with higher SOFA scores (Pearson $r = 0.29$), in particular samples with pro-ADM <2 corresponded to a median of SOFA score equal to 12.5, while samples with pro-ADM ≥ 2

corresponded to a median of SOFA score of 16.5 ($p = 0.29$) (Fig. 3).

Sepsis markers and survival

According to our preliminary data, none of the markers considered were able to predict survival during hospitalization. Specifically, univariate analysis showed no statistically significant differences between survivors and the deceased at 48 h (Table III).

DISCUSSION

In some particular conditions, sepsis can progress to septic shock, a frequent and serious complication with a high risk of mortality, despite the modern antibiotic treatment options available. However, the identification of reliable biomarkers that, together with analysis of clinical symptoms, enable specific detection of a severe systemic infection during hospitalization in the ICU may potentially increase the success of therapeutic interventions and secondary prevention strategies. Many infection

biomarkers are used to identify sepsis in current clinical practice, but their reliability has not been thoroughly tested (21). In this context, our results of a comparison of serum PCT, PRE-S and pro-ADM levels showed that they accurately differentiate between septic and non-septic patients. ROC curve analysis of patient data, divided into infection and non-infection groups, showed that all three markers had a p value ≤ 0.001 , with no significant difference between them, although PCT had the highest AUC, at 0.945 (95% CI 0.89–0.99). In recent years, some authors have proposed different panels of infection biomarkers for evaluating whether the combined measurement of PCT, PRE-S and pro-ADM in patients with sepsis substantially improves their early diagnosis and/or prognosis (13). Although this approach, integrating the different sites of production and molecular pathways of these biomarkers, seems reasonable, we observed that the combination of PCT, PRE-S and pro-ADM does not constitute good management of laboratory resources, as the costs of the additional test are not outweighed by a significant increase in diagnostic accuracy. In fact, our data confirm that PCT alone remains the best marker for an accurate diagnosis of sepsis, and that it may only therefore be useful to supplement this test with simultaneous PRE-S assessment in certain critical conditions (15). Our data confirm the literature that suggests a possible prognostic role for pro-ADM in septic states. These results need to be confirmed, in a larger sample, together with the lack of correlation between any of these markers and survival at 48 hours. It would be especially interesting for future research to confirm the role of PRE-S and pro-ADM in the diagnosis and prognosis of sepsis, as well as their potential utility in therapeutic monitoring and the differential diagnosis between bacterial, viral and fungal infections.

ACKNOWLEDGEMENTS

We thank Anna Forster for linguistic assistance

REFERENCES

1. Fleischmann C, Scherag A, Adhikari NK, et al. International Forum of Acute Care Trialists. Assessment of global incidence and mortality of hospital-treated sepsis: current estimates and limitations. *Am J Respir Crit Care Med* 2015; 193(3):259-72.
2. Singer M, Deutschman CS, Seymour CW, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA* 2016; 315(8):801-10.
3. Vincent JL, Opal SM, Marshall JC. Ten reasons why we should NOT use severity scores as entry criteria for clinical trials or in our treatment decisions. *Crit Care Med* 2010; 38(1):283-87.
4. Paul M, Shani V, Muchtar E, Kariv G, Robensht E, Leibovici L. Systematic review and meta analysis of the efficacy of appropriate empiric antibiotic therapy for sepsis. *Antimicrob Agents Chemoter* 2010; 54:4851-63.
5. Pierrakos C, Vincent JL. Sepsis biomarkers: a review. *Crit Care* 2010; 14(1):R15.
6. Chenevier-Gobeaux C, Borderie D, Weiss N, Mallet-Coste T, Claessens YE. Presepsin (sCD14-ST), an innate immune response marker in sepsis. *Clin Chim Acta* 2015; 450:97-103.
7. Simon L, Gauvin F, Amre DK, Saint-Louis P, Lacroix J. Serum procalcitonin and C-reactive protein levels as markers of bacterial infection: a systematic review and meta-analysis. *Clin Infect Dis* 2004; 39:206-17.
8. Endo S, Suzuki Y, Takahashi G, et al. Usefulness of presepsin in the diagnosis of sepsis in a multicenter prospective study. *J Infect Chemother* 2012; 18:891-97.
9. Chenevier-Gobeaux C, Borderie D, Weiss N, Mallet-Coste T, Claessens YE. Presepsin (sCD14-ST), an innate immune response marker in sepsis. *Clin Chim Acta* 2015; 450:97-103.
10. Hinson JP, Kapas S, Smith DM. Arenomedullin, a multifunctional regulatory peptide. *Endocr Rev* 2000; 21:138-67.
11. Struck J, Tao C, Morgenthaler NG, Bergmann A. Identification of an Adrenomedullin precursor fragment in plasma of sepsis patients. *Peptides* 2004; 25:1369-72.
12. Zheng Z, Jiang L, Ye L, Gao Y, Tang L, Zhang M. The accuracy of presepsin for the diagnosis of sepsis from SIRS: a systematic review and meta-analysis. *Ann Intensive Care* 2015; 5:48.

13. Enguix-Armada A, Escobar-Conesa R, García-De La Torre A, De La Torre-Prados MV. Usefulness of several biomarkers in the management of septic patients: C-reactive protein, procalcitonin, presepsin and mid-regional pro-adrenomedullin. *Clin Chem Lab Med* 2016; 54(1):163-68.
14. Christ-Crain M, Morgenthaler NG, Struck J, Harbarth S, Bergmann A, Müller B. Mid-regional pro-adrenomedullin as a prognostic marker in sepsis: an observational study. *Crit Care*. 2005; 9(6):R816-24.
15. Sargentini V, Ceccarelli G, D'Alessandro M et al. Presepsin as a potential marker for bacterial infection relapse in critical care patients. A preliminary study. *Clin Chem Lab Med* 2015; 53(4):567-73.

PROOF