

JOURNAL OF BIOLOGICAL REGULATORS & HOMEOSTATIC AGENTS

Volume 31, No. 2, April - June, 2017

CONTENTS

Editorials

P. Conti, Al. Caraffa, S.K. Kritas, G. Ronconi, G. Lessiani, E. Toniato and T.C. Theoharides. Mast cell, pro-inflammatory and anti-inflammatory: Jekyll and Hyde, the story continues	263
--	-----

Original articles

D. Chen, L.M. Bellussi, S. Cocca, J. Wang, G.C. Passali, X. Hao, L. Chen and D. Passali. Glycyrrhetic acid suppressed HMGB1 release by up-regulation of Sirt6 in nasal inflammation.....	269
S. Ebrahimpour, M. Shahbazi, A. Khalili, M.T. Tahoori, A. Zavarán Hosseini, A. Amari, B. Aghili, S. Abediankenari, H. Mohammadizad and M. Mohammadnia-Afrouzi. Elevated levels of IL-2 and IL-21 produced by CD4 ⁺ T cells in inflammatory bowel disease.....	279
B. Song, L.F. Wang, X.H. Fan, J.H. Zuo and Y.M. Huang. Expression of T-lymphoma invasion and metastasis factor on the occurrence of oral squamous cell carcinoma	289
A. Bryja, M. Dyszkiewicz-Konwińska, J. Budna, S. Ciesiółka, W. Kranc, S. Borys, M. Jeseta, O. Urbaniak, D. Bukowska, P. Antosik, M. Bruska, M. Nowicki, M. Zabel and B. Kempisty. Expression of cell mitotic progression proteins and keratinocyte markers in porcine buccal pouch mucosal cells during short-term, real-time primary culture	297
J.T. da Silva, B.G. Evangelista, R.A.G. Venega, M.E. Oliveira and M. Chacur. Early and late behavioral changes in sciatic nerve injury may be modulated by nerve growth factor and substance P in rats: a CCI long-term evaluation	309
J. Lechner, K. Huesker and V. Von Baehr. Impact of RANTES from jawbone on chronic fatigue syndrome	321

Letters to the Editor

L. Jia, K. Zhang, Z.G. Wang, L. Wang, SY. Yang and YP. Zheng. Proximal femoral nail antirotation internal fixation in treating intertrochanteric femoral fractures of elderly subjects.....	329
X-Y. Hong, X. Hong, W-W. Gu, J. Lin and W-T. Yin. Femoral nerve repair using an H-type nerve regeneration chamber in rats	335
F. Javed, A.A. Al-Kheraif, S.V. Kellesarian, F. Vohra and G.E. Romanos. Oral <i>Candida</i> carriage and species prevalence in denture stomatitis patients with and without diabetes	343
H-J. Shi, H-L. Wang, Y. Wang, H. Zhao, N. Xv, F. Liu and J-X. Sun. Combined 3D-tomography and 3D-power doppler for vascular imaging of the liver	347
A. Levchenko, O. Lukyanova, T. Borovik, M. Levchenko, D. Sevostianov and P. Sadchikov. The novel technique of microwave heating of infant formulas and human milk with direct temperature monitoring	353
ZH. Wu, X. Zhu, CK. Xu, YJ. Chen, L. Zhang and CL. Zhang. Effect of Xianling Gubao capsules on bone mineral density in osteoporosis patients	359
W. Kranc, J. Budna, M. Dudek, A. Bryja, A. Chachula, S. Ciesiółka, S. Borys, M. Dyszkiewicz-Konwińska, M. Jeseta, L. Porowski, P. Antosik, D. Bukowska, K.P. Brüßow, M. Bruska, M. Nowicki, M. Zabel and B. Kempisty. The origin, <i>in vitro</i> differentiation, and stemness specificity of progenitor cells	365
L. Liu, DZ. Liu, QP. Wang, ZL. Zhu, HM. Li and XY. Lu. Respiratory training during rehabilitation of acute organic fluorine-poisoned patients treated by non-invasive positive pressure ventilation	371
W. Żurawiński, K. Sosada, M. Muc-Wierzoń and T. Kokot. Impact of low-energy shock impulse on nitric oxide serum levels in healthy rabbits	377
XF. Lei, SZ. Jia, J. Ye, YL. Qiao, GM. Zhao, XH. Li and H. Chang. Application values of detection of serum CA199, CA242 and CA50 in the diagnosis of pancreatic cancer	383
XH. Guo, YF. Sun, SZ. Han, J. Miao, M. Cui and JB. Wang. Continuous blood purification in children with severe sepsis	389
J. Li, LM. Zhang and X. Huang. Urinary albumin, immunoglobulin G and beta-microglobulin in the diagnosis of diabetes in the elderly	395
H. Ismayilnadjteymurabadi and D. Konukoglu. The relationship between preptin, forkhead box protein O1 and mechanistic target of rapamycin levels in prediabetic patients	399
A. Grzanka, A. Damasiewicz-Bodzek, M. Szumska and A. Kasperska-Zajac. Decreased plasma kallikrein 5 concentrations in patients with chronic spontaneous urticaria	407
YJ. Tang, HT. Dou, QH. Zhang, JJ. Liu and XS. Yang. Expression of inflammatory cytokines in ovarian epithelial cancer and their correlation	413
M. Riaz, M. Shahid, A. Jamil and M. Saqib. <i>In vitro</i> antioxidant potential of selected aphrodisiac medicinal plants	419
F. Yousaf, M. Shahid, M. Riaz, A. Atta and H. Fatima. Immunomodulatory potential of <i>Anacyclus pyrethrum</i> (L.) and <i>Mucuna pruriens</i> (L.) in male albino rats	425
Z. Tang, XJ. Xu, XJ. He, ZS. Liang, WB. Liang, Y. Li and K. Gao. Analysis of hemorrhagic fever with renal syndrome and its pathogenic gene sequence based on geographic information system	431
L-J. Yan, P. Chang and R-H. Tian. A comprehensive evaluation of application of light-cured colored compomer for deciduous teeth	439
J-P. Yang, C-M. Hu, W. Zhong and Z-G. Wang. Primary diffuse large B-cell lymphoma of the frontal sinus	447
J. Yang. Clinical observation of the effects of the Suhuang Zhike capsule on acute bronchitis	453
G. Chang, LL. Xie, WY. Li, FF. Xia, PC. Huang, QR. Liu, CD. Zhang, TJ. Zhang and HF. Li. Application of oxaliplatin in combination with epirubicin in transcatheter arterial chemoembolization in the treatment of primary liver carcinoma	459
W. Chang, LL. Sun, N. Ma, ZW. Cui, RQ. Sun and SJ. Zhang. Correlation of PICCO/CT on cardiac output index value and prognosis in patients with heart disease	465
S. Taurone, M. Spoletini, F.M. Di Matteo, R. Mele, L. Tromba, F.R. Grippaudo, A. Minni and M. Artico. D'Andrea's disease (angiomegaly): a currently well-defined nosological entity	473
F. Bambini, A. Santarelli, A. Putignano, M. Procaccini, G. Orsini, D. Di Iorio, L. Memè, D. Sartini, M. Emanuelli and L. Lo Muzio. Use of supercharged cover screw as static magnetic field generator for bone healing, 2 nd part: <i>in vivo</i> enhancement of bone regeneration in rabbits	481
S. D'Ercole, M. Tieri, D. Fulco, D. Martinelli and D. Tripodi. The use of chlorhexidine in mouthguards	487
G. Guarino, F. Strollo, L. Carbone, T. Della Corte, M. Letizia, G. Marino and S. Gentile. Bioimpedance analysis, metabolic effects and safety of the association <i>Berberis aristata</i> / <i>Silybum marianum</i> : a 52-week double-blind, placebo-controlled study in obese patients with type 2 diabetes	495
P. Fallahi, S.M. Ferrari, E. Santini, S. Camastra, G. Frenzilli, M. Puccini, F. Goglia, A. Lanni, P. Marchetti and A. Antonelli. Both 3,5-diiodo-L-thyronine (T ₂) and t ₃ modulate glucose-induced insulin secretion	503
A. Notarnicola, G. Maccagnano, M.F. Gallone, I. Covelli, S. Tafuri and B. Moretti. Short term efficacy of capacitive-resistive diathermy therapy in patients with low back pain: a prospective randomized controlled trial	509
S. Fanali and R. Celletti. Biomechanical considerations on macromorphology of endosseous dental implants	517
S. Fanali, T. Villa, S. Lico and R. Celletti. Static and fatigue resistance of two types of implant/abutment connectors	523
P. Romita, C. Foti, L. Masciopinto, E. Nettis, E. Di Leo, G. Calogiuri, D. Bonamonte, G. Angelini, G. Dipalma, A. Ballini and F. Inchingolo. Allergic contact dermatitis to acrylates	529

EDITORIAL

**MAST CELL, PRO-INFLAMMATORY AND ANTI-INFLAMMATORY:
JEKYLL AND HYDE, THE STORY CONTINUES**

P. CONTI¹, AL. CARAFFA², S.K. KRITAS³, G. RONCONI⁴, G. LESSIANI⁵, E. TONIATO⁶
and T.C. THEOHARIDES⁷

¹*Immunology Division, Postgraduate Medical School, University of Chieti, Chieti, Italy;*

²*Department of Pharmacology, University of Perugia, Perugia, Italy;* ³*Department of Microbiology and Infectious Diseases, School of Veterinary Medicine, Aristotle University of Thessaloniki, Macedonia, Greece;* ⁴*UOS Clinica dei Pazienti del Territorio, Policlinico Gemelli, Rome, Italy;*

⁵*Center of Intensive Rehabilitation, "S. Agnese", Pineto (TE), Italy;* ⁶*Department of Medical, Oral and Biotechnological Sciences, University of Chieti, Italy;* ⁷*Molecular Immunopharmacology and Drug Discovery Laboratory, Department of Integrative Physiology and Pathobiology, Tufts University School of Medicine, Boston, MA USA*

Received April 2, 2017 - Accepted May 23, 2017

IL-1 family members include inflammatory and anti-inflammatory cytokines. They can be beneficial or detrimental, not only in cancer, but also in inflammatory conditions. Mast cells (MCs) originate from CD34+/CD117+/CD13+ pluripotent hematopoietic stem cells, express c-Kit receptor (c-Kit-R), which regulates the proliferation and sustain the survival, differentiation and maturation of MCs. They are immune cells involved in innate and adaptive immunity, allergy, autoimmunity, cancer and inflammation. MCs along with T cells and macrophages release interleukin (IL)-10, which is a pleiotropic immunoregulatory cytokine with multiple biological effects. IL-10 inhibits Th1 inflammatory cells, in particular TNF mostly generated by macrophages and MCs, and down-regulates IFN- γ , IL-1 and IL-6. IL-37 is a family member cytokine which binds IL-18 receptor α chain and inhibits inflammatory mediators including TNF, IL-1, IL-6, IL-33 and nitric oxide (NO). IL-37 similar to IL-10 inhibits MC inflammatory cytokines in several disorders, including asthma, allergy, arthritis, and cancer. Here we report a study comparing IL-10 with IL-37, two anti-inflammatory cytokines.

In 2004, in an article published in the journal "Trends in Immunology" entitled: "Mast cells: The Jekyll and Hyde of tumor growth", we reported that mast cells (MCs) can be both beneficial to tumor or detrimental to tumor (1). Today, this effect is confirmed in inflammatory skin diseases, as reported in an interesting article published by Reber LL. (Stephen J. Galli's group), in "JCI Insight" (2).

This dual behavior of MCs does not surprise us, since MCs forming part of the granulocyte family behave like their cousins, neutrophils, which, on the one hand, have a protective function expressing phagocytosis towards invading microbes and foreign bodies. On the other hand, after activation, they release from their granules highly inflammatory substances that can lead to death (3).

Key words: mast cell, inflammation, cytokines, chemokines, immunity

Mailing address:

Prof. P. Conti,
Immunology Division, Postgraduate Medical School,
University of Chieti-Pescara, Viale Unità d'Italia 73, 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.**

The immune system intervenes to defend us from external insults, such as bacteria and traumas, but in the intervention it can cause strong inflammatory reactions. Interleukin (IL)-1 family members mediate many conditions ranging from fever, arthritis, stroke, heart attacks, allergy and even cancer, among other diseases (4).

Here, in this review we attempt to give a brief view of several inflammatory cytokines inhibited by IL-37, and highlight implications of this as well as pointing out adverse reactions (5).

Mast cells

MCs were discovered 130 years ago; they originate from CD34+/CD117+/CD13+ pluripotent hematopoietic stem cells. MC precursors migrate in the circulation and complete the maturation process in their target tissue where they are influenced by various protein factors. One of the most important of these proteins is the c-Kit receptor (c-Kit-R), namely stem cell factor (SCF) which regulates the proliferation and sustains the survival, differentiation and maturation of MCs (6).

MCs participate in inflammatory reactions and are known to be almost exclusively linked to allergic reactions. They are immune cells involved in immune responses and inflammation. MCs are ubiquitous in the body, and play a key role in immune diseases, such as allergy, autoimmunity, asthma and cancer. They are located preferentially around blood vessels, in connective tissue and in intraepithelial locations, and are the first line of defense, mediating inflammation. MCs are more numerous close to the boundaries between the external environment and the internal milieu. They possess functional plasticity, since their phenotype is correlated with their anatomical location, and participate in tissue homeostasis.

MCs modulate and orchestrate the immune response and the pathogenesis of different inflammatory diseases. Normally, in allergy, MCs are associated with a detrimental role due to their allergic mediator release; but also they can have a protective role in various pathological conditions such as infections due to viruses, bacteria and fungi, and certain cancers.

Human MCs are rich source of proteases: tryptase and chymase which are capable to degrade extracellular matrix (7). They participate in innate and acquired immunity and are multifunctional immune cells, maintaining homeostasis under the control of peptides. They can be activated through cross-linking of their surface high affinity receptors for IgE (FcεRI), leading to immediate degranulation (in seconds), and release stored mediators such as histamine, beta-hexosaminidase, heparin, tryptase, chymase, kinogenases, carboxypeptidase A3 and TNF, which promote leukocyte infiltration in various inflammatory diseases. MCs do not circulate in the blood, they complete their differentiation and maturation in close proximity to blood vessels, lymphatic tissue, nerves, and secretory glands. Along with macrophages and dendritic cells, they play a critical role as sentinels against the invasion of microbes.

It obviously would be important if we could regulate mast cells in order to harness their useful potential when needed, and block the release of detrimental mediators involved in allergic and inflammatory diseases.

MCs synthesize *de novo* inflammatory compounds such as growth factors, leukotrienes, prostaglandins and NO. In addition, some cytokines/chemokines, are released later selectively without degranulation.

MC activation can be positively and negatively regulated in disease states and are critical cells of the pathogenesis of allergic reaction, CNS diseases, atherosclerosis, Alzheimer's disease, asthma, autoimmune disorders and cancer (8).

Mast cell activation

MCs express *c-kit* receptor, stem cell factor and the high affinity receptor ($K_d = 10^{-10}$ M) FcεRI for immunoglobulin E (IgE). Cross-linking of bound IgE by antigen activate protein tyrosine kinases, such as *Syk* and *Lyn*, which, in turn, trigger activation of a mitogen-activated protein (MAP) kinase cascade and a phosphatidylinositol-specific phospholipase C (PI-PLCγ). MAP kinase in MC nucleus activates cytokine gene expression with production of inflammatory cytokines (9). We now know that activation, migration and maturation of mast cells

involve several cytokines including TNF and IL-1 family members: IL-1, IL-18 and IL-33 which are strong mediators of inflammation, while IL-10, IL-4, IL-13, TGF β and IL-37 are inhibitory cytokines (10).

Interleukin 33

Pro-inflammatory cytokines are important in host defense. They may induce inflammation but also regulate the immune responses and metabolic activity.

IL-33 is a member of the IL-1 family of cytokines and has emerged as a “danger signal”. IL-1 family cytokines require a proteolytic process to be activated. IL-33 acts synergistically with the neuropeptide substance P in inducing TNF in cultured MCs. IL-33 activates a heterodimeric receptor complex comprised of IL-1 receptor-like 1 (IL-1RL1), along with its coreceptor IL-1 receptor accessory protein (IL-1RAcp), involved in inflammatory gene expression of MAPK and NF- κ B (11).

Since IL-37 inhibits IL-33, and MCs produce IL-33, it is therefore pertinent to study the inhibitory effect of IL-37 in MC activation.

IL-10

Coffman, et al. in 1993 described a cytokine synthesis inhibitory factor (CSIF), later called IL-10, which represents one of the most important inhibitory cytokines (12). IL-10 is a pleiotropic immunoregulatory cytokine with multiple biological effects on different cell types. The IL-10 family is composed of 5 related molecules IL-19, IL-20, IL-22, IL-24 and AK 155 (IL-26).

The mouse cDNA encoding IL-10 was originally isolated from a Th2 lymphocyte cDNA called D10-G4.1 stimulated with a mitogen. Thus, the cDNA encoding IL-10 is composed of 178 amino acid fragments (m.w. 18.5 kDa) with a hydrophobic sequence at the amino acid terminal.

LPS is a strong inducer of IL-10 and other IL-10 cytokine subfamily molecules in monocyte cultures, and exhibits substantial sharing of receptor complexes. It has been seen that after binding of antigen to receptor membrane activation, IL-10 is released from mature and immature T and B lymphocytes, and a variety of other cell types including keratinocytes, some tumor cell lines and

MCs which have the potential to regulate themselves in an autocrine manner (13).

The inhibitory effect of IL-10 is expressed mostly on T cells, macrophages and MCs (murine), which all participate in the inflammatory response. IL-10 inhibits Th1 inflammatory cells, in particular macrophages, representing an interesting therapeutic cytokine. The inhibitory effect is expressed on the production of inflammatory cytokine and chemokines, through the inhibition of nuclear factor κ B (NF κ B) and other molecules. IL-10 inhibits TNF generation and release, mostly by macrophages and MCs. In addition, IL-10 down-regulates IFN- γ , IL-1, and IL-6 which is also released by MCs, even though IL-10 fails to support the growth of MCs *in vitro*. Moreover, IL-10 does not affect the generation of proteases, but promotes the formation of c-Kit and Fc ϵ RI in MCs. We previously reported that human cord blood-derived mast cells and HMC-1 cell line cultured in IL-10 do not change the levels of tryptase compared to the untreated cells. Another inhibitory target for IL-10 are prostaglandins, in fact, IL-10, inhibits PGE2 and PGD2 in macrophages and MCs, respectively, without affecting histamine release.

In mice with a profound MC deficiency related to abnormalities in *Kit* expression (*Kit*-mutant MC-deficient mice), IL-10 can reduce skin pathology during the contact hypersensitivity reactions. However, the inhibition of MCs by IL-10 has been described only in mice and never in humans. It has been reported that MCs amplify rather than limit contact hypersensitivity reactions in *Kit*-independent MC-deficient mice. Moreover, other authors reported that MCs inhibit skin inflammation in a mouse model.

Interleukin-37 (IL-37)

The IL-I F7 cytokine (IL-37) is composed of 5 different variants, namely IL-IF7a, b, c, d, and e, among which IL-I F7b is the largest and binds IL-18 receptor α -chain (in this paper we refer IL-IF7b to IL-37) (14).

IL-37 binding IL-18 receptor α chain, acts as a natural cytokine which inhibits M1 generation of inflammatory mediators, including TNF, IL-1, IL-6, IL-33 and nitric oxide (NO). Innate immune cells,

such as granulocytes, NK cells, T cells, macrophages, dendritic cells, and MCs orchestrate inflammatory and allergic responses

In stimulated dendritic cells, IL-37 inhibits GM-CSF and M-CSF production and reduces the inflammatory response in rheumatoid arthritis (15).

It has been reported that IL-37 down-regulates CC chemokines, such as MCP-5/CCL12, and in neutrophils reduces CXC chemokines, including IL-8, BCA-1/CXCL13 and MIP-2/CXCL2.

IL-37 is an IL-1 family member expressed in human most cell types, such as monocytes, dendritic cells, endothelial cells and epithelial cells. It acts as a natural regulator of the inflammatory responses and may interfere with the TLR4 signaling pathway.

IL-37 is an intracellular regulator which modulates the signaling events initiated by FcεRI or c-Kit and TLR, and inhibits inflammatory cytokines generated by MCs, such as TNF, IL-1, IL-6 and IL-33 (16).

The inhibitory effect of IL-37 is exercised on cJun and p38 MAPK phosphorylation, pro-inflammatory signal STAT transcription factors, and p53, reducing cell differentiation and proliferation.

IL-37, binding IL-18 receptor alpha, inhibits IL-33 generated by MCs, abrogates proliferation, suppresses angiogenesis, induces apoptosis, and strongly reduces inflammation. IL-37 inhibits pro-inflammatory cytokines including IL-1, IL-18, IL-33, TNF, and some chemokines, including macrophage inflammatory protein-2 (MIP-2), by acting on MyD88, a gene which encodes a cytosolic adapter protein that plays a central role in the innate and adaptive immune response.

We recently reported that IL-37 suppresses the inflammatory responses in asthma, atherosclerosis, innate and adaptive immunity by affecting MCs (17).

It should be noted that IL-37 is added to the list of anti-inflammatory cytokines such as IL-10, IL-13, TGF-β and IL-4.

In conclusion, at present, we do not know whether MCs produce IL-37, but we certainly know that MCs produce IL-10 which protects against inflammation and, on the other hand, release pro-inflammatory compounds. However, whether MCs actually perform anti-inflammatory or immunosuppressive functions *in vivo* is controversial. The above studies

suggest that IL-10 and IL-37 could serve as a novel therapeutic target for the treatment of inflammatory disorders, including those mediated by MCs.

For now, there are many issues to be clarified, such as the side effects of IL-37 treatment, as well as the optimal concentration to be used, as high concentrations may not inhibit but even stimulate the inflammatory process. This is because, when IL-37 saturates all the receptors, the excess of protein may bind other unknown MC receptors, stimulating inflammation, since IL-37 is part of the IL-1 family members and may act as an inflammatory protein.

Research on IL-37 is still developing, but the findings hint that this inhibitory cytokine could one day form the basis for a powerful addition to current inflammatory therapies.

REFERENCES

1. Theoharides TC, Conti P. Mast cells: the Jekyll and Hyde of tumor growth. *Trends Immunol* 2004; 25(5):235-41.
2. Reber LL, Sibillano R, Starkl P, et al. Imaging protective mast cells in living mice during severe contact hypersensitivity. *JCI Insight* 2017; 2(9). doi: 10.1172/jci.insight.92900.
3. 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.
4. Dinarello CA. An expanding role for interleukin-1 blockade from gout to cancer. *Mol Med* 2014; 20(Suppl 1):S43-58. doi: 10.2119/molmed.2014.00232.
5. Carinci F, Lessiani G, Spinaz E, Kritas SK, Ronconi G, Caraffa A, Conti P. Mast cell and cancer with special emphasis on il-37 an anti-inflammatory and inhibitor of innate immunity: new frontiers. *J Biol Regul Homeost Agents* 2016; 30(4):945-50.
6. Galli SJ, Tsai M. IgE and mast cells in allergic disease. *Nat Med* 2012; 18(5):693-704. doi: 10.1038/nm.2755.
7. Galli SJ, Tsai M, Marichal T, Tchougounova E, Reber LL, Pejler G. Approaches for analyzing the roles of mast cells and their proteases *in vivo*. *Adv Immunol* 2015; 126:45-127. doi: 10.1016/bs.ai.2014.11.002.

8. Theoharides TC, Stewart JM, Taracanova A, Conti P, Zouboulis CC. Neuroendocrinology of the skin. *Rev Endocr Metab Disord* 2016; 17(3):287-94.
9. Galli SJ, Kalesnikoff J, Grimbaldston MA, Piliponsky AM, Williams CM, Tsai M. Mast cells as “tunable” effector and immunoregulatory cells: recent advances. *Annu Rev Immunol* 2005; 23:749-86.
10. Theoharides TC, Stewart JM, Tsilioni I. Tolerability and benefit of a tetramethoxyluteolin-containing skin lotion. *Int J Immunopathol Pharmacol* 2017; 1:394632017707610. doi: 10.1177/0394632017707610.
11. Shaik-Dasthagirisahab YB, Conti P. The role of mast cells in Alzheimer’s disease. *Adv Clin Exp Med* 2016; 25(4):781-7. doi: 10.17219/acem/61914.
12. Powrie F, Menon S, Coffman RL. Interleukin-4 and interleukin-10 synergize to inhibit cell-mediated immunity in vivo. *Eur J Immunol* 1993; 23(9):2223-29. Erratum in: *Eur J Immunol* 1994; 24(3):785.
13. Moore KW, de Waal Malefyt R, Coffman RL, O’Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 2001; 19:683-765.
14. Dinarello CA. Overview of the interleukin-1 family of ligands and receptors. *Semin Immunol* 2013; 25(6):389-93. doi: 10.1016/j.smim.2013.10.001.
15. Cavalli G, Koenders M, Kalabokis V, et al. Treating experimental arthritis with the innate immune inhibitor interleukin-37 reduces joint and systemic inflammation. *Rheumatology (Oxford)* 2016; 55(12):2220-29.
16. Conti P, Lessiani G, Kritas SK, Ronconi G, Caraffa A, Theoharides TC. Mast cells emerge as mediators of atherosclerosis: Special emphasis on IL-37 inhibition. *Tissue Cell* 2017 doi: 10.1016/j.tice.2017.04.002.
17. Conti P, Ronconi G, Caraffa A, Lessiani G, Duraisamy K. IL-37 a New IL-1 family member emerges as a key suppressor of asthma mediated by mast cells. *Immunol Invest* 2017; 46(3):239-50. doi: 10.1080/08820139.2016.1250220.

GLYCYRRHETINIC ACID SUPPRESSED HMGB1 RELEASE BY UP-REGULATION OF Sirt6 IN NASAL INFLAMMATION

D. CHEN^{1,2}, L.M. BELLUSSI³, S. COCCA³, J. WANG¹, G.C. PASSALI⁴, X. HAO¹,
L. CHEN¹ and D. PASSALI³

¹Department of Otolaryngology Head and Neck Surgery, Chinese PLA General Hospital, Chinese PLA Medical School, Beijing, China; ²Department of Neurosurgery, Medical Faculty of the Friedrich Alexander University of Erlangen-Nurnberg (FAU);

³Department of Medical-Surgical Sciences and Neurosciences, ENT Clinic, University of Siena, Siena, Italy; ⁴Sacred Heart University, ENT Clinic, Rome, Italy

Received October 12, 2016 – Accepted February 24, 2017

To extend our understanding of previous studies on the pathogenesis and mechanism of high mobility group box 1 (HMGB1) in chronic rhinosinusitis with nasal polyps (CRSwNP), here we show that Sirtuin 6 (Sirt6), one of the Sirtuin family members which are widely studied in aging, DNA repair, metabolism, inflammation and cancer, was expressed in normal nasal mucosa using immunohistochemical staining and Western blot assay. Sirt6 expression levels were decreased in CRSwNP tissue. Sirt6 expression levels were modulated by small interfering RNA transfection in human nasal epithelial cells (HNE). We found that depletion of Sirt6 suppressed the number of human nasal epithelial cell cilia, and dramatically induced HMGB1 translocation from nucleus to cytoplasm in the HNE cells. Glycyrrhizic acid (GA) and glycyrrhetic acid (GTA) are specific chemical compounds that may be isolated from the licorice plant. GTA has been shown to have anti-inflammatory and anti-allergic activity: it binds selectively to HMGB1 protein released extra-cellularly and inhibits its cytokine activities through a scavenger mechanism on the protein accumulation. In an *in vitro* study we used the 18- β -stereoisomer of GTA to enhance Sirt6 expression levels, inhibiting through this mechanism the translocation of HMGB1 protein from nucleus and reversing its extracellular accumulation stimulated by lipopolysaccharides. These findings reveal a previously unknown role for nasal mucosa steady-state conditions in the control of Sirt6 activity, and provide evidence for a relationship between HMGB1 and Sirt6 in CRSwNP, and promising benefits of glycyrrhetic acid for CRSwNP patients.

Normal sinonasal epithelial cells are a critical component of innate and adaptive immunity maintained by a balanced activation, but may also promote tissue injury in inflammatory conditions. Previous studies have reported that cytokines and small molecule proteins, such as S100 and PLUNC produced by epithelial cells, control local immune

responses as they possess anti-microbial and anti-biofilm properties (1-3). When epithelial barrier damage occurs, a set of molecules termed “damage-associated molecular patterns” (DAMPs) are released from the host itself in sustaining the inflammatory reaction. Endogenous molecule DAMPs, including surfactant proteins, S100 molecules, HMGB1, and

Key words: high mobility group box 1, glycyrrhetic acid, Sirt 6, nasal inflammation, rhinosinusitis

Mailing address:

Luisa Maria Bellussi, MD PhD
Department of Medical-Surgical Sciences and Neurosciences,
ENT Clinic, University of Siena,
Viale Bracci, 14, 53100 Siena, Italy
Tel.: +39 3356102667 - Fax: +39 0577585470
e-mail: l.bellussi@virgilio.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.

endogenous extracellular matrix (ECM) components, have been implicated in the inflammatory response during upper airway pathologies (4-8).

Our previous study, consistent with other studies on lower airway diseases (9) such as asthma and chronic obstructive pulmonary disease (COPD), presented a positive relationship between HMGB1 and CRSwNP (10). HMGB1 protein levels were increased in the nasal polyps and with significant over-expression in the cytoplasm of epithelial cells, inflammatory cells and also gland cells. We also found inflammatory mediator LPS induced cytosolic HMGB1 and extracellular HMGB1 increase in nasal epithelial cells *in vitro* (11), leading to the hypothesis that changes of HMGB1 may also be a key contributor to the inflammatory process of nasal epithelial cells. However, the regulation of HMGB1 in chronic rhinosinusitis requires further investigation.

Substantial evidence has shown a reduction in the level and activity of sirtuins, NAD⁺-dependent class III histone deacetylases closely correlated with chronic inflammatory conditions. As protein deacetylation represents the underlying release mechanism for HMGB1 (12-14), in this study we aimed to reveal whether Sirt6 is involved in the setting of CRSwNP and HNE cells. We also tried to determine whether Sirt6 expression and activity changes treated with small interfering RNA (siRNA) Sirt6 or 18- β -glycyrrhetic acid are consistent with shifts of HMGB1 from nucleus to cytoplasm and extra-cellularly in HNE cells.

Glycyrrhizic acid (GA) and glycyrrhetic acid (GTA) with their salts and esters are specific chemical compounds that may be isolated from the licorice root plant *Glycyrrhiza glabra*. GTA, the active triterpenoid metabolite of glycyrrizin, has anti-inflammatory, antiallergic and anticancer activity (15, 16).

In a randomized, double blind, perspective, placebo controlled trial, licorice extract prepared as topical gel and titrated based on glycyrrhizic acid (20.3 and 19.6%, respectively, in licorice extract and gel) was used to treat atopic dermatitis. The gel was effective in reducing the scores for erythema, oedema and itching over two weeks (17).

In a randomized controlled clinical study (18),

35 children with allergic rhinitis monosensitized to parietaria were examined and treated for seven days, respectively, with GTA administered as nasal spray (12 children), nasal corticosteroids (12 children) and placebo (11 children). HMGB1 levels in nasal mucus were higher in children with allergic rhinitis than in the control group, whereas they were significantly diminished ($p < 0.001$) in children treated with nasal GL and CS after 7 days of treatment. These studies provide evidence that HMGB1 protein could be considered a good marker of nasal mucosa inflammation and GTA nasal treatment could have glucocorticoid-like anti-inflammatory effects.

Considering the mechanism of the GTA anti-inflammatory effect, NMR and fluorescence studies indicate that glycyrrhizin binds directly to HMGB1 protein released extra-cellularly and inhibits its cytokine activities through a scavenger mechanism on the protein accumulation (19).

In this study we investigated the effects of 18- β -glycyrrhetic acid on Sirt6 expression and activity in primary HNE from CRSwNP patients.

MATERIALS AND METHODS

Subjects

Specimens were obtained from 36 patients with CRSwNP who were followed-up at the Department of Otolaryngology at the General Hospital of the People's Liberation Army, Beijing, China. Informed consent was obtained from all participants as part of an approved ongoing research protocol by the Ethics Committee of the Chinese PLA General Hospital. Clinical diagnostic criteria for CRSwNP were confirmed according to the European Position paper on Rhinosinusitis and Nasal Polyps 2012 guidelines (20). In this study, subjects were excluded if they had received any oral steroid or antihistamine 3 months before surgery, or suffered from fungal sinusitis, cystic fibrosis, primary ciliary dyskinesia (PCD) or severe asthma.

Additional inferior turbinate mucosal samples of 8 patients who had undergone septal surgery and had no evidence of any sinus disease were obtained as control samples.

Reagents and antibodies

HMGB1, Sirt6, β -actin, GAPDH antibodies and

FITC-IgG secondary antibodies were purchased from Abcam and Invitrogen. Lipopolysaccharide (LPS), protease type XIV, deoxyribonuclease and collagen type IV were purchased from Sigma (St Louis, MO, USA). All secondary antibodies used for Western blotting were purchased from Santa Cruz. HMGB1, Sirt6, GAPDH primers for RT-PCR were purchased from Metabion International AG. BEGM medium was purchased from Gibco Life Technologies. 18- β - stereoisomer of glycyrrhetic acid (GTA) (E0180500, Sigma, St Louis, MO, USA), the organic compound derived from Glycyrrhizic Acid or shredded licorice roots was used in primary HNE cell exposure experiments.

Primary human nasal epithelial cells culture

Removed nasal polyps tissue from CRSwNP patients with ages ranging from 20-55 years were freshly digested in 10 ml of DMEM/F12 medium with protease type XIV (0.1%) and deoxyribonuclease (0.1 mg/ml) for 18-24 h at 4°C. Before seeding, 48-well or 24-well plates were coated with collagen type IV in advance. Primary human nasal epithelial (HNE) cells were grown in BEGM medium with 10% FBS for 2 days and then grown in serum-free modified medium, as described previously.

siRNA transfection

Small interfering RNA (siRNA) targeting Sirt6 and siNeg were obtained from Cell Signaling Technology. Reverse transfection was performed by preparing a solution of 400 μ l of serum-free medium 10 μ l Lipofectamine 2000 and 4 μ l siRNA solution (100 nM) and incubating the mixture for 20 min at 37°C. siRNA-Lipofectamine 2000 complexes solution was added to the cell culture dish and primary HNE cells were incubated for 48 h at 37°C. Transfection efficiency was confirmed by real-time PCR using Sirt6 mRNA-specific primers. Viability was assessed using Trypan blue.

Immunostaining

Immunohistochemistry was used to detect the expression of HMGB1 and Sirt6 in nasal polyps and normal controls. Briefly, paraffin-sections were deparaffinized in xylene and rehydrated in descending ethanol series. Protein staining was performed using PV-9000 Kit, purchased from ZhongShan Goldenbridge Bio. Beijing. All sections were counterstained with hematoxylin and observed by light microscopy. Immunohistochemistry

staining was performed as previously described (11). HMGB1 and Sirt6 antibodies were used at a concentration of 1:300 and 1:50, respectively.

Western blotting

Tissue and cell pellet lysate were extracted with chilled RIPA buffer with protease inhibitor. Equal aliquots of cell culture supernatants were collected to determine the relative amounts of HMGB1 secreted into the culture media. Equal amounts of protein lysates were loaded and separated by 12% SDS-PAGE gels, and transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen, USA). The membranes were incubated with 5% nonfat milk solution in tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h, and then hybridized with the primary HMGB1, Sirt6, β -actin or GAPDH antibody dilutions (1:1000) in 5% BSA-TBST overnight at 4°C. Proteins were detected by HRP-conjugated secondary antibody and visualized by ECL detection. Quantity One software was used to quantify the optical density of each band.

Real time PCR analysis

Total RNA was extracted from cultured cells. The reverse transcription was performed using cDNA DyNAmo Kit (Biozym), and real-time PCR was performed using the SYBR green method. Primers (forward and reverse, respectively) specific for Sirt6, 5'-CCATCCTAGACTGGGAGGACT-3', 5'-GGATCTGCAGCG ATGTACCC-3'; GAPDH, 5'-TGAAGGTCGGAGTCAACGATTTGGT-3' and 5'-G AAGATGGTGATGGGATTTC-3'.

LPS and 18- β -glycyrrhetic acid exposure experiments

Primary HNE cells were subsequently exposed to 10 mM LPS for 2 days when cell confluence reached 80%, then treated with different indicated concentrations of GTA or without GTA for a further 3 days. The HNE cells and culture supernatant were harvested after treatment.

Cell viability assay

Primary HNE cells were plated in 96-well plates at 5.0×10^3 (100 μ l) and were allowed to attach overnight. Cells were treated with increasing concentration of 18- β -GTA for 72 h then incubated with MTT (5 mg/ml in phosphate-buffered saline; PBS) for 3 h. Afterwards, the formazan

crystals were dissolved in a mixture of ethanol/DMSO (1:1) and the absorbance of the obtained solutions was determined at 570 nm using TECAN F50 (USA).

Statistical analysis

Results were expressed as mean $\pm$ SD of at least three independent experiments. Statistical analysis was performed using SPSS13.0 statistical analysis software, and compared with unpaired *t*-test or Mann-Whitney test where appropriate. A *P*-value <0.05 was considered statistically significant.

RESULTS

HMGB1 expression was increased and Sirt6 expression was decreased in CRSwNP

Immunohistochemistry (IHC) showed cytosolic and nuclear HMGB1 in CRSwNP, which is primarily expressed in the nucleus of HNE cells in normal nasal mucosa. Nuclear localization of Sirt6 was also observed (Fig. 1). In contrast, Western blot analysis for Sirt6 in CRSwNP tissue revealed relatively lower

expression compared to normal tissue ($P<0.001$). These data suggest the potential clinical implication of Sirt6 in CRSwNP pathogenesis. Pro-inflammatory effects in CRSwNP may result in decreased Sirt6 expression and elevated HMGB1.

Sirt6 suppressed cilia differentiation in human nasal epithelial cells

We attempted to clarify the role of Sirt6 in the primary human nasal epithelial (HNE) cell differentiation. We knocked down the endogenous Sirt6 in HNE cells. Sirt6 levels were decreased about 60% using Sirt6 siRNA transfection, as assessed by RT-PCR (data not shown). Immunofluorescence staining for Sirt6 reconfirmed the localization of Sirt6 in the nucleus of HNE cells (Fig. 2a, right panel). Sirt6 knockdown clearly attenuated cilia quantity, as measured by expression of β -tubulin (Fig. 2a, left panel). Compared to the controls, β -tubulin and Sirt6 were significantly reduced from day 1 to day 3 ($P<0.001$), indicating that Sirt6 was necessary for HNE cell differentiation.

Decreased Sirt6 induced translocation of HMGB1 in

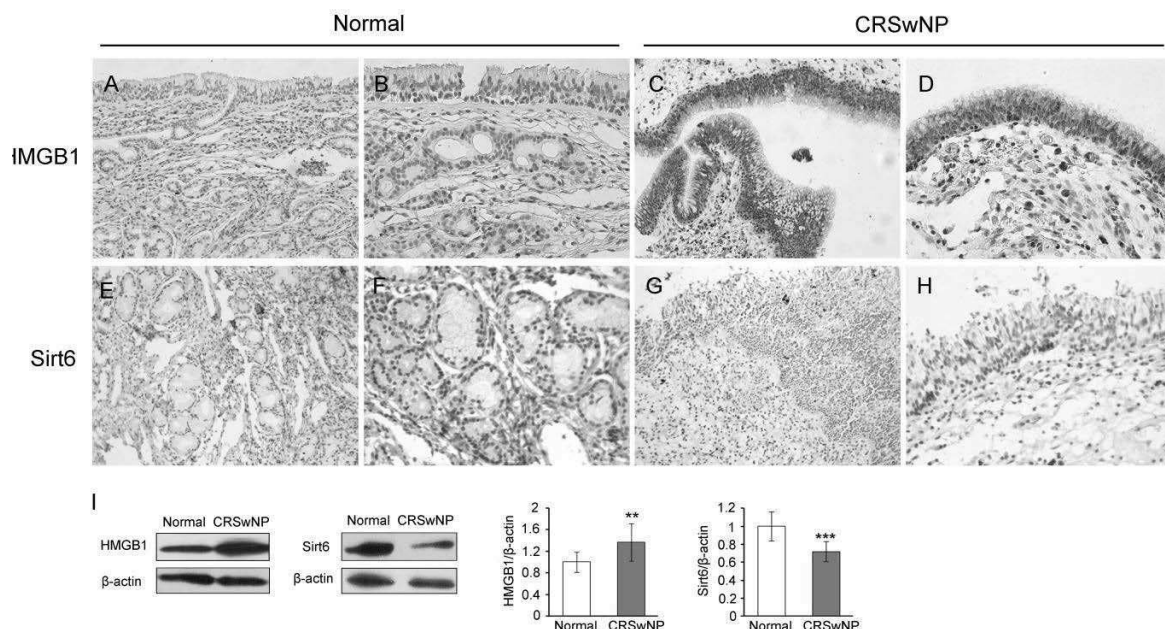


Fig. 1. HMGB1 and Sirt6 expression in tissue from patients with CRSwNP and normal nasal mucosa. **A-H)** Representative sections of specimens from 36 patients and 8 controls stained for HMGB1 and Sirt6. Positive staining of HMGB1 and Sirt6 appears as dark grey. Sections were counterstained with hematoxylin. Original magnification (A,C,E,G) $\times 100$, original magnification (B,D,F,H) $\times 400$. **I)** Western blot of HMGB1 and Sirt6 protein levels in CRSwNP and normal nasal mucosa. *as compared with normal, ** $P<0.01$, *** $P<0.001$, unpaired *t*-test

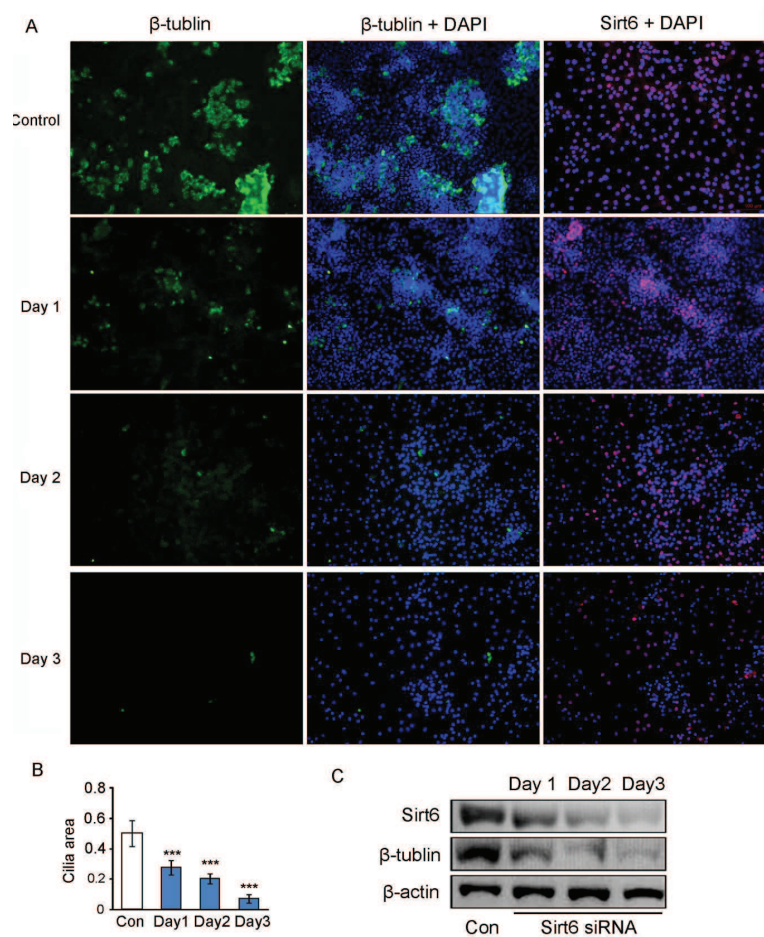


Fig. 2. SIRT6 suppressed cilia differentiation in HNE cells. (A) immunofluorescence image of epithelial cilia (green) and Sirt6 protein (red) in sirt6 knockdown HNE cells and control. Original magnification $\times 100$ (B) The percentage of cilia area from three independent experiments is shown. Open bar is transfection with control siRNA for 3 days. Filled bars are treated with Sirt6 siRNA. * as compared with control, *** $P < 0.001$, unpaired *t*-test.

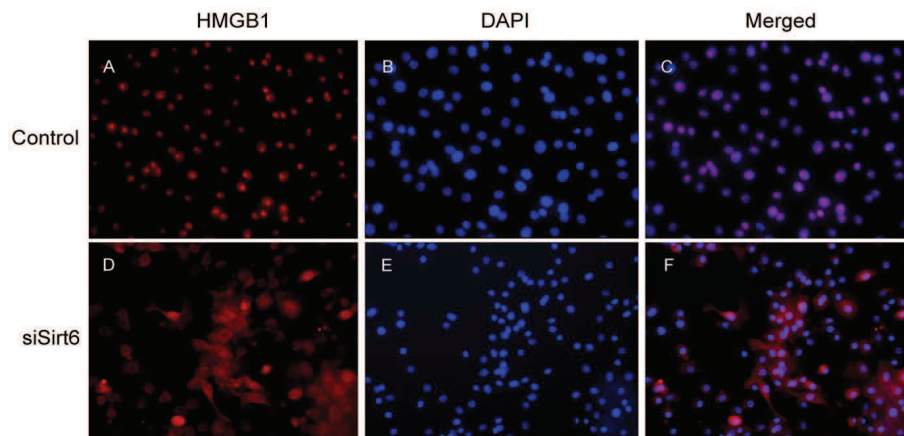


Fig. 3. Decreased Sirt6 induced translocation of HMGB1 in HNE cells. Localization of HMGB1 (red) in HNE cells treated with control siRNA (A-C) and Sirt6 siRNA (D-F). Original magnification $\times 200$

HNE cells

Intriguingly, immunofluorescence staining demonstrated that HMGB1 protein shifted from the nucleus to the cytoplasm after treatment with Sirt6 siRNA for 48 h (Fig 3). Thus, it appears that Sirt6 deacetylates HMGB1 in the nucleus in a normal situation. Decrease in Sirt6 levels or enzyme activity might precede the effects of acetylated nuclear HMGB1 transferring to the cytoplasm.

18- β -glycyrrhetic acid modulated HMGB1 and Sirt6 in HNE cells

Our previous study showed that intracellular HMGB1 shifted and released, and extracellular HMGB1 increased in HNE cells when simultaneously exposed to lipopolysaccharide (LPS), but the mechanism is not clear (11). Based on the above results of regulation of HMGB1 by Sirt6 down-regulation, we further used 18- β -GTA to reveal Sirt6 function in the inflammatory process. High concentration (10 μ M) of GTA was found toxic to HNE cells, so we used the range of 0.5-5 μ M in

our experiment (Fig. 4). In combination with GTA treatment, Sirt6 protein level was significantly up-regulated ($P<0.001$), and intracellular HMGB1 protein was also elevated accompanied by Sirt6 increase compared with the group treated with LPS ($P<0.001$). The level of released HMGB1 was increased upon 48-h LPS stimulation, consistent with our previous study, but this increase was attenuated significantly in the presence of GTA. Taken together, from these results we hypothesize LPS-primed release of HMGB1 was inversed by GTA activating Sirt6 expression. Activated Sirt6 may deacetylate HMGB1, and stabilize HMGB1 in the nucleus.

DISCUSSION

Drawing from previous studies indicating an important role of some DAMP family members in the underlying pathogenesis of patients with CRSwNP (1-3,6-8), and because of the limited data regarding the mechanism of DAMPs in CRSwNP, we focused on inflammatory mediator-induced nasal epithelial cell

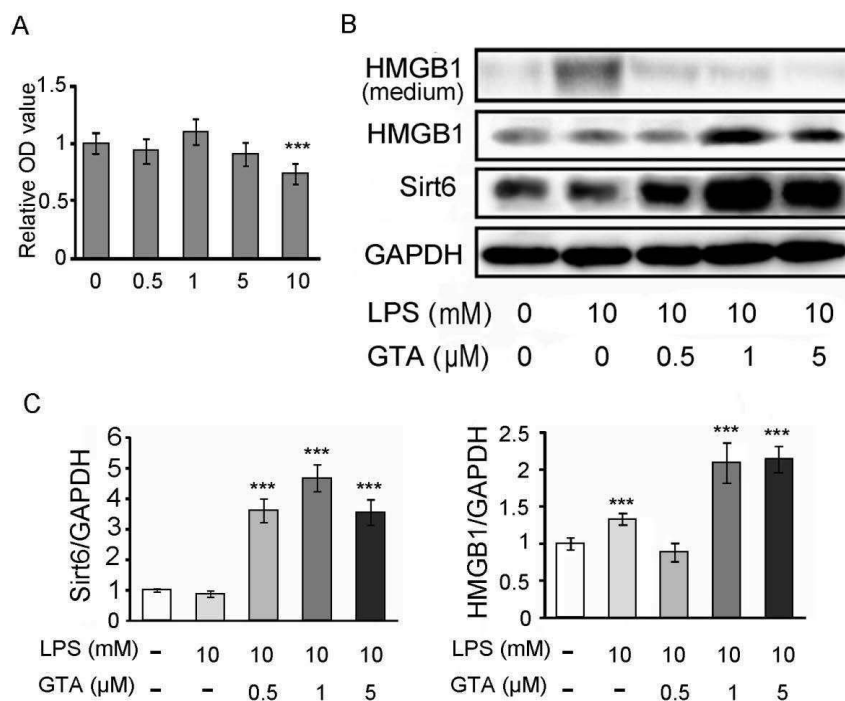


Fig. 4. 18- β -glycyrrhetic acid modulated HMGB1 and Sirt6 in HNE cells. **A)** Relative cell viability of HNE cells treated with (0, 0.5, 1, 5, 10 μ M) GTA for 3 days. **B-D)** Western blot analysis of HMGB1 and Sirt6 protein levels in LPS or GTA combination-treated HNE cells. * as compared with control, *** $P<0.001$, unpaired t -test

dysfunction via the Sirt6-HMGB1 signaling pathway. HMGB1 is a nuclear DNA binding protein with functions in the regulation and transcription. Secreted HMGB1 acts as a late pro-inflammatory cytokine in the pathogenesis of acute and chronic inflammatory diseases. Active HMGB1 secretion has long been known to be regulated by its acetylation, acetylation of chromatin, p300/CBP-associated factor and NF- κ B activation (21-22). Many studies have demonstrated that Sirt6 has an essential role in the deacetylation of proteins and the regulation of DNA stabilization and repair, cell proliferation and differentiation, life span, and genome stability (23-25).

Firstly, in our study, Sirt6 is predominantly expressed in the nucleus in the normal nasal mucosa. Sirt6 knockdown inhibited the differentiation of HNE cells, and induced cell death (data not shown). These results are in agreement with several studies that deficiency of Sirt6 inhibits chondrocyte proliferation also leads to shortened life span in the Sirt6 null mice (26).

Secondly, our current findings suggest reduced Sirt6 expression was accompanied by the increased translocation of HMGB1 to the cytoplasm in nasal polyps, which appears to signify that deacetylation is the vital regulator of HMGB1 release somehow linked with the regulation of HMGB1. In addition, Sirt6 knockdown also altered the localization of HMGB1 in HNE cells, which is consistent with decreased Sirt6 causing the relocation of HMGB1 during brain ischemia (27). In essence, Sirt6 can suppress inflammation in multiple tissues through positively deacetylating NF- κ B transcriptional activity, resulting in transcriptional repression of various inflammation-related genes and the production of proinflammatory cytokines IL-8, TNF- α and IFN- γ (25, 28). Hence, we believe that Sirt6 appeared more as a master regulator of maintaining the nuclear localization of HMGB1 through modifications in the chromatin structure, and had an anti-inflammatory effect in the pathogenesis of CRSwNP.

GTA, a medicinal herb, is an active triterpenoid metabolite of glycyrrhizic acid (GZA). GTA has been used for the prevention or treatment of viral infection, inflammation, pulmonary bronchitis and anti-allergic action due to its antitoxic and immunological regulatory properties (29-31). GZA

and GTA can form a complex with HMGB1 in the serum by binding HMGB1 directly to neutralize the endotoxic effect of HMGB1 during inflammation (32). Another derivative of GZA, CBX, is a licensed drug for esophageal ulceration and inflammation (33). It has been reported that CBX inhibited LPS-induced HMGB1 release associated with the blockade of ATP receptors in macrophage, as well as in neurons (34, 35).

In our study, although no change was found in LPS-induced Sirt6 protein levels on the first two days, combination with GTA up-regulated the Sirt6 protein levels, and also effectively reversed LPS-induced HMGB1 release. We assume these as possible mechanisms by which GTA could attenuate the inflammatory reaction or inhibit HMGB1 release, including effects on PI3K/Akt/GSK3 β signaling and glucocorticoid receptor activation, JNK and NF- κ B pathways in TNF- α activation or LPS-induced protein kinase R (PKR) activation (36, 37). The elevated cellular HMGB1 protein levels after GTA treatment may be related to increase of deacetylated HMGB1 in nucleus by increasing Sirt6 protein levels or Sirt6 activity. Indeed, further evidence is required to reveal which pathway of Sirt6 regulates HMGB1 in the nasal epithelial cells. Taken together, these results indicate that GTA-mediated upregulation of Sirt6 modulated the status of HMGB1 acetylation, which, in turn, has a critical role in the cellular response to inflammation through deacetylation-mediated regulation of HMGB1 release.

Sirt6 expression levels were modulated by small interfering RNA transfection in human nasal epithelial cells (HNE). We found that depletion of Sirt6 suppressed the number of human nasal epithelial cell cilia, and dramatically induced HMGB1 translocation from nucleus to cytoplasm in the HNE cells. Anti-inflammatory agent 18- β -glycyrrhetic acid strengthened the hypothesis that Sirt6 expression levels enhanced under 18- β -glycyrrhetic acid treatment could reverse extracellular HMGB1 accumulation which was stimulated by lipopolysaccharides *in vitro*. These findings reveal a previously unknown role for nasal mucosa steady-state conditions in the control of Sirt6 activity, and provide evidence for a

relationship between HMGB1 and Sirt6 in CRSwNP, and promising benefits of GTA for CRSwNP patients.

However, there are several principal limitations to our study. Firstly, the acetylated HMGB1 and deacetylated HMGB1 in nasal epithelial cells may be better detected by a larger tissue number than in our study. Secondly, epithelial cells from nasal polyps were under active inflammatory conditions, and such epithelial cells expressed low Sirt6 expression; this condition may lead to difficulty in changing Sirt6 levels during LPS stimulation. The timing of such stimulation and treatment would also bias our study. Furthermore, the effects of GTA, including the inflammatory cytokine expression, other sirtuin members and the potential pathways of GTA, need to be observed in further research. Nevertheless, the present data provide a new insight into the relationship between Sirt6 and GTA, GTA and HMGB1, and also the possible underlying pathogenic mechanisms in inflammation-induced repression of Sirt6 promoting HMGB1 nuclear-to-cytoplasmic translocation and release, thus maintaining inflammation.

REFERENCES

1. Wei Y, Xia W, Ye X, et al. The antimicrobial protein short palate, lung, and nasal epithelium clone 1 (SPLUNC1) is differentially modulated in eosinophilic and non-eosinophilic chronic rhinosinusitis with nasal polyps. *J Allergy Clin Immunol* 2014; 133:420-28.
2. Passali D, Sarafoleanu C, Manea C, et al. PLUNC proteins positivity in patients with chronic rhinosinusitis: a case-control study. *Sci World J* 2014; 853583.
3. Tieu DD, Peters AT, Carter RG, et al. Evidence for diminished levels of epithelial psoriasin and calprotectin in chronic rhinosinusitis. *J Allergy Clin Immunol* 2010; 125:667-75.
4. Richer SL, Truong-Tran AQ, Conley DB, et al. Epithelial genes in chronic rhinosinusitis with and without nasal polyps. *Am J Rhinol* 2008; 22:228-34.
5. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 2004; 5:987-95.
6. Lee HM, Kang HJ, Woo JS, Chae SW, Lee SH, Hwang SJ. Up-regulation of surfactant protein A in chronic rhinosinusitis. *Laryngoscope* 2006; 116:328-30.
7. Passali D, Astore S, Boccuzzi S, Loglisci M, Cannatelli A, Passali GC, Bellussi L. Values of SP-A protein in the nasal mucosa. *HNO* 2016; 64(9):671-5.
8. Ullah MA, Loh Z, Gan WJ, et al. Receptor for advanced glycation end products and its ligand high-mobility group box-1 mediate allergic airway sensitization and airway inflammation. *J Allergy Clin Immunol* 2014; 134:440-50.
9. Ferhani N, Letuve S, Kozhich A, et al. Expression of high mobility group box 1 and of receptor for advanced glycation end products in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2010; 181:917-27.
10. Chen D, Mao MF, Bellussi LM, Passali D, Chen L. Increase of high mobility group box chromosomal protein 1 in eosinophilic chronic rhinosinusitis with nasal polyps. *Int Forum Allergy Rhinol* 2014; 4:453-62.
11. Chen D, Bellussi LM, Passali D, Chen L. LPS may enhance expression and release of HMGB1 in human nasal epithelial cells in vitro. *Acta Otorhinolaryngol Ital* 2013; 33:398-404.
12. Rajendrasozhan S, Yang SR, Kinnula VL, Rahman I. SIRT1, an antiinflammatory and antiaging protein, is decreased in lungs of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2008; 177:861-70.
13. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 2002; 418:191-95.
14. Hwang JS, Lee WJ, Kang ES, et al. Ligand-activated peroxisome proliferator-activated receptor- δ and - γ inhibit lipopolysaccharide-primed release of high mobility group box 1 through upregulation of SIRT1. *Cell Death Dis* 2014; 5:e1432.
15. Yuan R, Lin Y. Traditional Chinese medicine: an approach to scientific proof and clinical validation. *PharmacolTherapeut* 2000; 86:191-98.
16. Khaksa G, Zolfaghari ME, Dehpour AR, Samadian T. Anti-inflammatory and anti-nociceptive activity of disodium glycyrrhetic acid and hemiphtalate. *Planta Med* 1996; 62:326-28.
17. Saeedi M, Morteza-Semnani K, Ghoreishi M-R. The treatment of atopic dermatitis with licorice gel. *J Dermatol Treat* 2003; 14:153-57.
18. Cuppari C, Salpietro A, Grasso L, Manti S, Loddo

- I, Ferrau V, Ciprandi G, Salpietro C HMGB1 and allergic rhinitis in children: preliminary results after corticosteroids or glycyrrhetic acid intranasal treatment. *J Pediatrics* 2012; 1 (2) <http://www.thechild.it/print.php?id=8>
19. Mollica L, De Marchis F, Spitaleri A, Dallacosta C, Pennacchini D, Glycyrrhizin binds to high-mobility group box 1. *Chem Biol* 2007; 14:431-41.
20. Fokkens WJ, Lund VJ, Mullol J, et al. European position paper on rhinosinusitis and nasal polyps 2012. *Rhinol Suppl* 2012; 23:1-298.
21. Bonaldi T, Talamo F, Scaffidi P, et al. Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. *EMBO J* 2003; 22:5551-60.
22. Ha T, Xia Y, Liu X, et al. Glucan phosphate attenuates myocardial HMGB1 translocation in severe sepsis through inhibiting NF-kappaB activation. *Am J Physiol Heart Circ Physiol* 2011; 301:848-55.
23. Fontana L, Partridge L, Longo VD. Extending healthy life span-from yeast to humans. *Science* 2010; 328:321-26.
24. Michishita E, McCord RA, Berber E, et al. SIRT6 is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin. *Nature* 2008; 452:492-96.
25. Kawahara TL, Michishita E, Adler AS, Damian M, Berber E, Lin M, McCord RA, Ongaigui KC, Boxer LD, Chang HY, Chua KF. SIRT6 links histone H3 lysine 9 deacetylation to NF-kappaB-dependent gene expression and organismal life span. *Cell* 2009; 136:62-74.
26. Piao J, Tsuji K, Ochi H, et al. Sirt6 regulates postnatal growth plate differentiation and proliferation via Ihh signaling. *Sci Rep* 2013; 3:3022.
27. Lee OH, Kim J, Kim JM, et al. Decreased expression of sirtuin 6 is associated with release of high mobility group box-1 after cerebral ischemia. *Biochem Biophys Res Commun* 2013; 438:388-94.
28. Bauer I, Grozio A, Lasiglie D, et al. The NAD⁺-dependent histone deacetylase SIRT6 promotes cytokine production and migration in pancreatic cancer cells by regulating Ca²⁺ responses. *J Biol Chem* 2012; 287:40924-37.
29. Arase Y, Ikeda K, Murashima N, et al. The long term efficacy of glycyrrhizin in chronic hepatitis C patients. *Cancer* 1997; 79:1494-500.
30. Cavone L, Muzzi M, Mencucci R, Sparatore B, Pedrazzi M, Moroni F Chiarugi A. 18β-glycyrrhetic acid inhibits immune activation triggered by HMGB1, a pro-inflammatory protein found in the tear fluid during conjunctivitis and blepharitis. *Ocul Immunol Inflamm* 2011; 19:180-85.
31. Kao TC, Wu CH, Yen GC. Glycyrrhizic acid and 18β-glycyrrhetic acid recover glucocorticoid resistance via PI3K-induced AP1, CRE and NFAT activation. *Phytomedicine* 2013; 20:295-302.
32. Sakamoto R1, Okano M, Takena H, Ohtsuki K. Inhibitory effect of glycyrrhizin on the phosphorylation and DNA binding abilities of high mobility group proteins 1 and 2 in vitro. *Biol Pharm Bull* 2001; 24:906-11.
33. Shearman DJ, Hetzel D. The medical management of peptic ulcer. *Annu Rev Med* 1979; 30:61-79.
34. Li W1, Li J, Sama AE, Wang H. Carbenoxolone blocks endotoxin-induced protein kinase R (PKR) activation and high mobility group box 1 (HMGB1) release. *Mol Med* 2013; 19:203-11.
35. Karatas H, Erdener SE, Gursay-Ozdemir Y, Lule S, Eren-Koçak E, Sen ZD, Dalkara T. Spreading depression triggers headache by activating neuronal Panx1 channels. *Science* 2013; 339:1092-95.
36. Kao TC, Shyu MH, Yen GC. Glycyrrhizic acid and 18beta-glycyrrhetic acid inhibit inflammation via PI3K/Akt/GSK3beta signaling and glucocorticoid receptor activation. *J Agric Food Chem* 2010; 58:8623-29.
37. Chang YL1, Chen CL, Kuo CL, Chen BC, You JS. Glycyrrhetic acid inhibits ICAM-1 expression via blocking JNK and NF-kappa B pathways in TNF-alpha-activated endothelial cells. *Acta Pharmacol Sin* 2010; 31:546-53.

ELEVATED LEVELS OF IL-2 AND IL-21 PRODUCED BY CD4⁺ T CELLS IN INFLAMMATORY BOWEL DISEASE

S. EBRAHIMPOUR¹, M. SHAHBAZI², A. KHALILF³, M.T. TAHOORI⁴, A. ZAVARAN HOSSEINI⁵, A. AMARI⁶, B. AGHILI⁷, S. ABEDIANKENARI³, H. MOHAMMADIZAD⁸ and M. MOHAMMADNIA-AFROUZI²

¹Infectious Diseases and Tropical Medicine Research Center, Babol University of Medical Sciences, Babol, Iran; ²Department of Immunology, School of Medicine, Babol University of Medical Sciences, Babol, Iran; ³Immunogenetics Research Center, Mazandaran University of Medical Sciences, Sari, Iran; ⁴Department of Immunology, Faculty of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran; ⁵Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran; ⁶Department of Immunology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran; ⁷Department of Microbiology and Immunology, School of Medicine, Qom University of Medical Sciences, Qom, Iran; ⁸Student Research Committee, Babol University of Medical Sciences, Babol, Iran

Received March 15, 2017 – Accepted April 21, 2017

CD4⁺ T cells are considered as a subset of cells that play a pivotal role in the development of inflammatory bowel disease (IBD). The aim of this study was to assess the levels of interleukin (IL)-2, IL-21 and their receptors produced by CD4⁺ T cells in patients with inflammatory bowel disease. Thirty-two patients with ulcerative colitis (UC) and mean age of 37.93±10.37 years, as well as 22 patients with Crohn's disease (CD) and mean age of 37.04±10.44 years, were studied. The healthy controls (HC) included 31 subjects with a mean age of 36.7±10.48 years. Peripheral blood mononuclear cells (PBMCs) were isolated from all the participants. The CD4⁺ T cells were isolated and the expression of IL-2 and IL-21 and also their receptors were examined by flow cytometry. The level of IL-2⁺ cells was significantly increased in UC patients compared with HC (40.71±6.04 vs 37.24±6.54, respectively, p=0.04). The level of IL-21⁺ cells was also significantly elevated in CD patients compared with HC (4.44±1 vs 3.83±0.74, respectively, p=0.02). Furthermore, we found a significant positive correlation between clinical activity index (CAI) and IL-21⁺ cells. According to the results, we hypothesize that the elevated level of IL-2⁺ and IL-21⁺ T cells and a positive correlation between IL-21⁺ cells with CAI in UC patients may contribute to the pathogenesis of disease. Moreover, the assessment of cells producing such cytokines constitutes a potential diagnostic and therapeutic strategy for IBD.

Inflammatory bowel disease (IBD) is a chronic recrudescent inflammatory condition of the gastrointestinal tract which is recognized as a serious cause of digestive system disease in children

and adults (1). Ulcerative colitis (UC) and Crohn's disease (CD) are two major clinicopathological subgroups of IBD (2, 3). IBD is a complex and multifactorial disorder, and the whole perception of

Key words: inflammatory bowel disease, CD4⁺ T cells, IL-2, IL-21

Mailing address:

Dr Mousa Mohammadnia-Afrouzi,
Department of Immunology, School of Medicine,
Babol University of Medical Sciences, Babol, Iran
Tel.: +98112151198
e-mail: m.mousa2016@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.

pathophysiology regarding IBD requires an extensive understanding of the immune responses. Recent studies on IBD are attributed to improper immunity (Th1, Th2, Th17) toward antigens present in the normal bacterial flora of the gut. Since cytokines related to Th1, such as IFN- γ , IL-2, and IL-12, as well as cytokines associated with Th17, such as IL-17A, IL-21 and IL-23, are significantly increased in CD patients, whereas the level of cytokines associated with Th2-, such as IL-4 and IL-13, is enhanced in UC patients (4). However, these explanations do not clarify which of these factors has a dominant role in the pathogenesis. CD4⁺ T cells have critical role in mounting effectiveness of immune response against pathogens. In addition, the classical effector subsets in immune response, such as Th1 and Th2, were identified as another one of subsets, consisting of Th9, Th17 and regulatory T cell (Tregs). Th1 cells secrete IL-2 and IFN γ and are involved in cellular immunity. These cells were associated with organ-specific autoimmunity (5). Th2 cells generate IL-4 and IL-5 and are engaged in humoral immune responses (6). Th17 cells are involved in various autoimmune diseases including multiple sclerosis (MS), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and IBD. They secrete IL-17, IL-21, and IL-22 (7). Regulatory T cells are the important cell types that mediate immune homeostasis and tolerance. The regulatory capacity, is influenced by several factors, like IL-2 as well as inhibitory cytokines, contains IL-10, TGF- β , or IL-35 (8). IL-2 is secreted by stimulated lymphocytes such as T cells and B cells. It is involved in immune activation and proliferation of T cells, regulating the activities of white blood cells responsible for immunity and enhancing the epithelial cell restitution which also has effects on other cells. Moreover, IL-2 promotes the differentiation of T cells into T cell effectors when the primary T cell is also stimulated by an antigen (9). Since CD is considered as a Th1- mediated disease, findings of cytokine profiles have shown increased IL-2 level in inflamed mucosa of Crohn's disease patients, although that is conflicting with ulcerative colitis (10). Different types of CD4⁺ T cells have also been reported to produce IL-21, though the higher level of secretion is observed by T helper-17 cells and natural

killer T cells. IL-21 has also upgrade effects in experimental autoimmune encephalomyelitis (EAE). Moreover, in some studies it has been shown that the development of B cell-mediated autoimmune disease is related to an enhanced expression of IL-21 and IL-21R, particularly in serum (11). Studies have also described IL-21 deficiency as a new cause of common variable immunodeficiency (CVID) and acute IBD (12). However, other observations suggested that IL-21 is involved in the pathogenesis of IBD and that blocking IL-21R signaling may have a therapeutic potential in IBD (13).

There are limited data on the levels of IL-2 and IL-21 in patients with IBD. In other words, the relationship between cytokines of CD4⁺ T cells with disease activity in IBD is still unclear. Therefore, the purpose of this study was to investigate the levels of IL-2 and IL-21 and their receptors producing CD4⁺ T cells in patients with IBD.

MATERIALS AND METHODS

Patients and controls

Thirty-two patients (16 males and 16 females) aged 37.93 ± 10.37 years (range: 18–65 years) with ulcerative colitis and twenty-three patients (12 males and 11 females) aged 37.04 ± 10.44 years (range: 18–65 years) with Crohn's disease were studied. The healthy control group (HC) included 31 age- and sex-matched healthy subjects. All patients with UC and CD were recently diagnosed cases who had not received any treatment. The disease was diagnosed for at least one month (range 1–4 months) with median disease duration of 2 months in UC and three months in CD. The mean UC clinical activity index (CAI) was 13.06 ± 2.01 (range: 10–17) according to Lichtiger et al. (14). Also, the mean Crohn's disease activity index (CDAI) was 11.6 ± 2.25 (range: 9–16) based on Harvey–Bradshaw Index (Simplified CD Activity Index) (15). UC and CD patients were free from other diseases with possible effects on the immune responses, consisting of autoimmune disorders.

The current research protocol was approved by the ethics committee of Babol University of Medical Sciences. Informed consent forms were signed by all the participants in the study.

Isolation of PBMCs and CD4⁺ T cells

Peripheral blood mononuclear cells (PBMCs) were isolated from 10 ml freshly drawn venous blood using density gradient centrifugation (Biowest, Nuaille, France) at 400 g for 30 min at room temperature. The cells in intermediate phase were suspended in RPMI-1640 medium supplemented with 100 mg/mL streptomycin, 100 U/mL penicillin, 10% heat-inactivated fetal calf serum (FCS) and 2 mM L-glutamine (Invitrogen Life Technologies, Carlsbad, CA, USA).

CD4⁺ T cells were isolated by MACS method using CD4⁺ CD25⁺ Treg cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany). Briefly, CD4⁺ cells were isolated by negative selection using antibody cocktail (CD8, CD14, CD15, CD16, CD19, CD36, CD56, CD123, TCR γ/δ and CD235a) conjugated microbeads. The purity of isolated CD4⁺ T cells was > 92%.

Flow cytometry

One million isolated CD4⁺ T cells were re-suspended in 1 mL RPMI-1640 medium and incubated for 4 h at 37°C, 5% CO₂ in the presence of 25 ng/ml of phorbol myristate acetate (PMA), 1 μ g/ml of ionomycin (both of Sigma Chemical Co., St. Louis, MO, USA), and 4 μ l BD GolgiStop (BD Biosciences, San Jose, CA, USA). PMA and ionomycin were used for quantitation of IL-2 and IL-21 producing CD4⁺ T cells, but anti-CD2/CD3/CD28 conjugated MACSiBeads (Miltenyi Biotech) were used for the evaluation of IL-2R and IL-21R producing CD4⁺ T cells.

After incubation, the cells were stained with APC-conjugated anti-CD4 (Clone RPA-T4), PerCP-Cy5.5-conjugated anti-CD25 (Clone BC96) and PE-conjugated anti-IL-21R (Clone 2SX21R) monoclonal antibodies at room temperature in the dark for 15 min. For intracellular cytokine staining, the cells were fixed and permeabilized with Cytofix/ Cytoperm (BD Biosciences) according to the manufacturer's instructions. The cells were then stained with PE-Cy7-conjugated anti-IL-2 (Clone MQ1-17H12) and PE-conjugated anti-IL-21 (3A3-N2) monoclonal antibodies. All the conjugated mouse anti-human monoclonal antibodies and isotype control were purchased from BD Biosciences. Isotype controls were given to enable correct compensation and confirm antibody specificity. The stained cells were analyzed by FACS Calibur flow cytometer (BD, San Jose, CA, USA), and the

results were analyzed using CellQuest (BD Biosciences) and FlowJo (version 7.6.1; Tree Star Inc., Ashland, OR, USA) software. A minimum of 10⁵ events at lymphocyte gate was analyzed.

Statistical analysis

All the tests were performed by sixth version of GraphPad Prism software for Windows (GraphPad software, La Jolla, CA, USA). The variables were presented as means and standard deviations (SD). The differences between three groups were evaluated using parametric or non-parametric tests as per normality of the data distribution. Correlation analysis was performed by Pearson's correlation coefficient. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

Clinical characteristics of participants

Demographic and clinical data of patients with ulcerative colitis and Crohn's disease as well as healthy controls are presented in Table I. There were no significant differences in total white blood cell count ($\times 10^3/\text{mL}$) among UC patient group (8.53 ± 1.62), CD cases (8.63 ± 1) and healthy control group (8.43 ± 1). The percentage of lymphocytes in UC patient group (27.4 ± 4.7) was significantly lower than the healthy control group (30.41 ± 4.6 , $p = 0.02$), but did not show a significant difference compared to HC in CD cases (28.43 ± 4.26).

Cytokines and their receptors in ulcerative colitis and Crohn's disease patients

The level of IL-2⁺ cells was significantly increased in UC patients (40.71 ± 6.04) compared with healthy controls (37.24 ± 6.54 , $p = 0.04$). Moreover, the level of IL-21⁺ cells was significantly elevated in CD patients (4.44 ± 1) compared to healthy subjects (3.83 ± 0.74 , $p = 0.02$). There were no significant differences in the level of IL-2⁺, IL-21R⁺ and IL-21R⁺ cells between two groups (UC and HC), although these levels in UC patients (40.71 ± 6.04 , 32.45 ± 3.99 and 26 ± 3.93 , respectively) were higher than the healthy group (37.24 ± 6.54 , 30.83 ± 3.26 and 25.12 ± 3.39 , respectively). Moreover there were no significant differences in the level of IL-2⁺ and

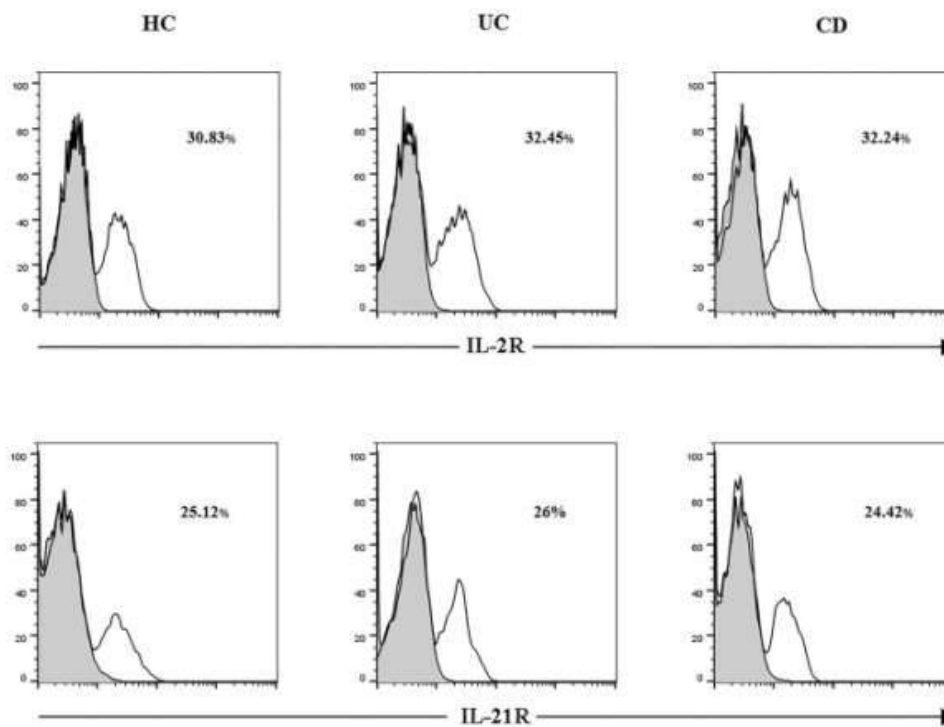


Fig. 1. The frequency of peripheral blood $IL-21^+$ $CD4^+$ cells and $IL-21R^+$ $CD4^+$ cells in patients and controls. $CD4^+$ cells were stimulated with anti- $CD2/CD3/CD28$ conjugated MACSiBead and were stained with labeled antibodies as described in methods. $IL-2R^+$ $CD4^+$ cell frequency is shown in the upper panel and percentage of $IL-21R^+$ $CD4^+$ cells is shown in the lower panel. HC: healthy control; UC: ulcerative colitis; CD: Crohn's disease.

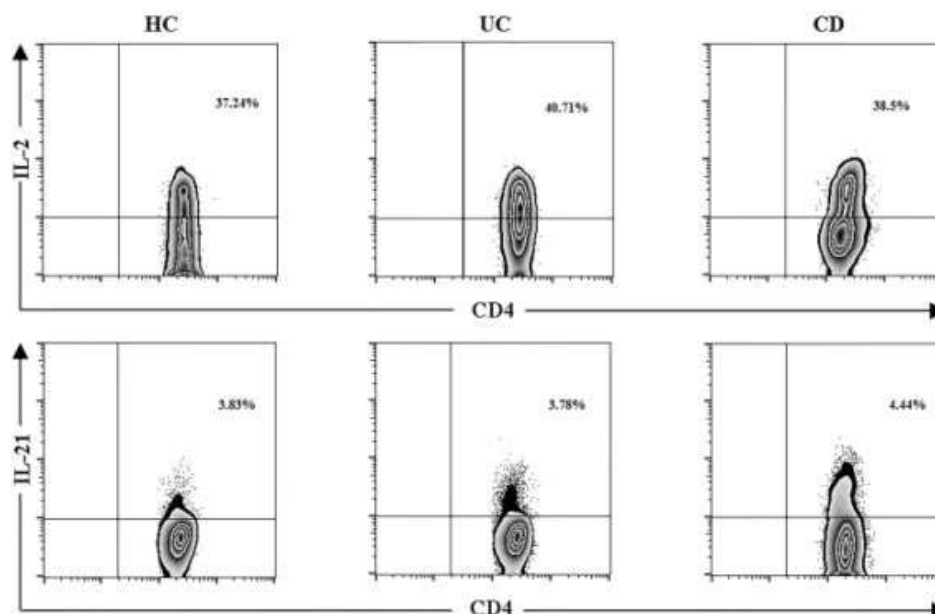


Fig. 2. The percentage of peripheral blood $IL-2^+$ $CD4^+$ cells and $IL-21^+$ $CD4^+$ cells in UC and CD patients and healthy controls (HC). $CD4^+$ cells from all subjects were stimulated with PMA, ionomycin and GolgiStop for 4 h, and then cells were stained with conjugated antibodies as mentioned in methods. The upper panel shows $IL-2^+$ $CD4^+$ cell frequency and the lower panel shows percentage of $IL-21^+$ $CD4^+$ cells. HC: healthy control; UC: ulcerative colitis; CD: Crohn's disease.

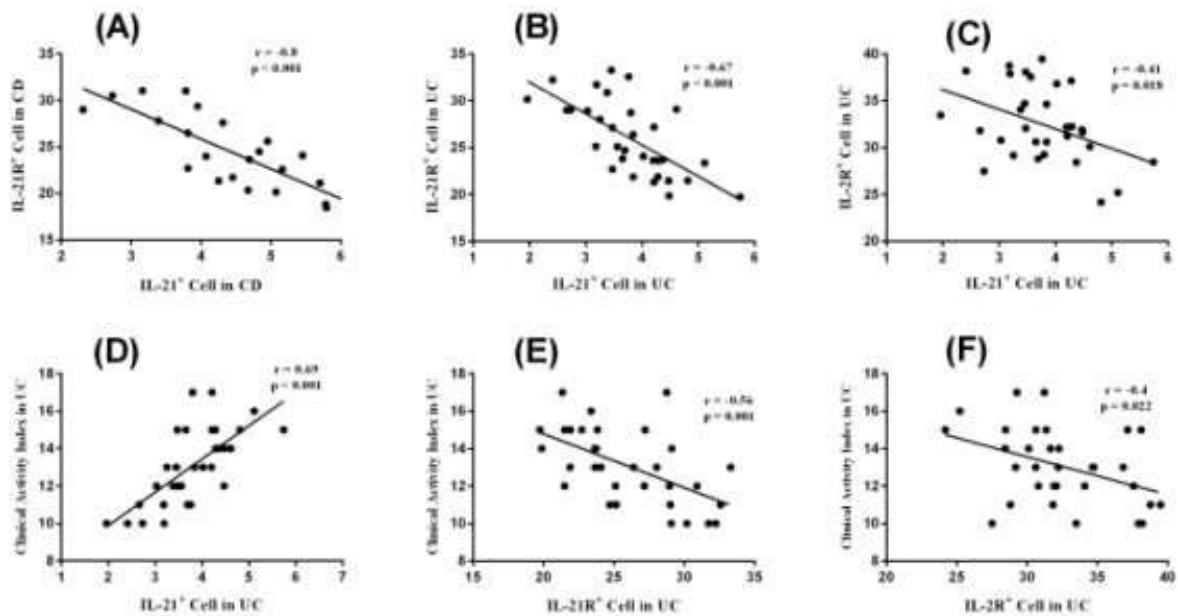


Fig. 3. Correlation between frequency of cytokines or cytokine receptors producing CD4⁺ cells together with CAI in UC and CD patients. There is negative correlation between IL-21R⁺ with IL-21⁺ in CD (A) and UC (B). Negative correlation is observed between IL-21⁺ with IL-2R⁺ in UC (C). Positive correlation is shown between IL-21⁺ and with CAI in UC (D). There is negative correlation between IL-21R⁺ and IL-2R⁺ with CAI in UC (E) and (F). HC: healthy control; UC: ulcerative colitis; CD: Crohn's disease.

Table I. Demographic and clinical data of patients included in the study.

Characteristic	UC patients	CD patients	Healthy controls
Number	32	23	31
Sex (male/female)	16/16	12/11	16/15
Age (years)	37.93±10.37	37.04±10.44	36.7±10.48
Disease duration (months)	2.2±1	2.7±1	-
WBC (×10 ³ /mL)	8.53±1.62	8.63±1	8.43±1
Clinical activity index (CAI)	13.06±2.01	11.6±2.25	

IL-2R⁺ cells between two groups (CD and HC), although these levels in CD patients (38.5 ± 4.36 , 32.24 ± 3.55 , respectively) were higher than healthy controls (Figs. 1-3).

Correlation between IL-21⁺ cells with IL-21R⁺ T cells in Crohn's disease patients

We observed that the level of IL-21⁺ cells was significantly negatively correlated to IL-21 R⁺ cells ($r = -0.8$, $p < 0.001$). There was no significant correlation between CDAI with any assessed cytokines or their receptors (Fig. 3).

Correlation of IL-21⁺ cells with IL-21R⁺ and IL-2R⁺ cells in ulcerative colitis patients

We achieved a significant negative correlation between the level of IL-21⁺ cells and IL-21 R⁺ cells ($r = -0.67$, $p < 0.001$). The level of IL-21⁺ cells was significantly negatively correlated to IL-2R⁺ cells ($r = -0.41$, $p = 0.018$) (Fig. 3).

Correlation of CAI with IL-21⁺, IL-21R⁺ and IL-2R⁺ cells in ulcerative colitis patients

There was a significant positive correlation between CAI and IL-21⁺ cells ($r = 0.69$, $p < 0.001$); furthermore, the significant negative correlation was found among CAI, IL-21R, and IL-2R⁺ cells ($r = -0.56$, $p = 0.001$ and $r = -0.4$, $p = 0.022$, respectively) (Fig. 3).

DISCUSSION

Inflammatory bowel disease is an immunological disease caused by erratic immune responses to gastrointestinal microflora. The two most common forms of IBD are ulcerative colitis and Crohn's disease, the pathogenesis of which is still somewhat unknown, although immune-mediated and autoimmune events may be involved. Many manuscripts have shown that T cell-mediated response plays an important role in the mechanism of IBD (16). Almost all these studies have reported that many inflammatory cells, such as antigen-presenting cells (APCs) and CD4⁺ T cells, present in the blood and intestinal mucosa of IBD patients produce IL-2, IL-21, and IL-21R, which are mainly

derived from activated CD4⁺ T cells. The cytokine responses in inflammatory bowel disease are the key pathophysiologic points in the disease initiation and extension (17). IL-2 has major roles in the immune system function and tolerance, via its direct effects on T cells. Some studies showed that cytokines could prevent autoimmune disorders by promoting the differentiation of Thymus-derived naive T cells into suppressor T cells (18). In our study, we observed the increasing number of IL-2⁺ cells in UC patients compared with healthy controls. Although, the IL-2 has a higher tendency to make contact with Treg cells as compared to other T-cell subsets, IL-2 is also as an autocrine growth factor for T lymphocyte, which causes proliferation and activated expansion of T lymphocytes (19). Thereby, the elevated level of IL-2-producing cells may increase the number of pathologically activated T cells in ulcerative colitis patients and lead to exacerbation of the disease. Moreover, decreased frequency and suppressive capacity of Treg cells were shown in UC patients (2). Similar to our findings, other reports presented that expression of IL-2 was increased in serum of IBD patients. In other words, they clearly showed that IL-2 also stimulates the function of some immune cells such as natural killer (NK) B cells and intervenes with the differentiation of Th17 cells, which have been proposed to play a key role in IBD (20). Furthermore, Therkelsen and colleagues revealed that in UC patients, the levels of IL-2, IL-5 and MIP-1 β were enhanced against controls (21). As a pro-inflammatory cytokine, IL-21 was the first regulator of lymphocyte functions. IL-21 is a product of CD4⁺ T and natural killer T cells (NKTs) that interfere in the immunity against tumor cells but full production of this cytokine has been correlated with the development of immune-inflammatory diseases (22, 23). The higher frequency of IL-21 producing CD4⁺ T cells and the correlation with IL-21R⁺ CD4⁺ T cells suggests that IL-21/IL-21R play a key role in CD. It was shown that IL-21 up-regulates the expression of transcription factors associated with Th1 response (24). Moreover, IL-21 seems to inhibit the Treg suppressive function and promotes the Th17 response (25). Therefore, the cytokine profile in CD has Th1/Th17 bias rather than Th2. Different

studies confirmed that IL-21 has a pathogenic role in the gut of patients with IBD, and also helps sustain the Th1 mucosal response in this disease. IL-21 enhances MMP secretion by fibroblasts without affecting gene expression (26). In a recent study, it was found that the level of IL-21 was significantly elevated in CD patients. In agreement with our findings, Monteleone et al. revealed that enhanced IL-21 was seen in both ileal and colonic CD. In other words, they have suggested that IL-21 contributes to the ongoing Th1 mucosal response in CD (27). Jian Ge et al. showed that there is enhanced production of IL-21 in the inflamed intestine of patients with UC (28). Also, other studies showed the involvement of IL-21- and IL-22-producing CD45RO⁺CD4⁺ T cells in CD. These patients had significantly higher levels of IL-21- and IL-22 in this disease (29).

CAI is the standard instrument for estimating clinical symptoms in UC patients. This research tool has been addressed by indices of quality of life for patients with ulcerative colitis (30). Several factors and elements, like cells and cytokines, may be associated with CAI. Some studies found that serum IL-12B levels were significantly correlated with CAI in UC patients. Thus, the cytokine can be used as a biomarker for the evaluation of disease activity in such patients (31). In other studies, a significant correlation was shown among CAI, serum concentrations of IL-6 and IL-17 in UC patients (32). In the present research, there was a significant positive correlation between clinical activity index and the level of IL-21⁺ cells in ulcerative colitis cases. Therefore, these findings confirm the key role of this cytokine in the immune-related pathogenesis of UC and also its contribution to the severity of clinical findings. In accordance with our results, studies on other autoimmune diseases, such as SLE, showed that patients with a higher activity index score (SLEDAI) had significantly higher amounts of serum IL-21 than those with a lower SLEDAI (33). The limitation of this study was the small number of patients that might have contributed to non-significant findings. Furthermore, as IBD was described as a group of disorders in which inflammation spreads into deep layers of tissues, the investigation of CD⁺ T cells in inflamed intestinal tissues is suggested.

Moreover, the evaluation of these cells and their other cytokines, such as IFN- γ , IL-13 and IL-17, is recommended.

In conclusion, we found evidence on the involvement of IL-2⁺ and IL-21⁺ cells in the pathogenesis of IBD. Our results indicated increased IL-2⁺ cells in UC patients and elevated IL-21⁺ cells in CD patients. Moreover, there was a positive correlation between IL-21⁺ cells and CAI in UC cases, indicating an important role of IL-21 in the severity of clinical findings of UC. These findings suggest that the levels of the above-mentioned cytokines constitute a promising diagnostic and therapeutic strategy for IBD.

ACKNOWLEDGEMENTS

All of the authors acknowledge the cooperation of the Student Research Committee, Babol University of Medical Sciences, Babol, Iran.

REFERENCES

1. Bamias G, Pizarro TT, Cominelli F. Pathway-based approaches to the treatment of inflammatory bowel disease. *Transl Res* 2016; 167:104-15.
2. Mohammadnia-Afrouzi M, Hosseini AZ, Khalili A, Abediankenari S, Amari A, Aghili B, Nataj HH. Altered microRNA expression and immunosuppressive cytokine production by regulatory T cells of ulcerative colitis patients. *Immunol invest* 2016; 45:63-74.
3. Imielinski M, Baldassano RN, Griffiths A, et al. Common variants at five new loci associated with early-onset inflammatory bowel disease. *Nat genet* 2009; 41:1335-40.
4. Xu X-R, Liu C-Q, Feng B-S, Liu Z-J. Dysregulation of mucosal immune response in pathogenesis of inflammatory bowel disease. *World J Gastroenterol* 2014; 20:3255-64.
5. Bluestone JA, Bour-Jordan H, Cheng M, Anderson M. T cells in the control of organ-specific autoimmunity. *J Clin Invest* 2015 ;125:2250-60.
6. Liao W, Lin JX, Leonard WJ. IL-2 family cytokines: new insights into the complex roles of IL-2 as a broad regulator of T helper cell differentiation. *Curr*

- Opin Immunol 2011; 23:598-604.
7. Veldhoen M. Th17 cells require you to chew before you swallow. *Immunity* 2017; 46:8-10.
8. Hasanjani Roushan MR, Bayani M, Soleimani Amiri S, Mohammadnia-Afrouzi M, Nouri HR, Ebrahimpour S. Evaluation of CD4⁺ CD25⁺ FoxP3⁺ regulatory T cells during treatment of patients with brucellosis. *J Biol Regul Homeost Agents* 2016; 30:675-82.
9. Mohammadnia-Afrouzi M, Shahbazi M, Damavandi SB, Ganji GF, Ebrahimpour S. Regulatory T-cell: Regulator of Host Defense in Infection. *J Mol Biol Res* 2017; 7:9-19.
10. Katz LH, Kopylov U, Fudim E, et al. Expression of IL-2, IL-17 and TNF-alpha in patients with Crohn's disease treated with anti-TNF antibodies. *Clin Res Hepatol Gastroenterol* 2014; 38:491-98.
11. Spolski R, Leonard WJ. Interleukin-21: Basic Biology And Implications For Cancer And Autoimmunity. *Annu Rev Immunol* 2008; 26:57-79.
12. Salzer E, Kansu A, Sic H, et al. Early-onset inflammatory bowel disease and common variable immunodeficiency-like disease caused by IL-21 deficiency. *J Allergy Clin Immunol* 2014; 133:1651-9.e12.
13. Liu Z, Yang L, Cui Y, et al. Il-21 enhances NK cell activation and cytolytic activity and induces Th17 cell differentiation in inflammatory bowel disease. *Inflamm Bowel Dis* 2009; 15:1133-44.
14. Lichtiger S, Present DH, Kornbluth A, et al. Cyclosporine in severe ulcerative colitis refractory to steroid therapy. *N Engl J Med* 1994; 330:1841-45.
15. Harvey R, Bradshaw J. A simple index of Crohn's-disease activity. *Lancet* 1980; 315:514.
16. Sanchez-Muñoz F, Dominguez-Lopez A, Yamamoto-Furusho JK. Role of cytokines in inflammatory bowel disease. *World J Gastroenterol* 2008; 14:4280-88.
17. Strober W, Fuss IJ. Pro-Inflammatory Cytokines in the Pathogenesis of IBD. *Gastroenterology* 2011; 140:1756-67.
18. Boyman O, Sprent J. The role of interleukin-2 during homeostasis and activation of the immune system. *Nat Rev Immunol* 2012; 12:180-90.
19. Malek TR, Bayer AL. Tolerance, not immunity, crucially depends on IL-2. *Nature Rev Immunol* 2004; 4:665-74.
20. Márquez A, Orozco G, Martínez A, et al. Novel association of the interleukin 2–interleukin 21 region with inflammatory bowel disease. *Am J gastroenterol* 2009; 104:1968-75.
21. Therkelsen SP, Hetland G, Lyberg T, Lygren I, Johnson E. Cytokine levels after consumption of a medicinal agaricus blazei murill-based mushroom extract, AndoSan™, in patients with Crohn's disease and ulcerative colitis in a randomized single-blinded placebo controlled study. *Scand J Immunol* 2016; 84(6):323-31.
22. Pallone F, Fina D, Caruso R, Monteleone G. Role of IL-21 in inflammatory bowel disease. *Expert Rev Clin Immunol* 2010; 6:537-41.
23. De Nitto D, Sarra M, Pallone F, Monteleone G. Interleukin-21 triggers effector cell responses in the gut. *World J Gastroenterol* 2010; 16:3638-41.
24. Tian Y, Zajac AJ. IL-21 and T cell differentiation: consider the context. *Trends Immunol* 2016; 37:557-68.
25. La Cava A. Tregs are regulated by cytokines: implications for autoimmunity. *Autoimmun Rev* 2008; 8:83-7.
26. Monteleone G, Caruso R, Fina D, et al. Control of matrix metalloproteinase production in human intestinal fibroblasts by interleukin 21. *Gut* 2006; 55:1774-80.
27. Monteleone G, Monteleone I, Fina D, et al. Interleukin-21 enhances T-helper cell type I signaling and interferon-γ production in Crohn's disease. *Gastroenterology* 2005; 128:687-94.
28. Ge J, Zhan G X, Liu J, et al. Elevated expression of interleukin-21 and its correlation to T-cell subpopulation in patients with ulcerative colitis. *Cent Eur J Immunol* 2015; 40:331-36.
29. Dige A, Støy S, Rasmussen TK, et al. Increased levels of circulating Th17 cells in quiescent versus active Crohn's disease. *J Crohn's and Colitis* 2013; 7:248-55.
30. Maleki I, Taghvaei T, Barzin M, Amin K, Khalilian A. Validation of the Persian version of the inflammatory bowel disease questionnaire (IBDQ) in ulcerative colitis patients. *Caspian J Intern Med* 2015; 6:20-4.
31. Lee HW, Chung SH, Moon CM, et al. The correlation of serum IL-12B expression with disease activity in patients with inflammatory bowel disease. *Medicine*

- (Baltimore) 2016; 95:e3772.
32. Mańkowska-Wierzbicka D, Swora-Cwynar E, Poniedziałek B, Adamski Z, Dobrowolska A, Karczewski J. Usefulness of selected laboratory markers in ulcerative colitis. *Eur Cytokine Netw* 2015; 26:26-37.
 33. Ahmed YM, Erfan DM, Hafez SF, Shehata IH, Morshedy NA. The association of single nucleotide polymorphism of interleukin-21 gene and serum interleukin-21 levels with systemic lupus erythematosus. *Egyptian J Med Human Gen* 2017; 18:129-36.

EXPRESSION OF T-LYMPHOMA INVASION AND METASTASIS FACTOR ON THE OCCURRENCE OF ORAL SQUAMOUS CELL CARCINOMA

B. SONG¹, L.F. WANG¹, XH. FAN², JH. ZUO¹ and YM. HUANG¹

¹Department of Oral and Maxillofacial Surgery, Binzhou Medical University Hospital, Binzhou, China; ²Ophthalmology Dept., Binzhou People's Hospital, Binzhou, China

Received February 16, 2017 – Accepted May 12, 2017

In recent years, many studies have found that tumor metastasis-related gene T-lymphoma invasion and metastasis-inducing factor 1 (TIAM1) had abnormal high expression in a variety of tumor cells; however, there are few studies regarding its expression in oral squamous cell carcinoma (OSCC). This study aimed to observe the expression of TIAM1 in OSCC and investigated its clinical significance. The expression of TIAM1 in tissues from 120 cases of OSCC and oral mucosa from 40 normal cases was detected by immunohistochemistry, and the relationship between the expression of TIAM1 and the clinicopathological parameters of OSCC was analyzed. The positive expression rate of TIAM1 in the OSCC tissues was significantly higher than that in the normal oral mucosa (92.5% vs 0%). With the decrease of histological differentiation of OSCC, the increase of tumor node metastasis (TNM) stage and the occurrence of lymph node metastasis, the TIAM1 staining positive rate was gradually increased, and the difference was statistically significant ($P < 0.05$). However, the expression of TIAM1 in the OSCC tissues was in no correlation with the gender and age of the patients. The expression of TIAM1 is closely related to the occurrence, development and metastasis of OSCC, and it can be used as a new marker for reflecting its biological behaviors.

Oral squamous cell carcinoma (OSCC) is a common mucosal epithelial tumor which has an incidence of 80% among oral and maxillofacial malignant tumors; the survival rate of patients with OSCC is lower than 60% (1). Currently, the efficacy of surgery-based comprehensive treatment is not favorable, and surgery can cause substantial damage to patients, which may directly affect their quality of life (2). Postoperative recurrence and metastases are important factors affecting the prognosis of OSCC. Therefore, exploring the molecular mechanism of the occurrence and development of OSCC and finding

the markers for genetic diagnosis and treatment targets are of great significance to the early diagnosis, treatment and prognosis determination (3).

T-lymphoma invasion and metastasis-inducing factor 1 (TIAM1) is a proto-oncogene isolated from the highly invasive variant of T-lymphoma cells, which is an important tumor metastasis-promoting gene. Belonging to the Db1 family, TIAM1 has multiple phosphorylation sites. The expression level of TIAM1 in normal human smooth muscle cells has been found to be moderate, and the expression level of TIAM1 lower in cardiomyocytes, bronchial

Key words: T-lymphoma invasion and metastasis factor 1, TNM stage, oral squamous cell carcinoma

Mailing address:

Dr Bing Song,
No.661 Huanghe 2nd Road,
Department of Oral and Maxillofacial Surgery,
Binzhou Medical University Hospital,
Binzhou, 256603, China
e-mail: songbsbing@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.

epithelial cells, hepatocytes, renal tubular epithelial cells and neurons, but no expression was found in other normal tissues (4). TIAM1 was highly expressed in breast cancer, bladder cancer, pancreatic cancer, melanoma and other malignant tumors, and it was closely related to tumor differentiation, invasion and metastasis (5). However, the expression of OSCC and its mechanism are rarely reported. In order to investigate the expression of TIAM1 in OSCC and its relationship with clinicopathological factors, this study investigated the distribution characteristics of TIAM1 in OSCC tissues by immunohistochemistry.

MATERIALS AND METHODS

General data

Specimens used in this study were from the OSCC patients who were admitted to the Department of Oral and Maxillofacial Surgery in the Binzhou Medical College Hospital, Shandong, China from May 2011 to May 2013. Tissue paraffin specimens were randomly collected from 120 cases, and the diagnosis followed the Chinese standards for cancer diagnosis and treatment. There were 78 males and 42 females aged from 35 to 63 years, 49 patients 50 years and over, and 71 patients over 50 years. According to the TNM staging standards of oral and maxillofacial tumor released by the International Anticancer Alliance (2002), the specimens were classified into 49 cases of tongue cancer, 33 cases of buccal cancer, 11 cases of gum cancer, 11 cases of oropharyngeal carcinoma, 9 cases of oral cancer, and 7 cases of carcinoma of the hard palate, and there were 81 cases of highly-differentiated squamous cell carcinoma and 39 cases of moderately and poorly differentiated squamous cell carcinoma. As to TNM stages, there were 28 cases of stage I, 49 cases of stage II, 22 cases of stage III and 21 cases of stage IV. There were 48 cases of positive lymph node metastasis and 72 cases of negative. Moreover, normal oral mucosa from 40 patients undergoing maxillofacial trauma surgery in the same department were taken as controls. All patients were at the first onset, had not previously undergone radiotherapy, chemotherapy or any other biological treatments, were confirmed by pathological diagnosis, and had complete clinical pathological data. This study was approved by the ethics committee of the Binzhou Medical University Hospital, and all the patients signed informed consent.

Reagents

The conventional immunohistochemical SP method was adopted. Immunohistochemistry kit (SP 9000 kit) and color development kit (DAB kit) were purchased from Beijing Zhongshu Biotechnology Co., Ltd., China. Rabbit anti-human TIAM1 polyclonal antibody was purchased from Santa Cruz Corporation, USA.

Immunohistochemical detection

Paraffin specimens were sliced into 4- μ m thick sections, were baked at 65°C for 4 h, conventionally dewaxed and hydrated, and blocked by 3% hydrogen peroxide solution. Then endogenous peroxidase was inactivated for 10 min and then the sections were rinsed by phosphate buffer solution (PBS) three times, 5 min each time. Antigens were repaired by microwave heating and rinsed by PBS three times, 5 min each time. Then the sections were sealed by normal sheep serum working fluid at 37°C for 10 min, and 60 μ L of rabbit anti-human TIAM1 polyclonal antibody which had been diluted in a ratio of 1:200 was added. They were transferred to a wet box and cultured at 4°C overnight for 16 h, after which, they were kept at a constant temperature of 37°C for 45 min. The sections were incubated at 37°C for 30 min after 60 μ L of En Vision mice-rabbit second antibody was added. Next, 60 μ L of S-A/HRP (reagent C) was added, followed by incubation at 37°C for 30 min in the wet box. Color development was performed using 0.04% DAB for 5 min, and reaction time was controlled under the microscope. Finally, the sections were stained by hematoxylin, dehydrated, and mounted by neutral gum. Positive sections were used as positive controls, and PBS was used to replace primary antibody as a blank control.

Determination of immunohistochemical results

All histopathological sections were observed by pathologists in an independent double-blinded way, and the semi-quantitative method was used to determine the results. The scores were determined based on the percentage of positive cells. When the number of positive cells was less than 5%, it was scored as 0 points, 6-25% as 1 point, 26-50% as 2 points, 51-75% as 3 points, and more than 75% as 4 points. Positive cells with no color were scored as 0 points, yellow as 1 point, brown-yellow as 2 points, and brown as 3 points. The calculation formula of the total score was: total score=percentage positive

cells×coloring degree of positive cells. The determination standards were as follows: 0 points was determined as negative (-), greater than 0 points as positive, 1 to 4 points as weak positive (+), 5 to 8 points as positive (++), and 9 to 12 points as strong positive (+++). Redetermination was required if the difference of the results of the two pathologists was more than 3.

Statistical analysis

The data were analyzed by SPSS ver. 20.0. The expression positive rate of the normal oral mucosa tissues and OSCC tissues were compared by χ^2 test, the categorical data of the pathological parameters was analyzed by non-parametric rank sum test. Difference was considered as statistically significant if $P < 0.05$.

RESULTS

Expression of TIAM1 in OSCC tissues and normal oral mucosa

The immunohistochemical staining results of TIAM1 in the normal human oral mucosa, OSCC tissue are shown in Fig. 1. Yellow-brown particles precipitation indicated that TIAM1 was positive. The nest of squamous carcinoma and the cytoplasm of cells in the tumor invaded sites were the major staining sites of TIAM1 with positive expression.

In the normal oral mucosa, the staining result of TIAM1 was negative (0/40, 0%), but 111 out of 120 OSCC tissues suggested positive staining (92.5%);

the difference was statistically significant ($P < 0.05$). In the OSCC tissues, there were 9 cases of “-” TIAM1 expression (7.5%), 26 cases of “+” (21.7%), 55 cases of “+ +” (45.8%), and 30 cases of “+++” (25.0%) (Table I).

Relationship between TIAM1 and clinicopathological features of OSCC

The expression of TIAM1 in 120 cases of OSCC tissues was in a correlation with age, gender, TNM stage and lymph node metastasis (Table II).

According to the results of non-parametric rank sum test, the expression of TIAM1 in the OSCC tissues was in no correlation with the gender ($P = 0.751$) and age ($P = 0.262$) of patients; the difference of expression of OSCC tissues with different differentiation degrees was significantly significant ($P = 0.019$) (Fig. 2).

There was a significant difference in expression of OSCC at different TNM stages (Fig. 3). The difference between stages I-II and III suggested $Z = 2.972$ and $P = 0.003$; the difference between stages I-II and IV suggested $Z = 3.471$ and $P = 0.001$; and the difference between stages I and II suggested $Z = 0.418$ and $P = 0.672$. TIAM1 expression in OSCC with or without lymph node metastasis also suggested a statistically significant difference ($P = 0.004$) (Fig. 4). Therefore, it could be concluded that the expression of TIAM1 was in a close correlation with the pathological grade, clinical stage and lymph node



Fig. 1. The expression of TIAM1 protein in the normal oral mucosa and OSCC tissues (DAB staining, X400). A: negative staining of TIAM1 protein in the normal oral mucosa; B: positive staining of TIAM1 protein in the highly differentiated OSCC tissues; C: positive staining of TIAM1 protein in the moderately and lowly differentiated OSCC tissues

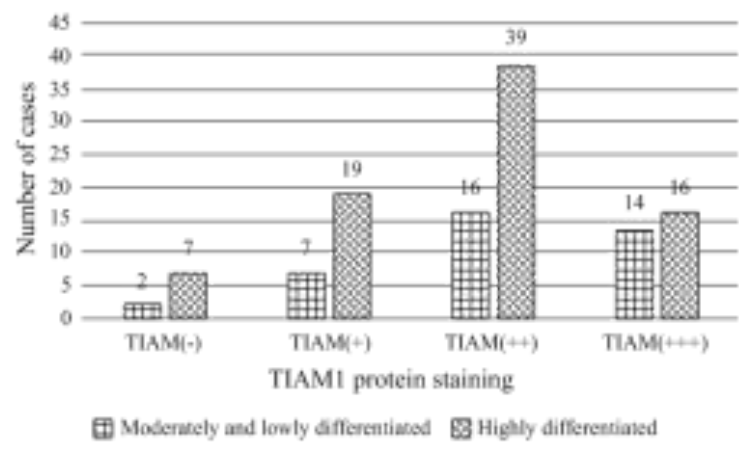


Fig. 2. Expression of *TIAM1* protein in OSCC tissues with different pathological grades

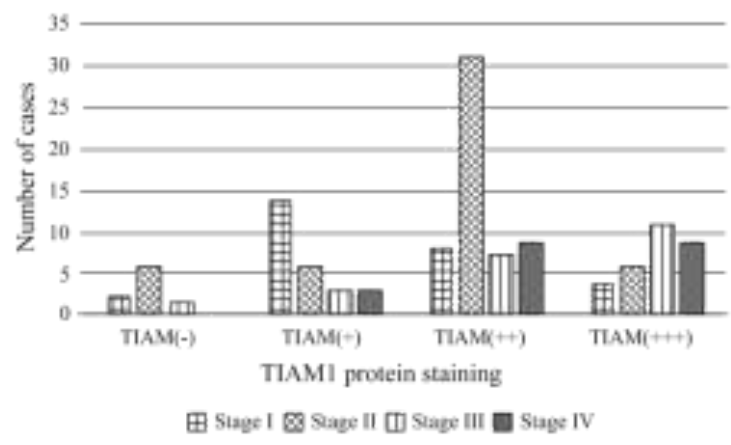


Fig. 3. Expression of *TIAM1* protein in OSCC tissues with different clinical stages

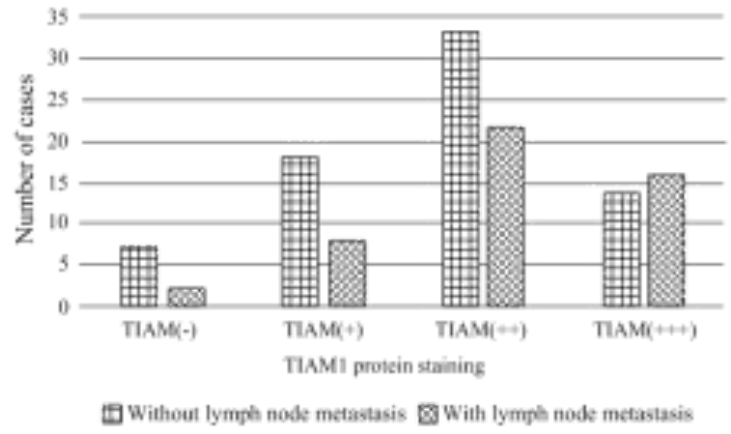


Fig. 4. Expression of *TIAM1* protein in OSCC tissues with or without lymph node metastasis

Table I. *The expression of TIAM1 in the normal oral mucosa and OSCC tissues.*

Category	TIAM1 expression			
	Negative	Weak positive	Positive	Strong positive
Normal oral mucosa (N=40)	40 (100.0)	0(0.0)	0(0.0)	0(0.0)
OSCC (N=120)	9(7.5)	26(21.7)	55(45.8)	30(25.0)

[N(%)];*In the comparison of the positive rate of TIAM1 between normal oral mucosa and OSCC tissues, $\chi^2=56.33$, $P<0.005$.*

Table II. *The correlation between the expression of TIAM1 protein in the OSCC tissues and clinical pathological parameters.*

Variables	N	TIAM1 expression				Z	P
		Negative	Weak positive	Positive	Strong positive		
Gender						0.322	0.751
Male	78	5	16	35	20		
Female	42	4	10	20	10		
Age (years)						1.134	0.262
>50	71	6	16	31	18		
≤50	49	3	10	24	12		
Histological differentiation						2.344	0.019
Moderate and low differentiation	39	2	7	16	14		
High differentiation	81	7	19	39	16		
TNM staging						16.437	0.001
Stage I	28	2	14	8	4		
Stage II	49	6	6	31	6		
Stage III	22	1	3	7	11		
Stage IV	21	0	3	9	9		
Lymphatic metastasis						7.625	0.004
Without	72	7	18	33	14		
With	48	2	8	22	16		

metastasis of OSCC. The positive expression of TIAM1 in the poorly differentiated OSCC was higher than that in the highly differentiated OSCC. The expression of TIAM1 in the OSCC at stage III and stage IV were higher than that at stage I-II. The expression of TIAM1 in the OSCC with lymph node metastasis was higher than that without lymph node metastasis.

DISCUSSION

The occurrence, development and metastasis of OSCC are related to cell proliferation caused by oncogene activation, inactivation of tumor suppressor genes and reduced apoptosis caused blocked apoptotic process (6). TIAM1 has been the

research focus in the field of tumor metastasis since it was isolated from T lymphoma cells of mouse and identified by Habets et al. (7) from the Dutch Cancer Institute. Generally, TIAM1 is not expressed or is lowly expressed in other normal tissues except the human brain and testicular tissues. Malliri et al. (8) found that the incidence of skin cancer in mice was significantly reduced and tumor progress was slow when TIAM1 gene was knocked out. Engers et al. (9) reported that prostate cancer patients with an overexpression of TIAM1 in the cancer tissues had a poor prognosis. In addition, the high expression of TIAM1 in the cells of breast cancer, pancreatic cancer, esophageal squamous cell carcinoma, head and neck squamous cell carcinoma and other tumors also has been confirmed, and the expression level was closely related to the invasion and metastasis ability of tumor cells (10).

This study found high expression of TIAM1 in the OSCC tissues and weak expression in the normal mucosal tissues, suggesting TIAM1 could be taken as an important marker for the detection of OSCC. The expression of TIAM1 was in no correlation with the gender and age of the patients, but was in an obvious correlation with the pathological level, clinical stages and lymph node metastasis. This result was consistent with the research of Wang et al. (11), which concerned the expression of TIAM1 in head and neck squamous cell carcinoma, and the study by Liu et al. (12) which focused on the clinicopathological relationship between TIAM1 and esophageal squamous cell carcinoma.

The present study suggested that TIAM1 is a useful indicator for evaluating the malignant degree of OSCC and may be used for reflecting the biological behaviors of OSCC. This work is helpful to the prediction of the infiltration, metastasis and prognosis of OSCC.

REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; 61(2):69-90.
2. Sinha N, Panda PK, Naik PP, Maiti TK, Bhutia SK. Abrus agglutinin targets cancer stem-like cells by eliminating self-renewal capacity accompanied with apoptosis in oral squamous cell carcinoma. *Tumour Biol* 2017; 39(5):1010428317701634.
3. Ding X, Wang X, Gong Y, Ruan H, Sun Y, Yu Y. KLF7 overexpression in human oral squamous cell carcinoma promotes migration and epithelial-mesenchymal transition. *Oncol Lett* 2017; 13(4):2281-89.
4. da Silva SD, Ferlito A, Takes RP, et al. Advances and applications of oral cancer basic research. *Oral Oncol* 2011; 47(9):783-91.
5. Jemal A, Siegel R, Xu J, Ward E. Cancer Statistics 2010. *CA Cancer J Clin* 2010; 60(5):277-300.
6. Zhou H, Kann MG, Mallory EK, Yang YH, Bugshan A, Binmadi NO, Basile JR. Recruitment of Tiam1 to semaphorin 4d activates rac and enhances proliferation, invasion, and metastasis in oral squamous cell carcinoma. *Neoplasia* 2017; 19(2):65-74.
7. Li H, Cui X, Chen D, Yang Y, Piao J, Lin Z, Yan G, Shen D. Clinical implication of Tiam1 overexpression in the prognosis of patients with serous ovarian carcinoma. *Oncol Lett* 2016; 12(5):3492-98.
8. Yang H, Cai YC, Cao Y, et al. The prognostic value of Tiam1 protein expression in head and neck squamous cell carcinoma: a retrospective study. *Chin J Cancer* 2015; 34(12):614-21.
9. Kalavrezos N, Bhandari R. Current trends and future perspectives in the surgical management of oral cancer. *Oral Oncol* 2010; 46(6):429-32.
10. Bourguignon LY, Zhu H, Shao L, Chen YW. Ankyrin-Tiam1 interaction promotes Rac1 signaling and metastatic breast tumor cell invasion and migration. *J Cell Biol* 2000; 150(1):177-91.
11. Minard ME, Kim LS, Price JE, Gallick GE. The role of the Guanine nucleotide exchange factor tiam1 in cellular migration, invasion, adhesion and tumor progression. *Breast Cancer Res Treat* 2004; 84(1):21-32.
12. Habets GG, Scholtes EH, Zuydgeest D, et al. Identification of an invasion -inducing gene, Tiam-1, that encodes a protein with homology to GDP-GTP exchangers for Rho-like proteins. *Cell* 1994; 77(4):537-49.
13. Malliri A, Rygiel TP, van der Kammen RA, et al. The rac activator Tiam1 is a Wnt-responsive gene that modifies intestinal tumor development. *J Biol Chem*

- 2006; 281(1):543-48.
14. Engers R, Mueller M, Walter A, Collard JG, Willers R, Gabbert HE. Prognostic relevance of Tiam1 protein expression in prostate carcinomas. *Br J Cancer* 2006; 95(8):1081-86.
 15. Qi Y, Huang B, Yu L, Wang Q, Lan G, Zhang Q. Prognostic value of Tiam1 and Rac1 overexpression in nasopharyngeal carcinoma. *ORL J Otorhinolaryngol Relat Spec* 2009; 71(3):163-71.
 16. Wang S, Li S, Yang X, Yang S, Liu S, Liu B, Liu J. Elevated expression of T-lymphoma invasion and metastasis inducing factor 1 in squamous-cell carcinoma of the head and neck and its clinical significance. *Eur J Cancer* 2014; 50(2):379-87.
 17. Liu H, Wang X, Shi G, Jiang L, Liu X. Tiam1 siRNA enhanced the sensitivity of sorafenib on esophageal squamous cell carcinoma in vivo. *Tumour Biol* 2014; 35(8):8249-58.

EXPRESSION OF CELL MITOTIC PROGRESSION PROTEINS AND KERATINOCYTE MARKERS IN PORCINE BUCCAL POUCH MUCOSAL CELLS DURING SHORT-TERM, REAL-TIME PRIMARY CULTURE

A. BRYJA¹, M. DYSZKIEWICZ-KONWIŃSKA², J. BUDNA³, S. CIESIÓŁKA³,
W. KRANC¹, S. BORYS¹, M. JESETA⁴, O. URBANIAK⁵, D. BUKOWSKA⁶,
P. ANTOSIK⁶, M. BRUSKA¹, M. NOWICKI³, M. ZABEL^{3,7} and B. KEMPISTY^{1,3}

¹*Department of Anatomy, Poznan University of Medical Sciences, Poznan, Poland;*

²*Department of Biomaterials and Experimental Dentistry, Poznan University of Medical Sciences, Poznan, Poland;*

³*Department of Histology and Embryology, Poznan University of Medical Sciences, Poznan, Poland;* ⁴*Department of Obstetrics and Gynecology, University Hospital and Masaryk, University, Brno, Czech Republic;* ⁵*Department of Immunology, Pathophysiology and Veterinary Preventive Medicine, Wrocław University of Environmental and Life Sciences, Wrocław, Poland;* ⁶*Institute of Veterinary Sciences, Poznan University of Life Sciences, Poznan, Poland;*

⁷*Department of Histology and Embryology, Wrocław Medical University, Wrocław, Poland*

Received March 17, 2017 – Accepted June 16, 2017

The porcine model is often used in clinical trials. The pig has many fundamental anatomic, physiological and nutritional similarities to humans. Additionally, the European Medicines Agency (EMA) demands the use large animals in clinical studies. Oral mucosa has received special attention due to its regenerative properties. Oral tissue is composed of several types of cells including fibroblasts and keratinocytes. The porcine oral mucosa/buccal pouch mucosa has a cellular structure with defined proliferation and differentiated capability. In this study, we investigated the expression pattern of porcine buccal pouch mucosal cell proliferation and differentiation markers such as Ki-67, proliferating cell nuclear antigen (PCNA), and involucrin. We observed a clear monolayer culture of spindle-shaped, porcine buccal pouch mucosal cells during 168 h of real-time *in vitro* culture. The RTCA assays revealed parametric and progressive increases in proliferation after 72 h of IVC. We found an altered proliferation index (PI) in the replicated groups of experiments except through the 144-168 h proliferation period. The RT-qPCR results demonstrated a significant increase in Ki-67 and PCNA expression after 48, 120, and 168 h of IVC as compared to other culture periods ($P < 0.001$). The involucrin mRNA displayed increased expression after 168 h of IVC as compared to other periods. We observed a lack of PCR product at 24 h in the case of Ki-67 and both before IVC (0h) and after 24 h of IVC for PCNA mRNA. When we analyzed the three transcripts together, we found the highest expression of involucrin during each of the culture periods. It has been suggested that Ki-67, PCNA, and involucrin may be successfully used as markers of porcine buccal pouch mucosal cell proliferation and differentiation capability *in vitro*.

Key words: pig, buccal pouch mucosal cells, cell cycle, Ki-67, PCNA, involucrin

Mailing address:

Bartosz Kempisty PhD,
Department of Histology and Embryology,
Department of Anatomy, Poznań University of Medical Sciences,
6 Śwignickiego St., 60-781 Poznań, Poland
Tel./Fax: +48 61 8546418/8546440
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.

The oral mucosa, a mucous membrane, is histologically similar to the mucous membrane of the esophagus, cervix, and vagina; however, it is completely different from the gastrointestinal mucosa. The above-mentioned mucosae consist of two specific tissue components including surface epithelium and connective tissue. It has been clearly recognized that the oral mucosa structure reveals several different functional adaptations (1). The main adaptation of the oral mucosa is the result of evolutionary changes that take place during the long process of tissue specific differentiation. However, the oral mucosa also displays some individual specifications, which are specimen-dependent and non-heritable changes. The oral mucosa present in the buccal pouch is characterized by stratified squamous non-keratinized epithelium. It is well recognized that the specialization of the oral mucosa epithelium is a complex process involving the differentiation and proliferation of epithelial keratinocytes (2). However, changes in keratinocyte marker expression in real-time cell proliferation in pigs have not yet been investigated. In this paper, the primary culture of porcine buccal pouch mucosal cells was performed to investigate the course of the keratinization process during short-term, real-time cellular proliferation.

Cellular and tissue engineering allow for the investigation of proliferative and differentiation potential of most of the progenitor cells. Buccal mucosa is composed of buccal mucosa fibroblasts (BMFs), which, after induction by external signals, influence differentiation potential of surrounding tissue (3). However, application of these two cellular populations, such as buccal pouch mucosal keratinocytes and fibroblasts, in advanced regenerative medicine based on tissues grafts requires further exploration.

Since epithelial cells from a variety of tissues, including oral keratinocytes, are organized into multiple layers, the assessment of proper proliferative and differentiated stem cell populations in the tissue and cell culture can be problematic. Recent experiments have shown that keratinocyte stem cells (KSCs) belong to a specific cell population within the whole culture. These populations are undifferentiated both morphologically and biochemically and, more

importantly, they have increased proliferative potential (4).

The presence of Ki-67 in the G_1 , S, G_2 , and mitosis phase, as well as its total absence in G_0 , of the cell cycle makes it possible to include this antigen as a main cell cycle marker (5). Although the molecular structure and role of Ki-67 in regulation of the cell cycle, as well as its role as a marker of cancer cell growth progression, is well understood, its functional significance in normal tissues with respect to growth, proliferation, and differentiation in the physiological condition still needs further investigation. Moreover, this study aimed to investigate the differential expression and distribution of Ki-67 and other markers in porcine buccal pouch mucosal cells in relation to possible keratinocyte and BMF differentiation during short-term, real-time cellular proliferation. The results of these experiments made it possible to establish stable primary cell culture of buccal pouch mucosal cells for future investigation of buccal pouch mucosal stem cell (SC) animal model.

As it was observed that oral mucosa is composed of progenitor and stem cells, which have a proven capacity of long-term cultivation and high proliferative potential, this study measured the mitotic activity by changes in real-time cellular proliferation. The mitotic activity was analyzed by the expression of Ki-67 and PCNA. The presence of non-keratinized and keratinized epithelium was determined by involucrin expression.

MATERIALS AND METHODS

Animals

For this study, a total of 35 pubertal crossbred Landrace gilts bred on a local commercial 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 Ethics Committee.

Cell isolation and culture

After collecting the material, 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 Na_2HPO_4 , 2 mM KH_2PO_4 , pH 7.4). The surface of the buccal pouch was surgically removed using sterile surgical blades. The cell suspension obtained from this digestion was filtered through mesh to remove non-dissociated tissue fragments and then incubated with 0.05% collagenase I (Sigma Aldrich, Madison, USA) for 25 min at 38°C in a shaking water bath. Additionally, dispersed tissue was treated with trypsin. 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 $\mu\text{g}/\text{mL}$) and 0.1% BSA. The epidermal side of the mucosa was gently scraped with a scalpel to detach the keratinocytes. The resultant cell suspension was filtered through a 400-micron mesh and then washed three times by centrifugation (10 min at 200 g) with supplemented DMEM. The final cell pellet was resuspended in DMEM supplemented with 10% fetal calf serum (FCS; Sigma Aldrich, Madison, USA) and 10,000 U mL^{-1} penicillin G, 10 mg mL^{-1} streptomycin, and 25 $\mu\text{g mL}^{-1}$ 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% CO_2 . Once the keratinocyte cultures attained 70–80% confluency, they were passaged by washing with PBS, digested with 0.025% trypsin/ethylenediaminetetraacetic acid (Cascade Biologics, Portland, USA), neutralized by a 0.0125% trypsin inhibitor (Cascade Biologics, Portland, USA), centrifuged, and resuspended at a seeding density of 2×10^4 cells/ cm^2 . 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/F12, Sigma-Aldrich, USA) as mentioned above. The cells were cultured for 0-168 h at 38.5°C under 5% CO_2 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 confocal microscopy and 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.

Confocal microscopic observations of protein expression and distribution

Both at recovery and during each step of *in vitro* culture (every 24 hours), mucosal cells were collected and fixed using an acetone-methanol (1:1, v/v) solution for 10 min at 20°C and washed three times in PBS/PVP (0.2%). To block non-specific binding, the samples were incubated in 3% BSA in PBS with 0.1% Tween-20 for 30 min at room temperature (RT). Mucosal cells were incubated for 1 h at RT with either a mouse monoclonal anti-Ki-67 antibody [H300] (Santa Cruz, CA, USA), mouse monoclonal anti-PCNA antibody [FL-261] (Santa Cruz, CA, USA), or mouse monoclonal anti-involucrin antibody [SY5] (Santa Cruz, CA, USA). The antibodies were diluted 1:500 in PBS with 1.5% BSA and 0.1% Tween-20. After several washes with PBS containing 0.1% Tween-20, the samples were incubated with MFP488-labeled donkey anti-rabbit IgG (against Ki-67 and PCNA) or MFP488-labeled goat anti-mouse IgG (against involucrin) (MoBiTec GmbH, Germany) diluted 1:500 in PBS with 0.1% Tween-20 for 1 h at RT. After washing in PBS with 0.1% Tween-20, the mucosal cells were stained with 0.1 $\mu\text{g}/\text{ml}$ 4,6-diamino-2-phenylindole (DAPI, UltraCruz Mounting Medium, sc-24941; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and examined by an LSN 510 confocal system with an Olympus Fluoview 10i microscope. All confocal microscopic images were analyzed using Imaris 7.2 (BitPlane, Zurich, Switzerland) software. The protein expression pattern was estimated as the fluorescence intensity measured by Imaris 7.2 program.

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 Ki-67, PCNA, and involucrin transcripts in each sample was standardized to the internal standards ACTB and PBGD. 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 oocyte mRNAs in each sample were calculated relative to PBGD and ACTB levels. To ensure the integrity of these results, an additional housekeeping gene, 18S, was used as an internal standard to demonstrate that PBGD and ACTB mRNAs were not differentially regulated in buccal pouch mucosal cell groups. The gene for 18S rRNA expression has been identified as an appropriate housekeeping gene for use in quantitative PCR studies. Expression of PBGD, ACTB, and 18S mRNA 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 real-time quantification of the proliferation index. The experiments were carried out in a minimum three replicates. The results quantifying the cell proliferation index were obtained using an RTCA system. The differences were considered to be significant at $*P<0.05$, $**P<0.01$, and $***P<0.001$ for the quantitative analysis of protein expression. The software program GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA, USA) was used for the statistical calculations.

RESULTS

After utilization of the fluorescence intensity index and confocal microscopic assays, we investigated the Ki-67, PCNA, and involucrin

protein expression and distribution among porcine buccal pouch mucosal cells cultured *in vitro* for 168 h. The highest expression of involucrin was observed at 72 of *in vitro* culture (IVC) as compared to 24, 48, 96, 120, 144 and 168 h ($P<0.001$). However, no differences were observed in expression of Ki-67 and PCNA among each of culture periods during one week of cellular IVC (Fig. 1). After 72 h of IVC, the involucrin was distributed in the cell cytoplasm and nucleus, whereas after 144 and 168 h, it was primarily distributed in cell cytoplasm (Fig. 2). Similarly, the Ki-67 was localized within cell nucleus at 72 h of IVC and only in the cytoplasm after 144 and 168 h of IVC (Fig. 3). The PCNA was distributed in the cell cytoplasm after 144 and 168 h of IVC (Fig. 4).

The real-time proliferation index (PI) was assayed in eight groups of buccal pouch cells, and the experiments were replicated two times. An increase was observed in the PI after 72 h of IVC (log phase) (Fig. 5A). In the first step of proliferation (0-24h), differences were found in PI in one investigated group of cells ($P<0.05$) (Fig. 5B). After 48 h of IVC, we found differences in PI in six of eight groups of cells ($P<0.05$ and $P<0.01$, for respected groups) (Fig. 5C). Between 48 and 72 h of IVC, the PI differed significantly in two groups of cells ($P<0.05$ and $P<0.001$, respectively) (Fig. 5D). Similarly, after 96 h of IVC, differences were observed in PI in two groups of cells ($P<0.05$) (Fig. 5E). After 120 and 144 h of IVC, an altered PI was found in only one group ($P<0.05$) (Fig. 5F and 5G). No differences were found in PI between 144 and 168 h of IVC (Fig. 5H). The proper spindle-shaped cell morphology during the multiple steps of IVC was measured after each 24 h. An increase in cell proliferation was seen using contrast-phase microscopy at 10x, 20x, and 40x magnification (Fig. 6).

RT-qPCR analysis was performed to examine Ki-67, PCNA, and involucrin mRNA expression in porcine buccal pouch mucosal cells cultured for 168 h. The highest expression of Ki-67 was found after 168 h of IVC as compared to 24, 72, 96, and 144 h ($P<0.001$) (Fig. 7A). However, changes were not observed in Ki-67 expression between 48, 120, and 168 h of IVC. Moreover, the Ki-67 transcript was not detected in cells before IVC (0

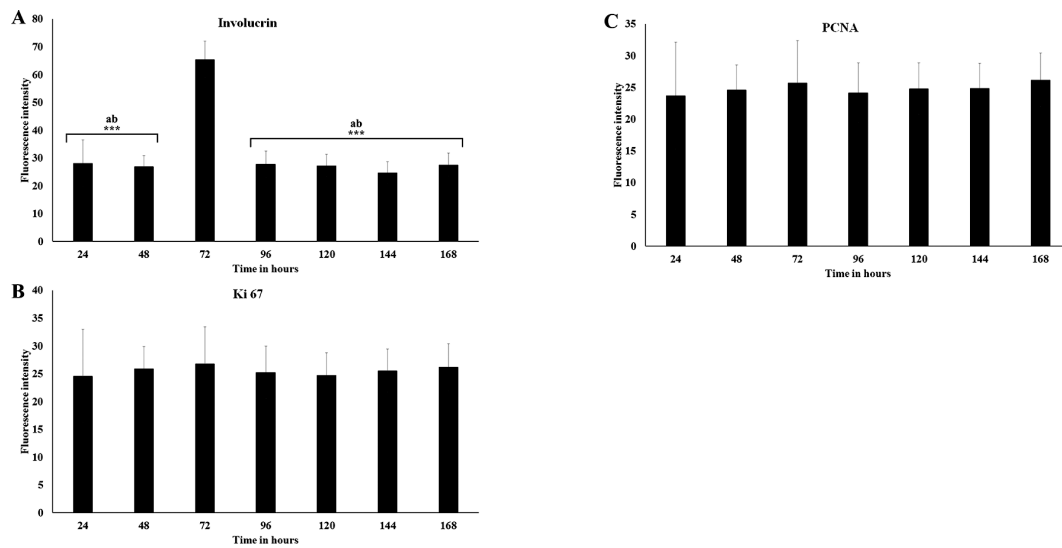


Fig. 1. Fluorescence intensity (FI) of involucrin, Ki-67, and PCNA protein expression. The fluorescence intensity (FI) of involucrin (A), Ki-67 (B), and involucrin (C) was analyzed using Imaris 7.2 (BitPlane, Zurich, Switzerland) software after 24 – 168 h of in vitro culture. Different superscripts display significant differences at the level of * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$). ab – different superscript representing statistical difference.

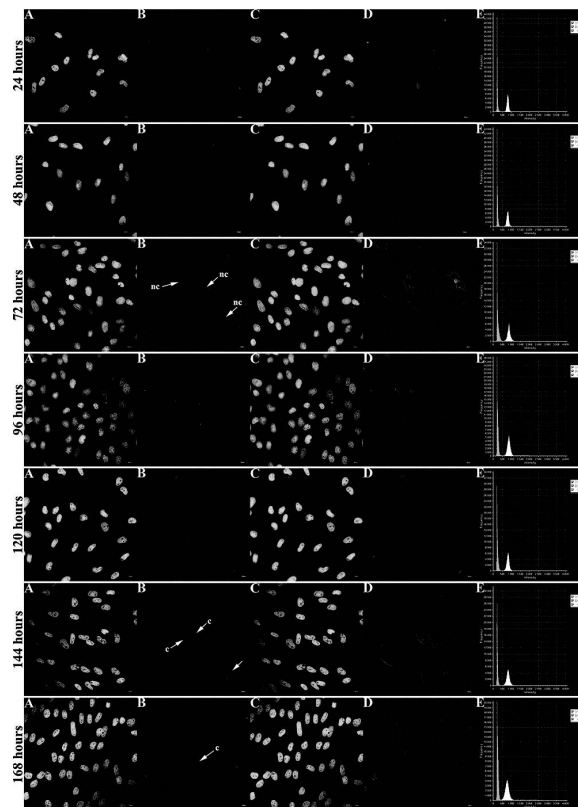


Fig. 2. Confocal microscopic observation of involucrin protein levels and cellular distribution in porcine buccal pouch oral mucosal cells during 168 h of culture. The porcine buccal pouch oral mucosal cells were collected from slaughtered animals, cultured for 168 h and, at each 24 h of culture, stained with DAPI in mineral oil followed by staining for porcine anti-involucrin. Cells were then treated with MFP488-labeled goat anti-mouse IgG (MoBiTec GmbH, Germany) diluted 1:500 in PBS with 0.1% Tween-20 for 1 h at RT. The scale bars represent 20 μm. a- DAPI, b- antibody stained cells, c- merge, d- Nomarski, e- histogram; n-nuclear; c-cytoplasmic.

h). Similarly, we observed increased expression of PCNA at 168 h of IVC as compared to 72, 96, and 144 h ($P<0.001$) (Fig. 7B). Additionally, differences were seen in PCNA mRNA expression at 48, 120, and 168 h of IVC ($P<0.05$). We did not detect the PCR product in templates collected at 0 and 24 h. The involucrin mRNA expression was increased at 168 h as compared to 24, 72, 96, 120, and 144 h of IVC ($P<0.01$), but the highest expression at 48 and 168 h was also observed ($P<0.001$) (Fig. 7C). Similar to Ki-67, we did not detect the involucrin

transcript in the template before IVC at 0 h. When compared Ki-67, PCNA, and involucrin mRNAs, involucrin had the highest expression compared to the other transcripts at 24, 72, 96, 144, and 168 h of IVC ($P<0.001$, $P<0.01$, $P<0.05$) (Fig. 7D). No differences were found in expression of these genes at 48 and 120 h of IVC.

DISCUSSION

Mammalian buccal pouch mucosal cells from

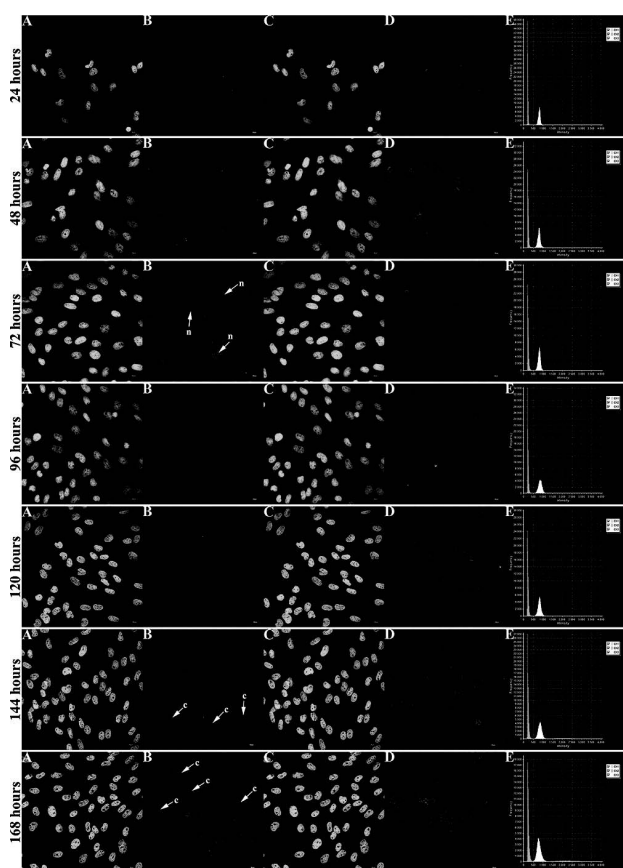


Fig. 3. Confocal microscopic observation of Ki-67 protein levels and cellular distribution in porcine buccal pouch oral mucosal cells during 168 h of culture. The porcine buccal pouch oral mucosal cells were collected from slaughtered animals, cultured for 168 h and, at each 24 h of culture, stained with DAPI in mineral oil following staining for porcine anti-Ki-67. Cells were then treated with MFP488-labeled donkey anti-rabbit IgG (MoBiTec GmbH, Germany) diluted 1:500 in PBS with 0.1% Tween-20 for 1 h at RT. The scale bars represent 20 μ m. a- DAPI, b- antibody stained cells, c- merge, d- Nomarski, e- histogram; n-nuclear; c-cytoplasmic.

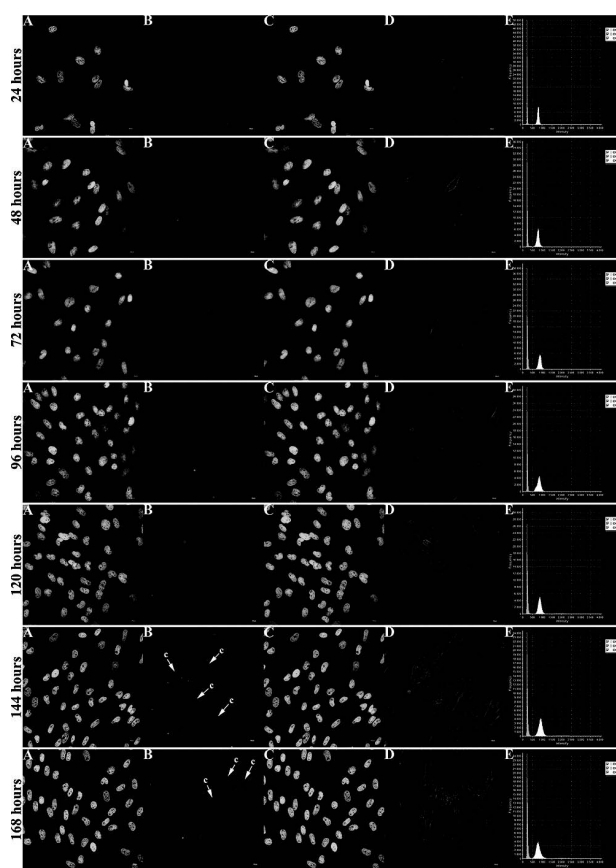


Fig. 4. Confocal microscopic observation of PCNA protein levels and cellular distribution in porcine buccal pouch oral mucosal cells during 168 h of culture. The porcine buccal pouch oral mucosal cells were collected from slaughtered animals, cultured for 168 h and, at each 24 h of culture, stained with DAPI in mineral oil following staining for porcine anti-PCNA. Cells were then treated with MFP488-labeled donkey anti-rabbit IgG (MoBiTec GmbH, Germany) diluted 1:500 in PBS with 0.1% Tween-20 for 1 h at RT. The scale bars represent 20 μ m. a- DAPI, b- antibody stained cells, c- merge, d- Nomarski, e- histogram; c-cytoplasmic.

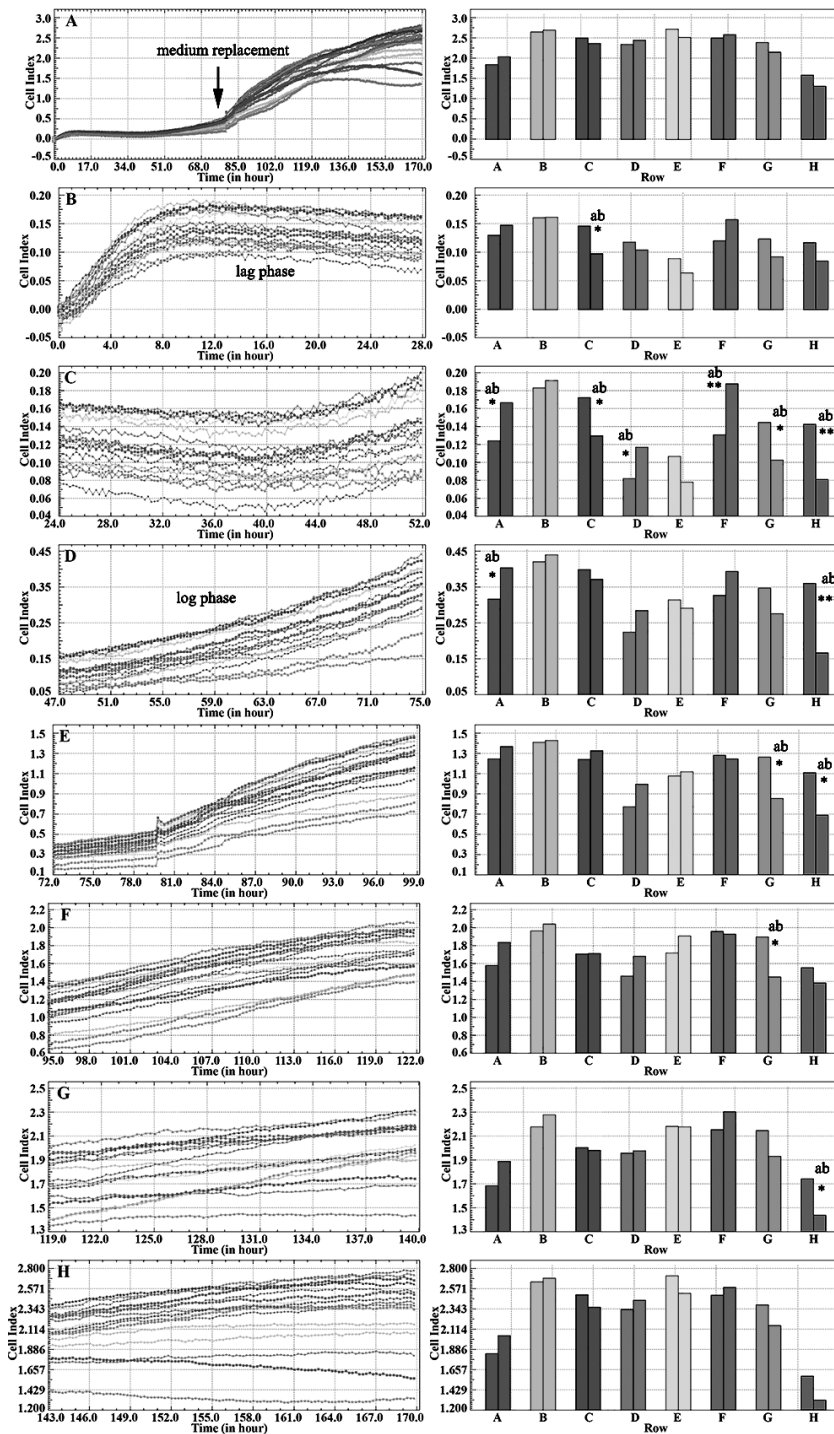


Fig. 5. Cell proliferation index (CI) of porcine buccal pouch mucosal cells cultivated for 168 h. Porcine buccal pouch mucosal cells were recovered from pubertal gilts and treated with collagenase for 10 min at 38.5°C. The cells were immediately transferred into an E-Plate 48 of a real-time cell analyzer (RTCA, Roche-Applied Science, GmbH, Penzberg, Germany). The experiment consisted of eight replicates involving the cultivation of the same population of collected cells. At each step, the cell proliferation index was assessed for real-time *in vitro* cultivation for the time periods of 0-168 h (A), 0-24 h (B), 24-48 h (C), 48-72 h (D), 72-96 h (E), 96-120 h (F), 120-144 h (G), and 144-168 h (H). The differences were considered to be significant at the level of * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. The different superscripts (ab) described significant differences.

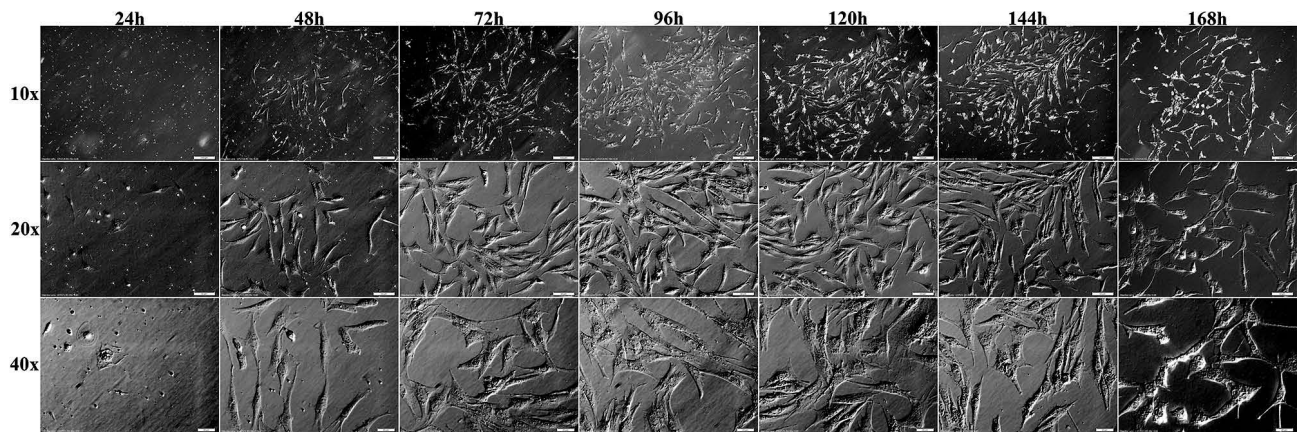


Fig. 6. Cellular proliferation, in vitro culture (IVC) monitoring, and Nomarski phase/contrast images. The porcine buccal pouch oral mucosal cells were cultured for 168 h using Dulbecco's Modified Eagle's Medium (DMEM/F12, Sigma-Aldrich, USA). Every 24 h of IVC, cells were monitored for proliferation index and morphological changes following achievement of confluency. Magnifications shown are 10x, 20x, and 40x.

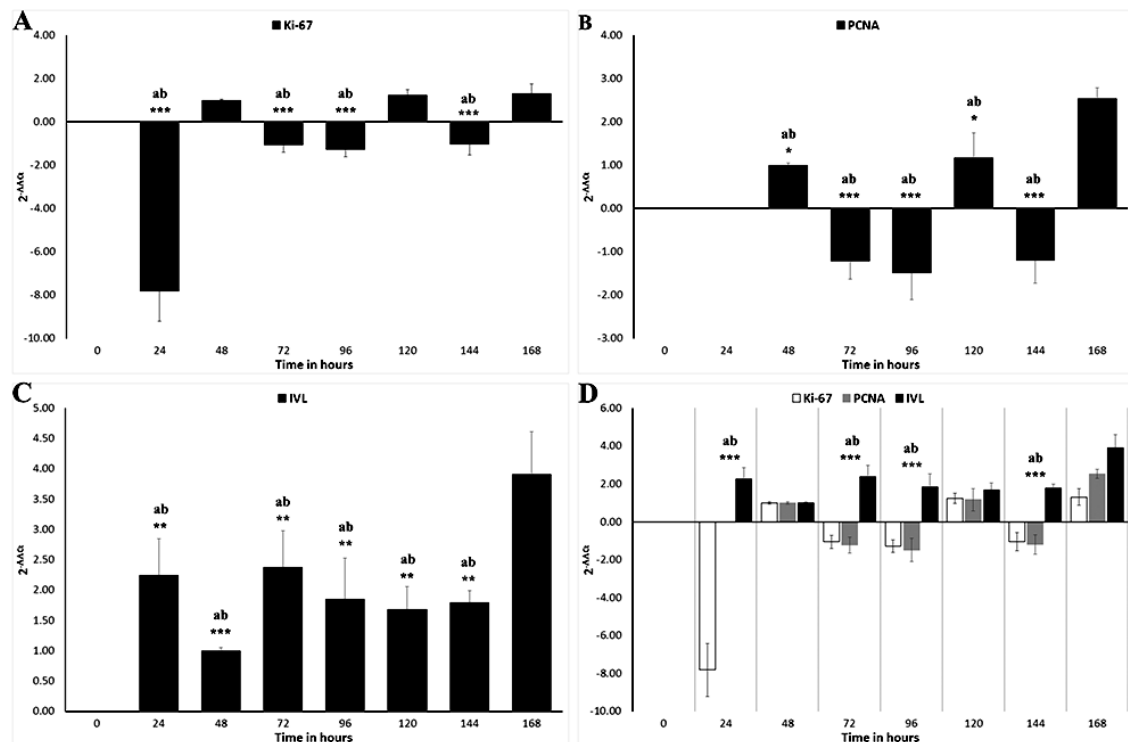


Fig. 7. Relative abundance of Ki-67, PCNA, and involucrin transcripts in porcine buccal pouch mucosal cells analyzed during 168 h of IVC. Porcine buccal pouch mucosal cells were isolated from pubertal gilts and immediately used to isolate RNA, which was reverse-transcribed into cDNA. The relative abundance of Ki-67 (A), PCNA (B), involucrin (C), and merge (D) mRNAs was evaluated by RT-qPCR analysis before and after each 24 h of IVC. The results are presented as the mean $\pm$ SEM with the level of significance shown as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ab – different superscript representing statistical difference.

Table I. *Oligonucleotide sequences used for RT-qPCR analysis.*

Transcript	Sequence (5'-3' direction)	Gene accession no.	Product size (bp)
PCNA	TTTGACGTATATGCCGAGA ATTTTATACTCTACAACAAGGGG	NM_001291925.1	293 bp
Ki-67	TGTCAGTCTCCACAACCAGAA TCTGTACTCCTGTTCTACGTT	XM_013983884.1	238 bp
Involucrin	TAAAGGGACAGCTGGAACAGG CTTTTCCAGGTGTTTCATCTCT	NM_214448.1	86 bp
PBGD	GAGAGTGCCCCTATGATGCT ATGATGGCACTGAACCTCT	NM_001097412.1	214 bp
β -actin	GGGAGATCGTGCGGGACAT CGTTGCCGATGGTGATGAC	DQ845171	141 bp
18S rRNA	GTGAAACTGCGAATGGCTC CCGTCGGCATGTATTAGCT	AB117609	105 bp

hamster, rat, mouse, and pig have been successfully used as models in biomedical research (6-8). In most cases, the purpose of the research was to investigate an ideal animal model of oral cavity carcinogenesis, drug delivery pathways, and/or oral mucosal sensitivity to drug exposure and treatment (9, 10). However, many of these studies concentrate on *in vivo* animal studies and/or *in vitro* experiments based on established cancer cell lines. Primary cell cultures of animal oral mucosa were rarely utilized. The primary culture of oral mucosal cells is most crucial in research focused on physiological and/or pathophysiological properties of oral cavity in both animals and humans. Recently, our experiments have concentrated on the biological properties of porcine buccal pouch mucosal cells as a model in biomedical research, particularly related to stemness specificity of these cells during primary, short-term cultivation. Current study includes research on Ki-67, PCNA, and involucrin mRNA and protein expression, protein distribution, and short-term cell proliferation in real-time.

Since Ki-67 is a well-known cell cycle progression marker, its expression is directly accompanied by real-time cell proliferation. In this study, we observed Ki-67 protein expression was higher after 144 and 168 h of IVC, although this result was not

statistically significant. The translocation of Ki-67 from the nucleus after 72 h to the cytoplasm after 144 and 168 h of IVC suggested that this protein may be localized in the nucleus and cytoplasm. Moreover, we postulated the differential distribution of this protein may be a result of a ligand-receptor complex formation accompanied by cell cycle progression. This observation is confirmed by the correlation between increased Ki-67 mRNA levels and the proliferation index as measured during real-time cellular proliferation after 168 h of IVC. There is no data that indicates the role of Ki-67 in physiological and/or pathophysiological properties of buccal pouch mucosal cells either in humans or animals. However, there are experiments investigating the role of Ki-67 in induction and growth of cancer, drug transport and its administration, but these data were typically related to oral mucosa (11, 12).

Recently, Sa et al. have searched for an ideal animal model for clinical research of oral mucosa (13). They investigated histological characteristics based on six morphological criteria and two immunohistochemical markers, such as Ki-67 and cytokeratin 19. Their research included rat, rabbit, cavy, sheep, dog, pig, and human oral mucosa. After immunohistochemical examination, a significant distribution of the Ki-67 protein in the suprabasal

layers of human and canine mucosa was observed. Additionally, the most proliferative cells were observed in the basal layer in both investigated species. The results suggested that human and dog oral mucosa display significant histological similarities, which makes using this a potential model in biomedical research.

On the other hand, Ki-67 has also been investigated as a marker of proliferative activity in esophageal, gastric, intestinal, and colon mucosal epithelial cells (14-17). Moreover, it is recognized that Ki-67 may be one of the mediators of immune activation after HIV infection (18, 19). Similar to our experiments, Khan et al., used primary and/or immortalized keratinocytes as a model to investigate the histological architecture of cultured cells versus oral mucosal tissue (20). They also examined the maturation, proliferation, and differentiation ability of keratinocytes in an IVC system (organotypic culture) compared to normal oral mucosa. The RT-qPCR was used to analyze E-cadherin, desmoglein-3, plakophilin, cytokeratin's (CK1, CK5, CK6, CK10, CK13), involucrin, and Ki-67 mRNA expression profiles. Immunohistochemical experiments indicated comparable results between keratinocyte *in vitro* proliferation and differentiation and normal oral mucosal tissue properties. The keratinocyte gene expression profile did not differ significantly when compared to normal tissue. Taking into account these results, in addition to our observations, we propose that the Ki-67 protein is not only the main cell cycle progression marker, but it is also involved in keratinocyte proliferation and differentiation during the formation of normal histological architecture of oral mucosa and/or buccal pouch mucosa in both animals and humans.

Currently, there is no data that ties PCNA expression in porcine buccal pouch mucosal cells to tissue proliferation and/or differentiation capability. However, several experiments have focused on hamster buccal pouch carcinogenesis. Lin et al. first described the PCNA expression and immune localization, as well as the nucleolus organizer region (NOR), in hamster buccal pouch mucosa after cancer induction (21). They found NOR was mostly distributed in the basal cell layer of

normal epithelium, whereas PCNA was similarly expressed in normal epithelium of hyperplastic tissue. Moreover, PCNA had increased expression in squamous cell carcinoma and significant expression in malignant cancer. The authors concluded that PCNA expression patterns correlates with NOR, and PCNA may be successfully used as a positive cancer cell cycle progression marker in a hamster model of carcinogenesis.

Similarly, in our experiments, the increased expression of PCNA mRNA and protein after 144 and 168 h of IVC was determined. Moreover, we observed cytoplasmic localization of PCNA after these culture periods, which may be associated with formation of peptide complexes in response to the higher proliferative activity of porcine buccal pouch mucosal cells. In research performed by Vinothkumar et al., a hamster model for oral buccal pouch mucosal carcinogenesis was used to investigate the proliferation and differentiation activities of mucosal epithelial cells in relation to cell death, survival and/or cell cycle progression (22). They used 7,12-dimethylbenz[a]anthracene (DMBA) as the method of cancer induction in buccal pouch mucosal cells. In addition to the inflammatory response genes, apoptotic, and angiogenic markers analyzed in the study, the expression of cell cycle progression proteins such as PCNA, cyclin D1, and c-fos were also measured. They concluded that geraniol reflected anti-inflammatory, anti-angiogenic, and anti-proliferative activity as the expression of marker genes was significantly decreased in the buccal pouch carcinogenesis hamster model. That supports the observation made by Sulfikkarali et al. where the effect of DMBA-induced hamster buccal pouch mucosal cells was also investigated (23). The results of this study confirmed previous observations that DMBA-induced oral epithelial carcinogenesis is significantly orchestrated by an altered expression pattern of PCNA. In conclusion, since PCNA is a well-known cell proliferation marker, the expression pattern of this gene was successfully used in research related to DMBA-induced hamster buccal pouch carcinogenesis. Furthermore, the anti-proliferative activity of several chemopreventive agents such as ferulic acid and andrographolide was recently

investigated in relation to a PCNA expression profile (24, 25).

Unfortunately, no data exist relating to an involucrin expression pattern and immunolocalization in buccal pouch mucosal cells of either animals or humans. However, involucrin expression, which is also involved in keratinization and epithelialization of oral mucosa, was recently investigated in human oral epithelium explants. Sundqvist et al. used a human buccal epithelial cell explant model, which was *in vitro* cultured in medium supplemented with MCDB 153 (26). They observed that transforming growth factor beta-1, used as media supplement, did not affect cell migration, cross linked envelope formation, or involucrin expression. Moreover, the supplementation of media with FBS, 12-O-tetradecanoylphorbol-13-acetate, and/or Ca^{2+} lead to squamous differentiation, inhibition of migration, increases in surface area, and higher involucrin expression. Finally, they concluded that human buccal epithelial cells undergo substantial modifications in *in vitro* proliferation and differentiation, which may be characterized as a significant specificity and plasticity of epithelial tissue. Together with our results, we postulate that oral buccal mucosal/epithelial cells show increased cell/tissue specialization with significant stemness capability, determined by the cell's ability to proliferate and differentiate *in vitro*.

Studies performed by Satoh et al. used reconstructed oral mucosa and a T3M-1 squamous cell carcinoma system cultured with 3T3 fibroblasts (27). Their experiments indicated that the Ki-67 index was higher in the T3M-1 system cultured with 3T3 fibroblasts. Additionally, the fibroblast culture significantly increased the expression of involucrin in T3M-1 cells. In conclusion, they observed that 3T3 fibroblasts increased the expression of involucrin, which is used as a keratinocyte differentiation marker. Moreover, T3M-1/3T3 seems to be a well-defined system for culture of differentiated squamous cell carcinoma. Our results indicate that the involucrin mRNA level was significantly increased after 168 h of IVC. However, the protein expression was higher after 72 h of IVC, which was significantly accompanied by progressive (logarithmic) growth of cells in real-time. After 72 h, involucrin was distributed both in

the nucleus and cytoplasm, whereas after 144 and 168 h of IVC, only cytoplasmic localization was detected. Taking into account these results, we propose that involucrin may not only be a marker of oral buccal cell keratinization/stratification/carcinogenesis but is also involved in oral epithelial/mucosal cell proliferation and differentiation in *in vitro* culture models.

In conclusion, all investigated genes (Ki-67, PCNA, and involucrin) are involved in the physiological/pathophysiological processes of oral epithelium and buccal pouch mucosal cells in pigs. They may also be used as markers of oral mucosal cell proliferation and differentiation *in vitro*, which, in some aspects, can be recognized as stemness capability markers.

REFERENCES

1. Squier CA, Kremer MJ. Biology of oral mucosa and esophagus. J Natl Cancer Inst Monogr 2001;7:15.
2. Lindberg K, Rheinwald JG. Three distinct keratinocyte subtypes identified in human oral epithelium by their patterns of keratin expression in culture and in xenografts. Differentiation 1990; 45:230-41.
3. Moharamzadeh K, Brook IM, van Noort R, Scutt AM, Thornhill MH. Tissue-engineered oral mucosa: a review of the scientific literature. J Dent Res 2007; 86:115-24.
4. Nakamura T, Endo K, 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.
5. Zamboni AC. Use of the Ki67 promoter to label cell cycle entry in living cells. Cytometry A 2010; 77:564-70.
6. Nagini S. Of humans and hamsters: the hamster buccal pouch carcinogenesis model as a paradigm for oral oncogenesis and chemoprevention. Anticancer Agents Med Chem 2009; 9:843-52.
7. Merne ME, Syrjanen KJ, Syrjanen SM. Systemic and local effects of long-term exposure to alkaline drinking water in rats. Int J Exp Pathol 2001; 82:213-19.
8. Kulkarni U, Mahalingam R, Pather SI, Li X, Jasti B. Porcine buccal mucosa as an *in vitro* model: relative

- contribution of epithelium and connective tissue as permeability barriers. *J Pharm Sci* 2009; 98:471-83.
9. Chen YK, Lin LM. DMBA-induced hamster buccal pouch carcinoma and VX2-induced rabbit cancer as a model for human oral carcinogenesis. *Expert Rev Anticancer Ther* 2010; 10:1485-96.
 10. Zhao X, Pang L, Qian Y, et al. An animal model of buccal mucosa cancer and cervical lymph node metastasis induced by U14 squamous cell carcinoma cells. *Exp Ther Med* 2013; 5:1083-88.
 11. Dwivedi N, Chandra S, Kashyap B, Raj V, Agarwal A. Suprabasal expression of Ki-67 as a marker for the severity of oral epithelial dysplasia and oral squamous cell carcinoma. *Contemp Clin Dent* 2013; 4:7-12.
 12. Bartlett JA, van der Voort MK. Understanding the oral mucosal absorption and resulting clinical pharmacokinetics of asenapine. *AAPS PharmSciTech* 2012; 13:1110-15.
 13. 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.
 14. Zhou X, Ma X, Wang Z, Sun C, Wang Y, He Y, Zhang H. Radiation-induced hyperproliferation of intestinal crypts results in elevated genome instability with inactive p53-related genomic surveillance. *Life Sci* 2015; 143:80-88.
 15. Kim A, Ahn SJ, Park dY, Lee BE, Song GA, Kim GH, Lauwers GY. Gastric crypt dysplasia: a distinct subtype of gastric dysplasia with characteristic endoscopic features and immunophenotypic and biological anomalies. *Histopathology* 2016; 68:843-49.
 16. Wang HQ, Liu AJ. Esophageal granular cell tumors: Case report and literature review. *World J Gastrointest Oncol* 2015; 7:123-27.
 17. Hormann N, Brandao I, Jackel S, Ens N, Lillich M, Walter U, Reinhardt C. Gut microbial colonization orchestrates TLR2 expression, signaling and epithelial proliferation in the small intestinal mucosa. *PLoS One* 2014; 9:e113080.
 18. Li H, Goepfert P, Reeves RK. Short communication: Plasmacytoid dendritic cells from HIV-1 Elite Controllers maintain a gut-homing phenotype associated with immune activation. *AIDS Res Hum Retroviruses* 2014; 30:1213-15.
 19. Mehandru S, Deren S, Kang SY, et al. Behavioural, mucosal and systemic immune parameters in HIV-infected and uninfected injection drug users. *J Addict Res Ther* 2015; 6:1-8.
 20. Khan E, Shelton RM, Cooper PR, Hamburger J, Landini G. Architectural characterization of organotypic cultures of H400 and primary rat keratinocytes. *J Biomed Mater Res A* 2012; 100:3227-38.
 21. Lin M, Li B, Zhou M, Duan K. The expression of PCNA and NOR in carcinogenesis procession of hamster buccal pouch mucosa. *Hua Xi Kou Qiang Yi Xue Za Zhi* 2000; 18:371-73.
 22. Vinothkumar V, Manoharan S, Sindhu G, Nirmal, Vetrivelvi V. Geraniol modulates cell proliferation, apoptosis, inflammation, and angiogenesis during 7,12-dimethylbenzaanthracene-induced hamster buccal pouch carcinogenesis. *Mol Cell Biochem* 2012; 369:17-25.
 23. Sulfikkarali N, Krishnakumar N, Manoharan S, Nirmal RM. Chemopreventive efficacy of naringenin-loaded nanoparticles in 7,12-dimethylbenz(a)anthracene induced experimental oral carcinogenesis. *Pathol Oncol Res* 2013; 19:287-96.
 24. Prabhakar MM, Vasudevan K, Karthikeyan S, Baskaran N, Silvan S, Manoharan S. Anti-cell proliferative efficacy of ferulic acid against 7, 12-dimethylbenz(a)anthracene induced hamster buccal pouch carcinogenesis. *Asian Pac J Cancer Prev* 2012; 13:5207-5211.
 25. Singh AK, Manoharan S, Vasudevan K, Rajasekaran D, Manimaran A, Suresh K. Anti-cell proliferative and anti-angiogenic potential of andrographolide during 7,12- dimethylbenz(a)anthracene induced hamster buccal pouch carcinogenesis. *Asian Pac J Cancer Prev* 2013; 14:6001-5.
 26. Sundqvist K, Liu Y, Arvidson K, Ormstad K, Nilsson L, Toftgard R, Grafstrom RC. Growth regulation of serum-free cultures of epithelial cells from normal human buccal mucosa. *In Vitro Cell Dev Biol* 1991; 27A:562-68.
 27. Satoh S, Toda S, Inokuchi A, Sugihara H. A new in vitro model for analyzing the biological behavior of well-differentiated squamous cell carcinoma. *Pathol Res Pract* 2005; 201:27-35.

EARLY AND LATE BEHAVIORAL CHANGES IN SCIATIC NERVE INJURY MAY BE MODULATED BY NERVE GROWTH FACTOR AND SUBSTANCE P IN RATS: A CHRONIC CONSTRICTION INJURY LONG-TERM EVALUATION

J.T. DA SILVA^{1,2}, B.G. EVANGELISTA², R.A.G. VENEGA², M.E. OLIVEIRA²
and M. CHACUR²

¹Department of Neural and Pain Sciences, School of Dentistry, University of Maryland, Baltimore, United States of America; ²Department of Anatomy, Institute of Biomedical Science-III, University of Sao Paulo, Sao Paulo, Brazil

Received January 14, 2017 – Accepted May 16, 2017

Chronic constriction injury (CCI) simulates the symptoms of chronic nerve compression, which is characterized by allodynia and hyperalgesia. Nerve growth factor (NGF) is released after nerve injury by immune and Schwann cells and transported in retrograde fashion to the dorsal root ganglion (DRG), resulting in increased synthesis of Substance P (SP) and the triggering of neuropathic pain. Here we performed long-term evaluation of allodynia and hyperalgesia in a CCI model, and evaluated the effects of NGF and SP on the peripheral and central nervous systems. Most previous studies have shown deficits and molecular changes 14 days after surgery, however, the long-term effects have not been evaluated. We performed Randall-Selitto, Von Frey, Hargreaves and acetone tests for the entire 56 days post-surgery. Several of these deficits increased 14 to 56 days after CCI and we measured a constant increase in NGF levels in the DRG and spinal cord over the course of the experiment. In contrast, SP optical density maintained enhanced expression in DRG tissue from 14 to 56 days after CCI, whereas it was significantly increased only 56 days post-surgery in spinal cord. We perform long-term evaluation of symptoms associated with CCI and measure associated molecular changes. Moreover, by characterizing the behavioral signatures of this model, our work supports future studies.

The International Association for the Study of Pain defines peripheral neuropathic pain (PNP) as “pain caused by a lesion or disease of the peripheral somatosensory nervous system”. PNP is a complex syndrome and can be induced by trauma, compression, neurotoxins, inflammation, as well as immune and metabolic diseases. In chronic constriction injury (CCI) (1), a widely used peripheral nerve injury model of chronic neuropathic pain, four catgut ligatures are placed around the sciatic nerve.

This procedure leads to changes in cell structure, function, biochemical properties and connections. Clinical manifestations of this procedure include the development of pain in the innervation site of the damaged nerve and allodynia (pain due to a stimulus that does not usually provoke pain) or hyperalgesia (increased pain from a stimulus that usually provokes pain) extending beyond the innervation site of the damaged nerve (2, 3).

The specialized primary sensory neurons

Key words: chronic constriction injury, pain, nerve growth factor, substance P, allodynia, hyperalgesia

Mailing address:

Joyce Teixeira da Silva, PhD
Department of Neural and Pain Sciences,
School of Dentistry, University of Maryland,
Room 8404, Baltimore, United States of America
Tel.: +1(410)706 4049
e-mail: JTeixeira@umaryland.edu

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.

that respond to actual or potential tissue injury (nociceptors) express receptors for neurotrophic factors, either nerve growth factor (NGF) or glial cell-derived neurotrophic factor (GDNF) (4, 5). Endogenous NGF levels are elevated in certain chronic pain conditions (6, 7) and a single administration of NGF is sufficient to trigger transition to a persistent pain state in humans that lasts for weeks (8-10). NGF and its cognate receptor TrkA undergo retrograde transport to the DRG, resulting in increased synthesis of neuropeptides [e.g., substance P (SP), brain-derived neurotrophic factor (BDNF)], and transport of ion channels from the DRG to peripheral tissue and spinal cord (11). Further evidence that NGF is involved in neuropeptide regulation is the observation that SP levels are elevated in the target fields of sensory neurons after chemical (6-hydroxy-dopamine) or surgical destruction of NGF-dependent sympathetic neurons innervating the same structures (12, 13). SP is synthesized in the DRG and transported to the terminals of C-fibers located in the dorsal horn of the spinal cord and in the skin (14). SP can be released in response to increased NGF (15) and is associated with the ectopic neural activity in neuropathic pain models (16). Therefore, NGF and SP contribute to the development of hyperalgesia and allodynia after nerve injury (17).

Most studies look at pain mechanisms in relatively early stages (i.e., within the first 2-4 weeks) following injury (18-20), limiting possible findings related to time-dependency and chronicity of the pain process. In the present study, we try to characterize the long-term symptoms of chronic pain using different behavioral tests to show allodynic and hyperalgesic patterns over time in the CCI model. In addition, we sought to elucidate the roles of NGF and SP levels at 14 and 56 days post-surgery in the DRG and spinal cord.

MATERIALS AND METHODS

Animals and CCI-induced neuropathic pain

Male Wistar rats weighing between 250 and 300 g were used in all experiments. They were housed in cages and maintained in climate and light-controlled rooms

under a 12:12 h light/dark cycle with standard rodent food pellets and water ad libitum. Animals were handled three days before the experiments to enable habituation to the experimental conditions and the groups were blind to the experimenters. All animals were tested in the light cycle at the same time of day (9:00 am– 12:00 am). All procedures were approved by the Institutional Animal Care Committee of the University of São Paulo (protocol number 11 – book number 03). Efforts were made to minimize the number of animals used and their suffering, the number of animals per group was 10 (21).

For the induction of neuropathic pain, chronic constriction of the sciatic nerve was performed as previously described by Bennett and Xie in 1988. In short, rats were anesthetized with halothane (Cristalia, Minas Gerais, Brazil) (1, 22) and the right sciatic nerve was exposed at the level of the middle of the thigh by blunt dissection of the biceps femoris. Proximal to the sciatic trifurcation (about 7 mm), the nerve was freed of adhering tissue and four ligatures (4.0 chromic gut) were tied loosely around it with about 1-mm spacing. Great care was taken to tie the ligatures, such that the diameter of the nerve was seen to be just barely constricted. The incision was closed in layers. In sham-operated rats, the sciatic nerve was exposed but left unaffected and served as the naive control group. Each rat was closely observed during the recovery from anesthesia and then returned to the home cage where it was monitored closely over the subsequent 24 h.

Nociceptive testing

All behavioral tests were performed before any procedure at (baseline – BL), 14, 28, 42 and 56 days post-surgery. To evaluate mechanical hyperalgesia, we used the Randall and Selitto test (23). An Ugo-Basile pressure apparatus (Ugo Basile, Comerio, Italy) was used and an increasing force (16 g/s) was applied to the right hindpaw. The force needed to induce paw withdrawal was recorded as the nociceptive threshold. The von Frey test was used to assess low-threshold mechanical nociceptive thresholds. Briefly, a logarithmic series of seven calibrated Semmes-Weinstein monofilaments (Von Frey hair test, Stoelting, USA) was applied to the middle of the plantar surface of the right hindpaw, for a maximum of 10 s to determine the threshold intensity of the stiffness stimulus required to elicit a paw withdrawal response. Log stiffness of the

hairs is determined by ranges starting at 4,56N (3.630 g); 3.61 (0.407 g); 3.84 (0.692 g); 4.17 (1.479 g); 4.93 (8.511 g); 5.18 (15.136 g) and 5.46 (28.840 g). During each trial, the series of filaments was presented following an up-down procedure as described and previously validated by Chaplan (24), and the 50% response threshold was calculated for each rat. Thermal hyperalgesia was assessed using the Plantar Test with the Hargreaves Apparatus (25). The rats were placed in a Plexiglas chamber and allowed to acclimatize for about 30 min. A light beam was focused on the mid-plantar surface of the right hindpaw using the Hargreaves apparatus. The photocell sensor detects the withdrawal of the hind limb and automatically disconnects the beam. The time latency of the withdrawal was recorded and the cut-off time for this test was 25 s. The final result was determined by averaging three consecutive trials separated by 5 minutes. Tests for cold sensitivity (acetone test) were performed as described by Choi et al. (1994) (26) and the animals were placed in Plexiglas boxes and allowed to acclimatize for 30 min. Acetone (50 mL) was applied in the plantar portion of the right hindpaw with a syringe without touching the needle and a 2-minute video was recorded. Responses (licking, biting and sniffing) were counted with a timer and the total response time is given in seconds.

Western blot

Briefly, neuropathic (CCI 14 and 56 days), sham and naive (56 days) rats were sacrificed by decapitation under isoflurane anesthesia, and the ipsilateral DRG (L4-L6) and spinal cord (lumbar portion) were quickly removed and homogenized in an extraction buffer containing 100 mM Tris, pH 7.4, 10 mM EDTA, 2 mM PMSF and 10mg/mL aprotinin. The extracted proteins (75 µg per sample) were subjected to 12% acrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were treated for 2 h at room temperature with 5% blocking buffer containing powdered full fat milk. They were incubated overnight at 4°C with a rat monoclonal primary antibody against NGF (F30, 1:1000; Santa Cruz Biotechnology, Texas, USA) and a rabbit monoclonal primary antibody against SP (1:1000; Millipore, Darmstadt, Germany). A peroxidase-conjugated anti-rat antibody 1:5000 (ZIMED Laboratories Inc., San Francisco, CA, USA) and anti-rabbit 1:5000 (GE Healthcare, Pittsburgh, PA, USA) were used as secondary

antibodies. The bands were normalized by measuring the optical density of β -actin (1:10000, Sigma-Aldrich, St. Louis, MO) using samples from control animals as the standard for normalization. The specifically bound antibody was visualized using a chemiluminescence kit (Amersham Biosciences, Piscataway, NJ). The blot was analyzed using NIH-Scion Image 4.0.2 and quantified by optical densitometry of the developed autoradiographs (Scion Corporation, Frederick, MD). Figures were created with Adobe Photoshop CS.

Statistics

Behavioral data were reported as mean and SEM or as a box-and-whisker plot, and they were compared using 1-way or 2-way analysis of variance (ANOVA) with post hoc by the Bonferroni test. Western blot data were compared using 1-way ANOVA with post hoc by the Bonferroni test. All statistics were performed using GraphPad Prism 5 software (GraphPad Software Inc., CA, USA). A value of $p \leq 0.05$ was considered significant.

RESULTS

Sensorial modalities are differently affected in CCI-induced nociception

The CCI injury caused a significant increase in pain-related behavior. For all behavioral tests analyzed, the impaired thresholds started 14 days after injury and remained until 56 days post-surgery (Figs. 1-4). The sham or naive (control groups) did not modify the nociceptive threshold in all behavioral tests. The nociception was localized, since nociceptive threshold measurements of the non-surgical limb (the contralateral hindpaw) did not show statistical differences (data not shown).

For mechanical hyperalgesia, we observed a statistical difference between 14 and 56 days following CCI-injury. The mechanical hyperalgesia 56 days after the induced lesion was higher than at 14 days after surgery (Fig. 1). In tactile allodynia, higher nociceptive behavioral was observed at 28 days post-surgery when compared with other time points in CCI animals (Fig. 2). The reaction to acetone stimulation (cold allodynia) such as licking, biting and sniffing were significantly increased in the CCI group 42 and 56 days after surgery compared to previous evaluations (Fig. 3). On the other

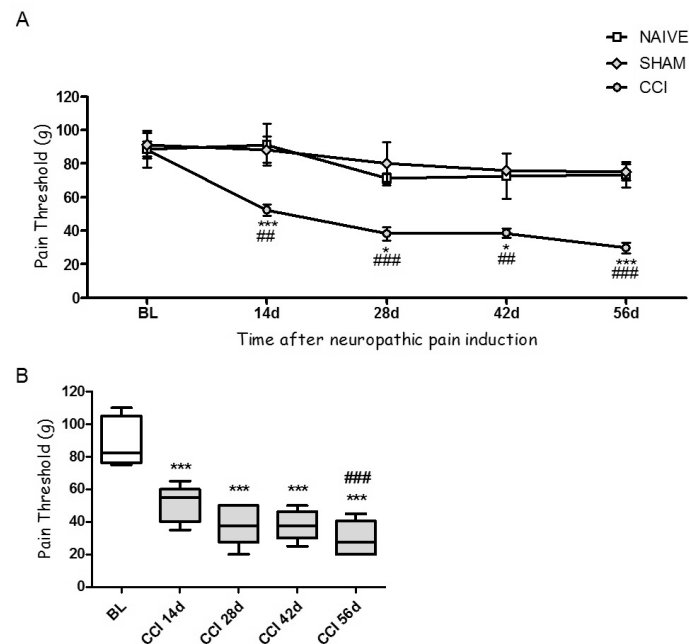


Fig. 1. Enhanced mechanical hyperalgesia was persistent in the CCI model until 56 days post-surgery (A). * denotes significant differences compared to naive ($p \leq .05$ and 0.001) and # denotes significant differences compared to sham ($p \leq .01$ and $.001$) using 2-way ANOVA and Bonferroni test. CCI showed higher mechanical hyperalgesia at 56 days compared to 14 days post-surgery (B). * denotes significant differences compared to baseline ($p \leq 0.0001$) and # denotes significant differences compared to CCI 14 days ($p \leq 0.0001$) using 1-way ANOVA and Bonferroni test.

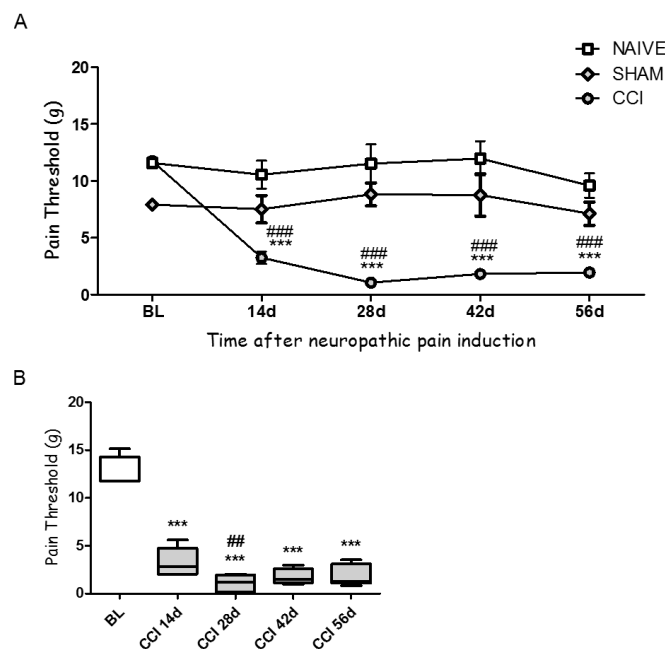


Fig. 2. Tactile threshold was decreased in the CCI group compared to control groups over time (A). * denotes significant differences compared to naive ($p \leq 0.001$) and # denotes significant differences compared to sham ($p \leq 0.001$) using 2-way ANOVA and Bonferroni test. CCI showed higher tactile allodynia at 28 days compared to 14 days post-surgery (B). * denotes significant differences compared to baseline ($p \leq 0.0001$) and # denotes significant differences compared to CCI 14 days ($p \leq 0.001$) using 1-way ANOVA and Bonferroni test.

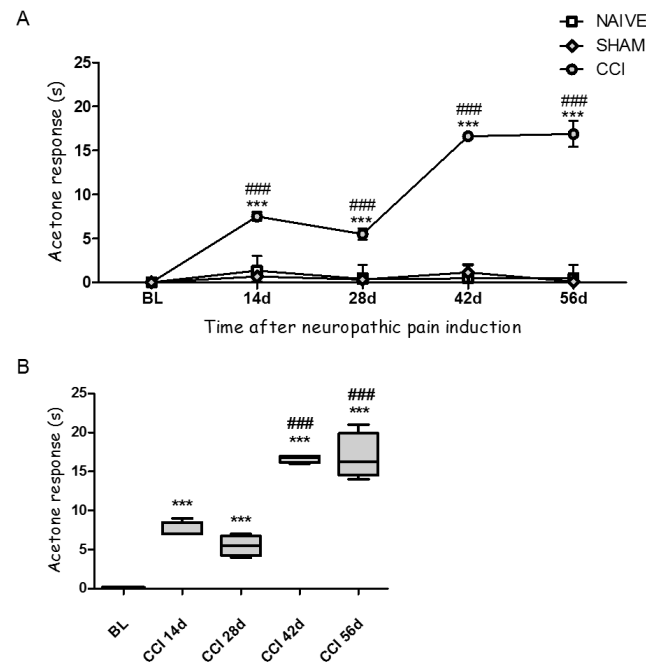


Fig. 3. Enhanced acetone response was observed in the CCI model at 56 days post-surgery (A). * denotes significant differences compared to naive ($p \leq 0.001$) and # denotes significant differences compared to sham ($p \leq .001$) using 2-way ANOVA and Bonferroni test. CCI showed a threefold increase in reaction time at 42 and 56 days compared to 14 and 28 days after surgery (B). * denotes significant differences compared to baseline ($p \leq 0.0001$) and # denotes significant differences compared to CCI 14 and 28 days ($p \leq 0.0001$) using 1-way ANOVA and Bonferroni test.

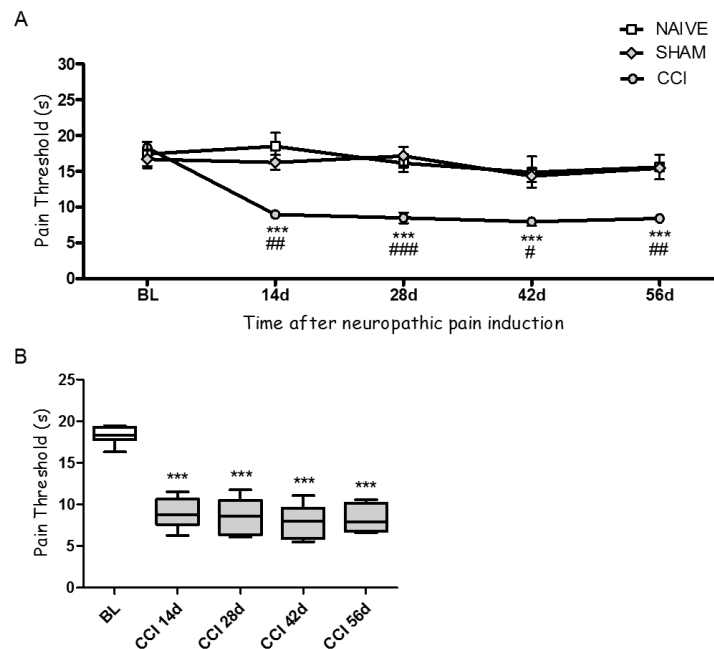


Fig. 4. Thermal threshold was decreased in the CCI group compared to control groups over time (A). * denotes significant differences compared to naive ($p \leq 0.001$) and # denotes significant differences compared to sham ($p \leq 0.05, 0.01$ and 0.001) using 2-way ANOVA and Bonferroni test. CCI showed a constant thermal hyperalgesia threshold over time compared to baseline and no difference between CCI evaluations (B). * denotes significant differences compared to baseline ($p \leq 0.0001$) using 1-way ANOVA and Bonferroni test.

hand, regarding thermal threshold, the CCI group had no difference between time points for the whole period analyzed (Fig. 4). We also compared baseline and post-CCI evaluations in B figures ($p \leq 0.0001$).

NGF levels increased over time in the DRG and spinal cord in the CCI model

A single NGF-positive band was observed in DRG and spinal cord extracts from all groups analyzed (Fig. 5). Western blot analysis of the DRG in the injured side showed that the optical density of NGF bands of CCI rats was significantly increased compared to controls and they did not change over time ($p \leq 0.001$ and 0.0004 for naive and $p \leq 0.05$ and 0.001 for sham, Fig. 5A). However, a higher intensity of optical density was observed at 56 days after CCI compared to other groups, but there was no statistical difference between 14 vs 56 days post-surgery. Regarding the lumbar region of the spinal cord, we showed that the optical density of NGF bands of CCI rats was significantly increased compared to controls (sham and naive). In this case, we also observed increased NGF at 56 days compared to 14 days post-surgery ($p \leq 0.05$, Fig. 5B). No differences were observed for NGF between control groups and for β -actin between control and CCI groups at any of the regions analyzed (Fig. 5).

Substance P is increased over time in the DRG but is enhanced only in the later stage in spinal cord

A single SP-positive band was observed in DRG and spinal cord extracts from all groups analyzed (Fig. 6). Western blot analysis of the DRG in the injured side showed that the optical density of SP bands of CCI rats was significantly increased compared to controls (sham and naive). However, greater enhancement over control groups was observed at 56 days after CCI injury compared with animals 14-days post CCI injury ($p \leq 0.05$ vs 0.001 for sham and naive, Fig. 6A) in DRG. Regarding the lumbar region of the spinal cord, we did not observe any significant difference in SP optical density 14 days post-surgery compared to control groups (sham and naive - Fig. 6B). However, we observed an increase in SP optical density 56 days after CCI compared to all groups of

animals ($p \leq 0.05$ for naive and sham and $p \leq 0.001$ for 14 days post-CCI). No differences were observed for SP optical density between control groups and for β -actin between control and CCI groups at any of the regions analyzed (Fig. 6).

DISCUSSION

Several behavioral tests are commonly used to evaluate different sensorial modalities involved with neuropathic pain processes, including exposure to warm, cold, mechanical and tactile stimuli. These tests elucidate common symptoms underlying this pathology (20, 27). In the CCI model, impaired nociceptive sensibility has been observed during chronic pain conditions (18, 20). Previous authors showed a decrease in mechanical threshold (approx. 10 grams) and an increase in cold allodynia scores after 5 or 15 days post-surgery (18). Similar results observed by Santos et al. (2012) and Hughes et al. (2007) showed a decrease in thermal threshold (approx. 10 seconds) 14 days following CCI (19, 20). A previous study examining rats 30 days after CCI revealed, with the Von Frey test, a mechanical threshold of 10 g and a 300 g threshold using the Randall Sellito test (28). However, these studies only examined early stages (i.e., within the first 2–4 weeks) and thus their conclusions have limited relevance to human pathology, which can involve chronic neuropathic pain over many years. In the present study, we found a decreased average mechanical threshold at 56 days post CCI (29 grams) compared to 14 days (57 grams) post CCI using the Randall & Sellito test, and the average threshold for baseline measurements was 90 grams (Fig. 1). In tactile allodynia using Von Frey filaments, we observed a greater statistical difference 28 days post-surgery (1.05 grams vs 3.25 grams 14 days after CCI) and the decreased threshold was continuous until 56 days compared to baseline (11 grams - Fig. 2). These results suggest the most impaired mechanical and tactile thresholds occurred during later periods (28 and 56 days post-surgery).

Meanwhile, we found distinct responses for thermal sensitivity using cold allodynia and the Hargreaves tests. Cold allodynia induced by acetone

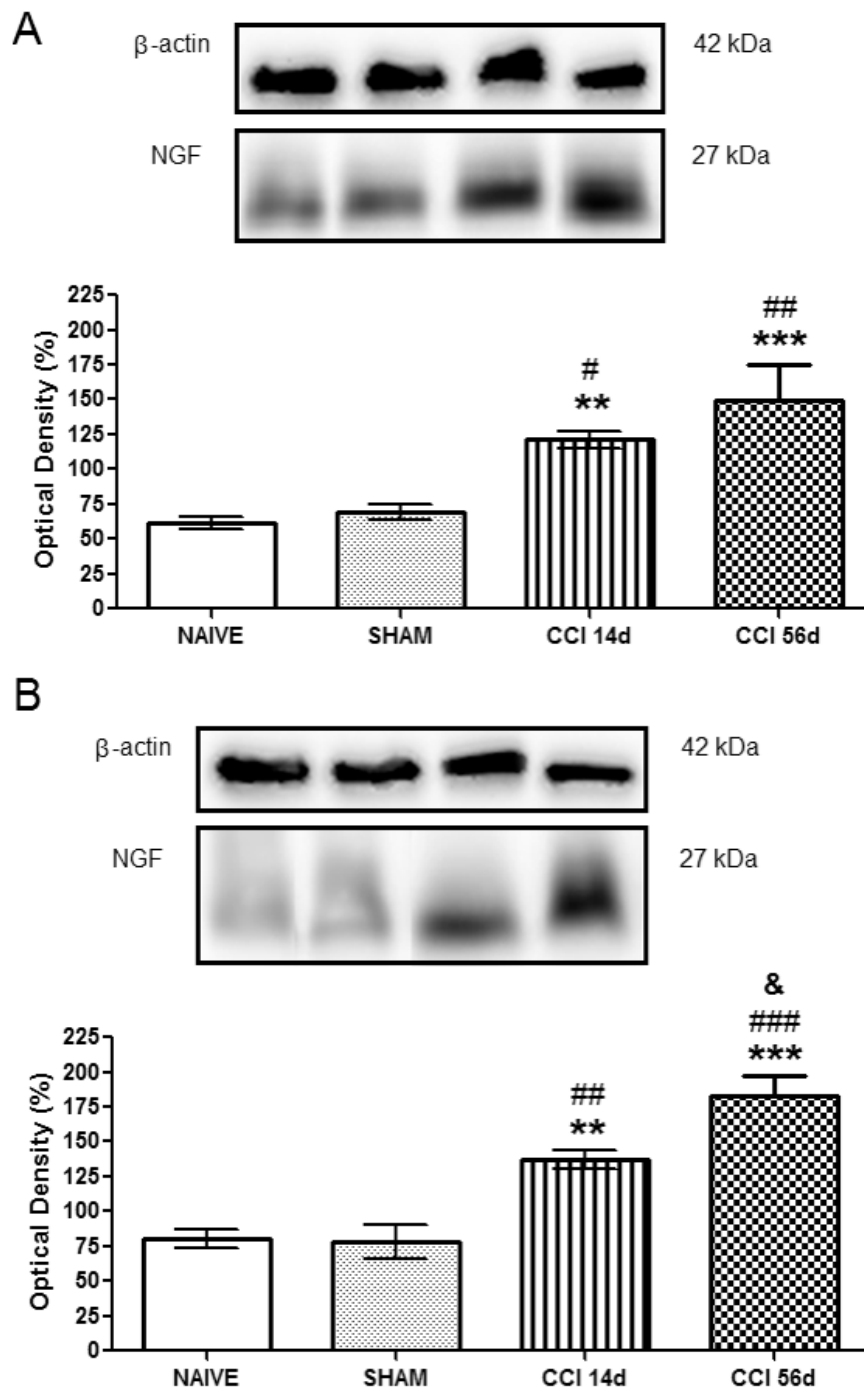


Fig. 5. Upregulated NGF immunoreactivity in the DRG and spinal cord of CCI rats. Western blot analysis of the DRG (**A**) and lumbar level of spinal cord (**B**) revealed a 42 kDa protein band specific for β -actin and a 27 kDa protein band specific for NGF. The optical density of the NGF was increased after 14 and 56 days in DRG and spinal cord. However, this protein band was enhanced 56 days post-surgery in spinal cord compared to 14 days. * denotes significant differences compared to naive ($p \leq 0.001$ and 0.0004 for DRG analysis and $p \leq 0.001$ and 0.0001 for spinal cord analysis). # denotes significant differences compared to sham ($p \leq 0.05$ and 0.001 for DRG analysis and $p \leq 0.001$ and 0.0001 for spinal cord analysis). & denotes significant differences compared to CCI after 14 days ($p \leq 0.05$ for spinal cord analysis). All data were analyzed using 2-way ANOVA and Bonferroni test.

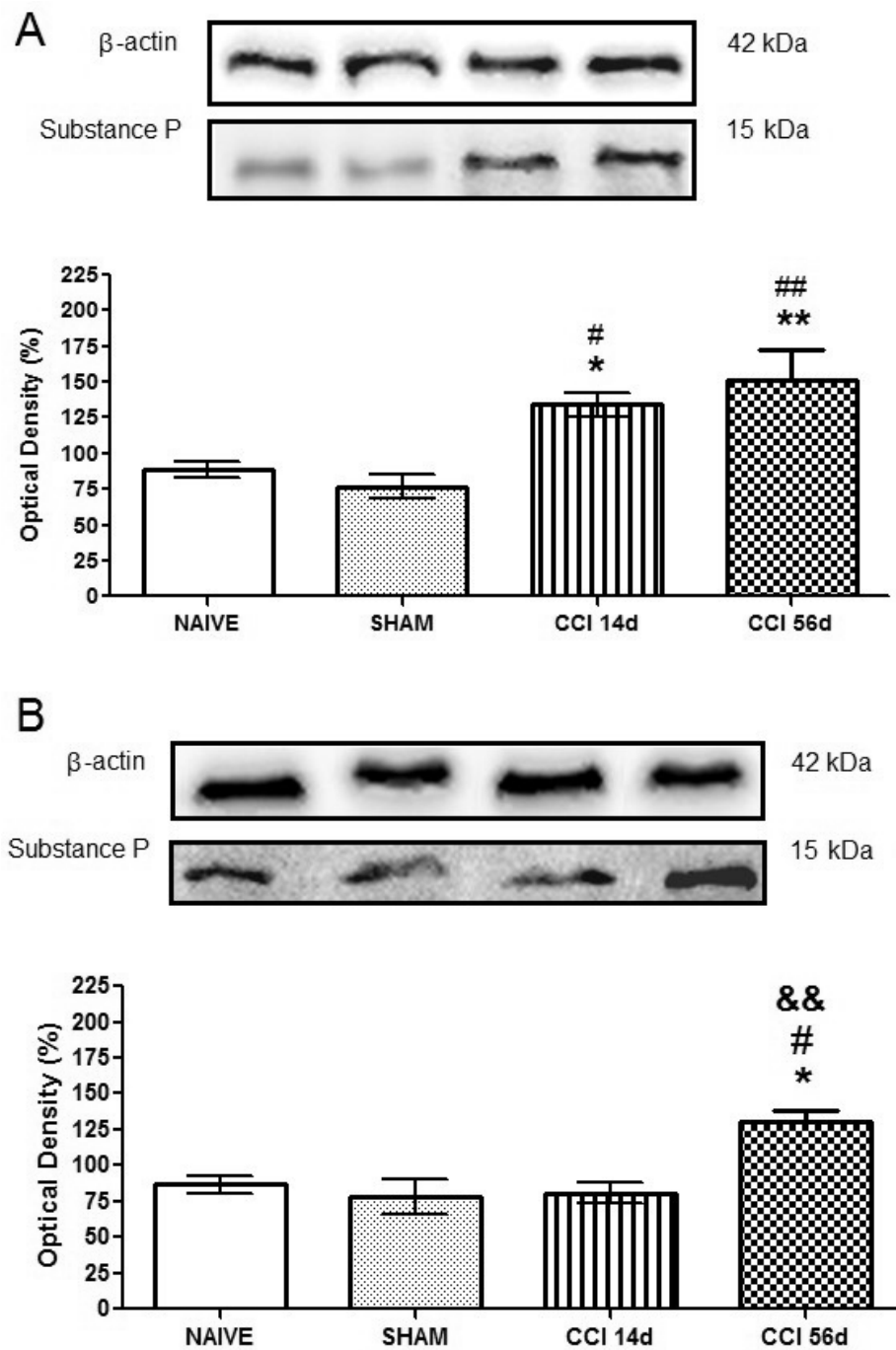


Fig. 6. Upregulated SP immunoreactivity in the DRG and spinal cord of CCI rats. Western blot analysis of the DRG (**A**) and lumbar level of spinal cord (**B**) revealed a 42 kDa protein band specific for β -actin and a 15 kDa protein band specific for SP. The optical density of the SP was increased after 14 and 56 days in DRG, however, this protein band was enhanced only 56 days post-surgery in the spinal cord. * denotes significant differences compared to naive ($p \leq 0.05$ and 0.001 for DRG analysis and $p \leq 0.05$ for spinal cord analysis). # denotes significant differences compared to sham ($p \leq 0.05$ and 0.001 for DRG analysis and $p \leq 0.05$ for spinal cord analysis). & denotes significant differences compared to CCI after 14 days ($p \leq .001$ for spinal cord analysis). All data were analyzed using 2-way ANOVA and Bonferroni test.

application increased reaction time in CCI animals 14 days after injury (7 s vs 1 s of reaction time in the control groups) and we observed a threefold increase in reaction time over 42 and 56 days compared to 14 and 28 days after this surgery (Fig. 3). Otherwise, the Hargreaves test showed a constant low nociceptive threshold in CCI (< 9 seconds) compared to control groups (around 18 s) for the duration of the study period (Fig. 4). We decided to look at nociceptive mediators in regions belonging to pain pathways, including those of the peripheral (DRG) and central nervous system (spinal cord), with the goal of understanding our behavioral results and their correlations with certain nociceptive mediators.

Consistently, Western blot analysis of the DRG and spinal cord from neuropathic rats revealed an increase of NGF 14 and 56 days after surgery (50% and 80%, respectively for DRG and 56% and 92%, respectively for spinal cord) compared to control groups. When we compared 14 vs 56 days, we observed that NGF optical density was greater at 56 days compared to 14 days post-CCI in spinal cord (36%, $p \leq 0.05$), and we did not observe any statistical time differences in DRG post-CCI. These data are consistent with studies that showed that NGF is an important regulator of nociceptor sensitization following tissue injury in several models, and a potential therapeutic target (29, 30). CCI-induced hyperalgesia, both mechanical and thermal for all evaluations, might be due, at least in part, to the ability of NGF to bind to TrkA and to be transported to DRG neurons, thus changing the expression of nociceptive neuropeptides, such as Substance P and CGRP, and inducing BDNF expression, which could drive this long-lasting sensitization (4, 31). SP regulates pain transmission and sensitization by acting on neurokinin-1 receptor (NK-1), which is expressed in postsynaptic dorsal horn neurons and in DRG neurons coexpressing the capsaicin receptor TRPV1 (transient receptor potential vanilloid subtype 1), a critical receptor for the generation of heat hyperalgesia (32).

Consistent with previous studies, we found a constant increase in SP level in the DRG 14 and 56 days after injury, which was required for the generation and maintenance of heat hyperalgesia observed in our results using the Hargreaves test. Regarding the delayed increase in SP in the spinal cord only at 56

days post-injury, we suggest this may be the result of cold allodynia modulation, since we also found a delayed increase in reaction time in cold allodynia in the CCI group 42 and 56 days post-surgery compared to early-stages. These data corroborate a previous study showing unchanged SP after 28 days in the same model (33). Moreover, Hubbard et al. demonstrated that SNI (spared nerve injury) rats showed glutamate increases in the ACC (anterior cingulate cortex), which correlated with cold hypersensitivity 1 month post-SNI (34). SP is also known to function as a modulator of the excitatory effects of glutamate (35). Further studies will be required to show this correlation, since the SP and glutamate can be acting in supraspinal regions to increase nociceptive sensitivity in chronic neuropathy.

In conclusion, the present study showed that the CCI in the sciatic nerve has different time-related responses in behavioral tests and to nociceptive mediators. We observed impaired mechanical and tactile thresholds in later periods. The steadily decreasing hypersensitivity in thermal tests persisted for a significant duration post-surgery, and a threefold increase in reaction time in cold allodynia was observed in the later measurements. Therefore, this model has broad application in neuropathic pain studies because of its relevance to chronic pain states observed in both humans and animals, and the behavioral aspects of the approach may elucidate several questions. NGF and SP are critical mediators in pain-related behavior and maintenance of chronic pain processes, in addition to their role in peripheral and central sensitization (11).

Our study suggests the involvement of NGF and SP in modulating time-dependent behavioral responses and shows how these responses are distinct in different anatomical regions. Further studies will be necessary to reveal potential interactions between NGF and SP during the response to pain. Consequently, the current research aims to enhance our understanding of neuropathic pain processes and to provide novel therapeutic targets.

ACKNOWLEDGEMENTS

This study was supported by FAPESP (2012/24408-6 and 2014/24533-0 and 2016/14675-8), CNPq, CAPES (Brazil). No competing financial interests exist.

REFERENCES

1. Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain* 1988; 33(1):87-107.
2. Klusakova I, Dubovy P. Experimental models of peripheral neuropathic pain based on traumatic nerve injuries - an anatomical perspective. *Anna Anat* 2009; 191(3):248-59.
3. Woolf CJ, Mannion RJ. Neuropathic pain: etiology, symptoms, mechanisms, and management. *Lancet* 1999; 353(9168):1959-64.
4. Malcangio M, Ramer MS, Boucher TJ, McMahon SB. Intrathecally injected neurotrophins and the release of substance P from the rat isolated spinal cord. *Eur J Neurosci* 2000; 12(1):139-44.
5. Bennett DL, Michael GJ, Ramachandran N, et al. A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. *J Neurosci* 1998; 18(8):3059-72.
6. Iannone F, De Bari C, Dell'Accio F, Covelli M, Patella V, Lo Bianco G, Lapadula G. Increased expression of nerve growth factor (NGF) and high affinity NGF receptor (p140 TrkA) in human osteoarthritic chondrocytes. *Rheumatology* 2002; 41(12):1413-8.
7. Sarchielli P, Mancini ML, Floridi A, et al. Increased levels of neurotrophins are not specific for chronic migraine: evidence from primary fibromyalgia syndrome. *J Pain* 2007; 8(9):737-45.
8. Dyck PJ, Peroutka S, Rask C, et al. Intradermal recombinant human nerve growth factor induces pressure allodynia and lowered heat-pain threshold in humans. *Neurology* 1997; 48(2):501-5.
9. Svensson P, Cairns BE, Wang K, Arendt-Nielsen L. Injection of nerve growth factor into human masseter muscle evokes long-lasting mechanical allodynia and hyperalgesia. *Pain* 2003; 104(1-2):241-7.
10. Rukwied R, Mayer A, Kluschina O, Obreja O, Schley M, Schmelz M. NGF induces non-inflammatory localized and lasting mechanical and thermal hypersensitivity in human skin. *Pain* 2010; 148(3):407-13.
11. Mantyh PW, Koltzenburg M, Mendell LM, Tive L, Shelton DL. Antagonism of nerve growth factor-TrkA signaling and the relief of pain. *Anesthesiology* 2011; 115(1):189-204.
12. Lindsay RM, Harmor AJ. Nerve growth factor regulates expression of neuropeptide genes in adult sensory neurons. *Nature* 1989; 337(6205):362-64.
13. Kessler JA, Bell WO, Black IB. Interactions between the sympathetic and sensory innervation of the iris. *J Neurosci* 1983; 3(6):1301-7.
14. Otten U, Goedert M, Mayer N, Lembeck F. Requirement of nerve growth factor for development of substance P-containing sensory neurones. *Nature* 1980; 287(5778):158-59.
15. Kessler JA, Black IB. Nerve growth factor stimulates the development of substance P in sensory ganglia. *Proc Natl Acad Sci U S A* 1980; 77(1):649-52.
16. Cameron AA, Cliffer KD, Dougherty PM, Garrison CJ, Willis WD, Carlton SM. Time course of degenerative and regenerative changes in the dorsal horn in a rat model of peripheral neuropathy. *J Comp Neurol* 1997; 379(3):428-42.
17. Costa GM, de Oliveira AP, Martinelli PM, da Silva Camargos ER, Arantes RM, de Almeida-Leite CM. Demyelination/remyelination and expression of interleukin-1beta, substance P, nerve growth factor, and glial-derived neurotrophic factor during trigeminal neuropathic pain in rats. *Neurosci Lett* 2016; 612:210-18.
18. Camara CC, Araujo CV, de Sousa KK, et al. Gabapentin attenuates neuropathic pain and improves nerve myelination after chronic sciatic constriction in rats. *Neurosci Lett* 2015; 607:52-58.
19. Hughes DI, Scott DT, Riddell JS, Todd AJ. Upregulation of substance P in low-threshold myelinated afferents is not required for tactile allodynia in the chronic constriction injury and spinal nerve ligation models. *J Neurosci* 2007; 27(8):2035-44.
20. Santos FM, Silva JT, Giardini AC, et al. Neural mobilization reverses behavioral and cellular changes that characterize neuropathic pain in rats. *Mol Pain* 2012; 8:57.
21. Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 1983; 16(2):109-10.
22. da Silva JT, Santos FM, Giardini AC, et al. Neural mobilization promotes nerve regeneration by nerve growth factor and myelin protein zero increased after sciatic nerve injury. *Growth Factors* 2015; 33(1):8-13.

23. Randall LO, Selitto JJ. A method for measurement of analgesia activity on inflamed tissue. *Arch Int Pharmacodyn Ther* 1957; 111:209-19.
24. 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.
25. Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 1988; 32:77-88.
26. Choi Y, Yoon YW, Na HS, Kim SH, Chung JM. Behavioral signs of ongoing pain and cold allodynia in a rat model of neuropathic pain. *Pain* 1994; 59(3):369-76.
27. Li Y, Zhang H, Kosturakis AK, Jawad AB, Dougherty PM. Toll-like receptor 4 signaling contributes to Paclitaxel-induced peripheral neuropathy. *J Pain* 2014; 15(7):712-25.
28. M'Dahoma S, Barthelemy S, Tromilin C, et al. Respective pharmacological features of neuropathic-like pain evoked by intrathecal BDNF versus sciatic nerve ligation in rats. *Eur Neuropsychopharmacol* 2015; 25(11):2118-30.
29. Wild KD, Bian D, Zhu D, Davis J, Bannon AW, Zhang TJ, Louis JC. Antibodies to nerve growth factor reverse established tactile allodynia in rodent models of neuropathic pain without tolerance. *J Pharmacol Exp Ther* 2007; 322(1):282-7.
30. Zhu Y, Mehta K, Li C, Xu GY, Liu L, Colak T, Shenoy M, Pasricha PJ. Systemic administration of anti-NGF increases A-type potassium currents and decreases pancreatic nociceptor excitability in a rat model of chronic pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 2012; 302(1):28.
31. Donnerer J, Schuligoi R, Stein C, Amann R. Upregulation, release and axonal transport of substance P and calcitonin gene-related peptide in adjuvant inflammation and regulatory function of nerve growth factor. *Regul Pept* 1993; 46(1-2):150-4.
32. Zhang H, Cang CL, Kawasaki Y, Liang LL, Zhang YQ, Ji RR, Zhao ZQ. Neurokinin-1 receptor enhances TRPV1 activity in primary sensory neurons via PKCepsilon: a novel pathway for heat hyperalgesia. *J Neurosci* 2007; 27(44):12067-77.
33. Casals-Diaz L, Vivo M, Navarro X. Nociceptive responses and spinal plastic changes of afferent C-fibers in three neuropathic pain models induced by sciatic nerve injury in the rat. *Exp Neurol* 2009; 217(1):84-95.
34. Hubbard CS, Khan SA, Xu S, Cha M, Masri R, Seminowicz DA. Behavioral, metabolic and functional brain changes in a rat model of chronic neuropathic pain: a longitudinal MRI study. *Neuroimage* 2015; 107:333-44.
35. De Biasi S, Rustioni A. Glutamate and substance P coexist in primary afferent terminals in the superficial laminae of spinal cord. *Proc Natl Acad Sci U S A* 1988; 85(20):7820-4.

IMPACT OF RANTES FROM JAWBONE ON CHRONIC FATIGUE SYNDROME

J. LECHNER¹, K. HUESKER² and V. VON BAEHR³

¹*Clinic for Integrative Dentistry, Munich, Germany;* ²*Endocrinology and Immunology Department, Institute for Medical Diagnostics, Berlin, Germany;* ³*Compartment of Immunology and Allergology, Institute for Medical Diagnostics, Berlin, Germany*

Received February 18, 2017 – Accepted June 8, 2017

This study elucidates the question of whether chronic inflammation in the jawbone contributes to the development of Chronic Fatigue Syndrome (CFS). Fatty degenerative osteonecrosis in jawbone (FDOJ) may contribute to CFS by induction of inflammatory mediators. We examined seven cytokines by multiplex analysis in jawbone samples from two groups of patients. In order to clarify neurological interrelations, specimens from 21 CFS patients were analyzed from areas of previous surgery in the retromolar wisdom tooth area. Each of the retromolar jawbone samples showed clinically fatty degenerated and osteonecrotic medullary changes. As control, healthy jawbone specimens from 19 healthy patients were analyzed. All fatty necrotic and osteolytic jawbone (FDOJ) samples showed high expression of RANTES and fibroblast growth factor (FGF)-2. FDOJ cohorts showed a 30-fold mean overexpression of RANTES and a 20-fold overexpressed level of FGF-2 when compared to healthy controls. As RANTES is discussed in the literature as a possible contributor to inflammatory diseases, we hypothesize that FDOJ in areas of improper and incomplete wound healing in the jawbone may hyperactivate signaling pathways. Constituting a hidden source of “silent inflammation” FDOJ may represent a hitherto unknown cause for the development of CFS.

Depressive syndromes are not only associated with inflammatory diseases of the central nervous system (CNS), such as encephalitis and bacterial meningitis, but also with peripheral inflammatory processes (1). The term “sickness behavior” refers to characteristic behavioral changes that develop in the course of an infection. At the molecular level, these changes are due to the action of proinflammatory cytokines in the brain, potentially underlying the development of chronic fatigue syndrome (CFS) (2). Current research suggests that dysregulate immune functions may play a role in CFS pathogenesis. Interestingly, recent evidence points to an association of peripheral and central neuroinflammation to cytokine-chemokine

networks, which may play a central role in the pathogenesis of neuropathic syndromes (3). As CFS symptoms are largely unspecific and no diagnostic test has so far been established, CFS diagnosis is often difficult and uncertain (4). The present study aims to investigate chronic inflammatory processes of the jawbone in patients with a diagnosis of CFS. The effect of fatty-degenerative osteolysis in jawbone (FDOJ) on chronic silent inflammation has been the subject of previous studies, using Luminex multi-analyte profiling technology (5). The current study addresses the question of whether FDOJ areas in CFS patients produce inflammatory messengers, activating signaling pathways that may be related to CFS development.

Key words: chemokine RANTES, chronic fatigue syndrome, osteopathy of jawbone, multiplex-analysis, signaling pathways

Mailing address:

Johann Lechner, M.D., PhD,
Clinic for Integrative Dentistry
Gruenwalder Str. 10A - 81547 Munich, Germany
Tel.: +49 89 6970055 - Fax: +49 89 6925830
e-mail: drlechner@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.

MATERIALS AND METHODS

Study population

This study was performed as a randomized patient-centered controlled trial. Samples and collected data were derived directly from daily practice during normal medical treatment. Data was analyzed retrospectively. FDOJ tissue samples from 21 patients with CFS were collected. CFS was diagnosed by neurologists and physicians, based on clinical criteria for chronic fatigue syndrome. Following clinically necessary surgical FDOJ removal, conspicuous FDOJ samples of 21 CFS patients were tested for inflammatory mediators (11 cases in upper and 10 in lower FDOJ areas). All patients provided written informed consent. Patients taking any medications due to CFS complaints were not excluded from the study. Inclusion criteria were (a) therapy-resistant CFS symptomatology and (b) local diagnosis of FDOJ in edentulous jaw sites. Mandatory inclusion criteria were (c) the availability of two-dimensional orthopantomograms (2D-OPG) and (d) cone beam three-dimensional (digital volume tomograms DVT) images. A further inclusion criterion was the measurement of the bone density of the jawbone with transalveolar ultrasound technology (TAU) (8). The use of bisphosphonate medication was the main exclusion criterion for both groups. Demographic data from the CFS cohort showed an average age of 56.4 years, ranging from 30 to 79 years, and a gender ratio of 8:13 (female:male). In 12 of these patients, RANTES could also be measured in the serum in a gender ratio of 8:4 (female:male). The second cohort was a group of patients with healthy jawbone (HJB) samples, which were taken in the form of drill cores during normal dental implantation surgery. The inclusion criteria for this group were no radiologically distinctive features in 2D-OPG and inconspicuous TAU measurements of bone density in the implantation range. The age range of the control group consisting of 19 patients without FDOJ, was 38–71 years, with an average age of 54 years (SD=12.4 years), and a gender ratio of 11:8 (female:male).

Morphology and histology of fatty degenerative samples of jawbone

The osteopathies of the bone and, in particular, the jawbone are subject to different definitions and classifications. FDOJ lesions are a phenomenon that,

to date, is perceived by few specialists in medicine and dentistry. It has become increasingly apparent that intravascular coagulation plays a significant role in the pathogenesis of the osteonecrosis. The term FDOJ is used to describe a condition influenced by a wide range of factors (6). There are clear differences between FDOJ and the classical form of acute or chronic osteomyelitis. FDOJ is similar to silent inflammation or subclinical inflammation with typical signs of subliminal inflammation and painlessness. FDOJ results from an ischemic deficiency in chronic trophic disorder with fatty-degenerative spongy disintegration. Morphologically, FDOJ presents itself as greasy lumps of tissue, which are easily curetted from the medullary cavity of the jawbone (Fig. 1). Bouquot et al. described FDOJ-induced necrotic and cancellous bone as follows: “Hollow cavities containing soft tissue that has undergone fatty dystrophic changes and delamination of the bony sheath of the inferior alveolar nerve” (6). Fig. 1 shows such a specimen with the predominantly fatty transformation of the jawbone (left panel). The often impressive extent of FDOJ lesions is indicated by the contrast agent in the X-ray image (right panel), which was introduced immediately after surgical FDOJ curettage and removed following the X-ray.

A characteristic definition of FDOJ can be inferred from over a thousand histological findings at the corresponding author's clinic. Notably, the number of fat cells is consistently and strikingly increased in all FDOJ samples. Typical signs of inflammation, especially that of an inflammatory cell response, are absent. The histologic examination of the curetted tissue demonstrates ischemia, necrotic adipocytes, and myxoid degeneration. Aseptic, ischemic osteonecrosis (AIO) appears as the type of bone lesion that most closely resembles FDOJ (7). According to previous research (6), FDOJ represents lesions similar to those found in long bones primarily defined as “bone marrow edema” and “chronic nonsuppurative osteomyelitis”.

Sampling of FDOJ tissue

The current treatment of FDOJ lesions consists of curettage of the bony cavity. To elucidate a possible causative link between FDOJ and CFS at the Munich Clinic for Integrative Dentistry, Germany, 21 patients with CFS who were diagnosed with FDOJ had surgery on the affected area of the jaw. After local anesthesia and

folding back of a mucoperiosteal flap, the cortical layer was removed. All 21 patients exhibited FDOJ inside the bone marrow, similar to the samples described in the literature (5-7).

Processing of necrotic tissue samples and multiplex analysis

The analysis was taken from 40 samples of jawbone: 21 samples of FDOJ in patients with CFS plus 19 healthy jawbone samples as a control group. At the Institute for Medical Diagnostics, Berlin, inspected by DAKKS (Deutsche Akkreditierungsstelle GmbH, accredited to DIN EN ISO/IEC 17025:2005 and DIN EN ISO 15189:2007), the samples were homogenized by mechanical force in 200 μ L of cold protease inhibitor buffer (Complete Mini Protease Inhibitor Cocktail; Roche Diagnostics GmbH, Penzberg, Germany). The homogenate was then centrifuged for 15 min at 13,400 rpm. Next, the supernatant was collected and centrifuged for further 25 min at 13,400 rpm. In the 15 supernatants of tissue homogenate, regulated on activation, normal T-cell expressed and secreted (RANTES), also known as chemokine C-C motif ligand 5 (CCL5), FGF-2, interleukin- (IL-)1 receptor antagonist (ra), IL-6, IL-8, monocyte chemoattractant protein-1 (MCP1), and tumor necrosis factor- α (TNF- α) were measured. The measurement was performed using the Human Cytokine/Chemokine Panel I (MPXHCYTO-60K; Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions and analyzed using Luminex 200 with xPonent software (Luminex Co, Austin, TX, USA).

Analysis of RANTES in serum

In 14 patients of the CFS-FDOJ cohort, RANTES was additionally analyzed in serum. The measurement was performed using the Human Cytokine/Chemokine Panel I (MPXHCYTO-60K, Millipore GmbH, 65824 Schwalbach, Germany) according to the manufacturer's instructions and analysed using Luminex[®] 200[™] with xPonent[®] Software (Luminex, Austin, TX, USA). For the measurement of RANTES in serum, samples were pre-diluted 1:100 in sample buffer according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using IBM SPSS, version 19 (IBM Corporation, Armonk, NY, USA). All

data was presented as a mean $\pm$ standard mean error. The two-sided unpaired *t*-test was used in determining the differences in the groups while a Spearman coefficient was used in examining the correlation presence among the cytokines analyzed. Data was considered significant where the value was <0.05 .

RESULTS

Immune mediators were analyzed in FDOJ samples from CFS patients ($n=21$) and in drilled explants from normal jawbone of healthy controls ($n=19$). This comparison showed significantly elevated levels in FDOJ samples versus control tissue for fibroblast growth factor (FGF-2; 762 ± 314 vs 27.6 ± 36 , $p < 0.0001$), interleukin 1 receptor antagonist (IL1-ra; 718 ± 435 vs 197 ± 569 , $p < 0.01$), monocyte chemoattractant protein 1 (MCP-1; 123 ± 205 vs 20.3 ± 20.5 , $p < 0.05$), and regulated on activation, normal T-cell expressed and secreted / C-C-chemokine ligand 5 (RANTES/CCL5; 5199 ± 2648 vs 150 ± 131 $p < 0.0001$). At the same time, there was a significant decrease of interleukin 6 (IL-6; 5.4 ± 14.7 vs 101 ± 81.7 , $p < 0.0001$). No significant differences were observed for interleukin 8 (IL-8; 12.6 ± 15.5 vs 7.5 ± 3.9 , $p = 0.17$) and tumor necrosis factor alpha (TNF-alpha; 5.3 ± 23.3 vs 11 ± 3.2 , $p = 0.3$). Reference values for healthy jawbone tissue were not available in the literature.

Based on these distributions, we focused our study on the highly overexpressed chemokine RANTES being the most significantly overexpressed signaling pathway in further discussion. Overall, the changes in cytokine distribution between healthy and FDOJ tissue show a prominent increase in RANTES with a concomitant decrease of acute cytokines IL-6 and (by non-significant trend) TNF-alpha (Fig. 2).

In an effort to address any possible effects of RANTES expression in FDOJ on the pathogenesis of CFS, we examined RANTES serum levels in the CFS cohort. Evaluation of 13 CFS patients available for serum sampling revealed a mean RANTES level of 42.6 ± 17.2 ng/mL, with 11 of 13 patients exceeding the published normal range of < 29 ng/mL (9). Comparison of matched serum and FDOJ samples indicated a significant correlation of RANTES tissue

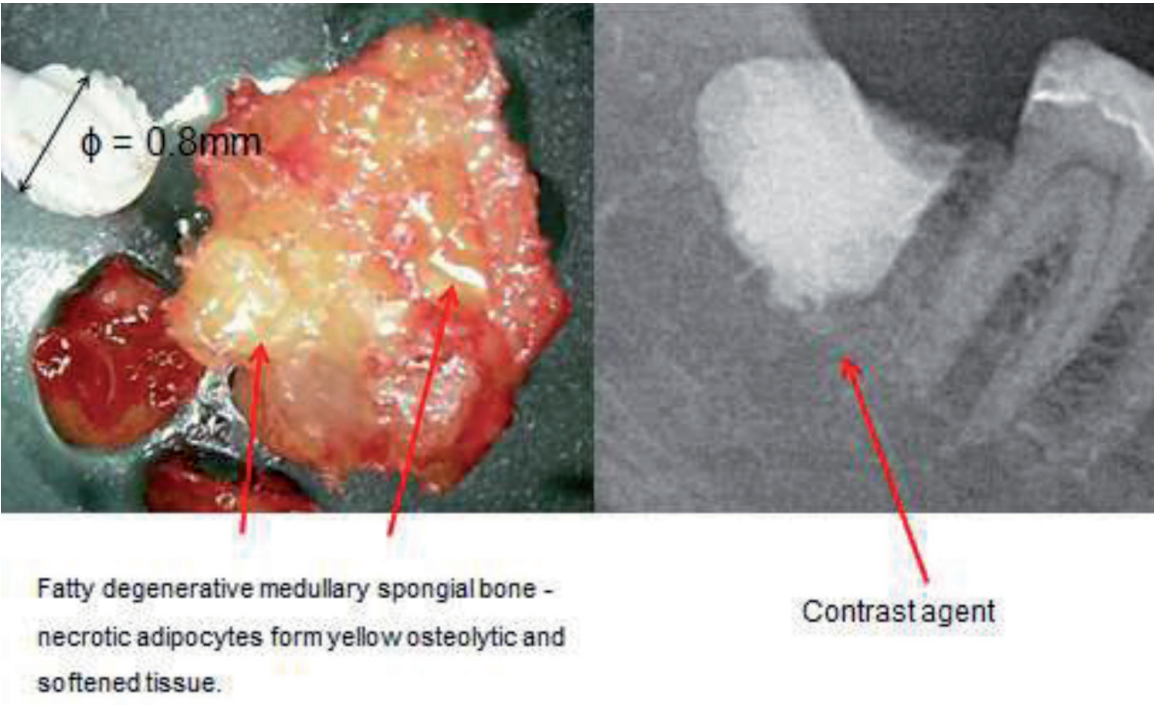


Fig. 1. FDOJ sample of fatty and osteolytic degenerated bone marrow; head of the drill with 0.8 mm diameter shows the extent of FDOJ (left panel) and contrast medium in FDOJ cavity after curettage (right panel).

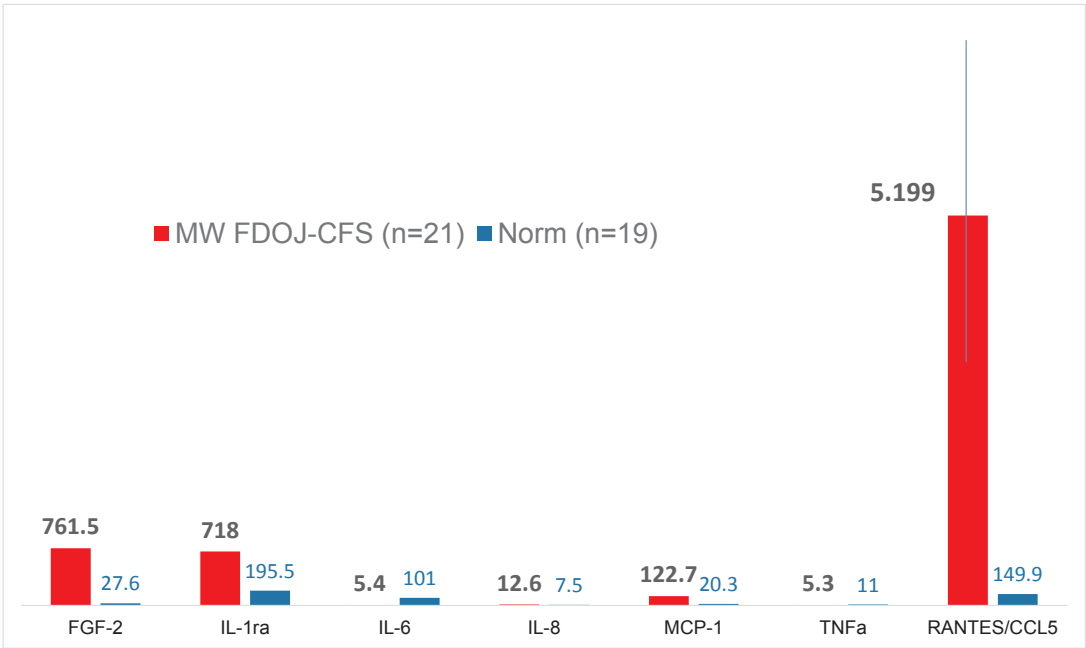


Fig. 2. Distribution of 7 cytokines in healthy jawbone (blue columns/ n = 19) vs in FDOJ specimens of the CFS cohort (red columns/n = 21) in pg/ml.

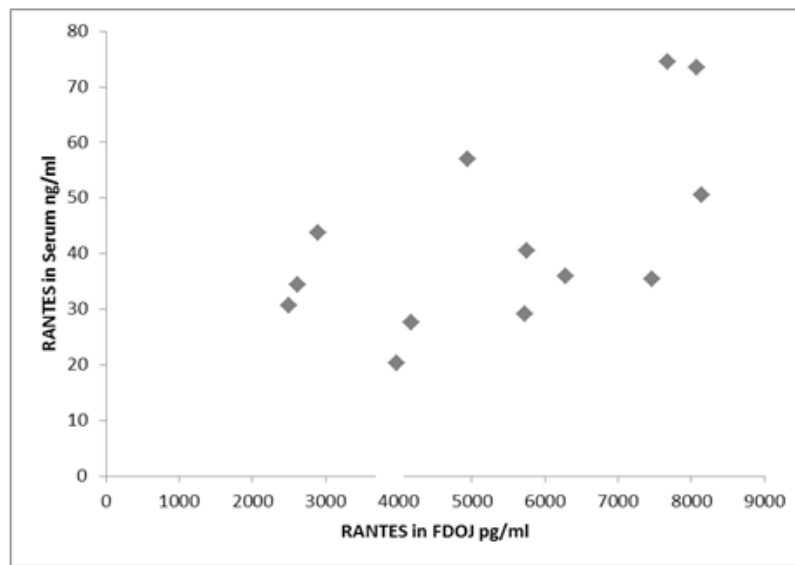


Fig. 3. RANTES levels in serum from the CFS cohort ($n=13$) compared to RANTES levels in FDOJ.

levels in FDOJ to serum levels ($r=0.59$, $p<0.05$), pointing to FDOJ tissue as a source for the increase of systemic RANTES (Fig. 3).

DISCUSSION

Our data show increased RANTES levels both in FDOJ tissue and the serum of CFS patients. Considering the potential systemic effects of elevated RANTES levels we hypothesize that RANTES induced immunological and neurological changes contribute to CFS pathogenesis, rendering FDOJ a hitherto unrecognized risk factor for CFS. In obese patients RANTES promotes ischemia by reducing blood flow and capillary density (9, 10). Recent data show that ischemia associated chemokine expression increases local and systemic secretion of inflammatory cytokines (11). CFS is associated with elevated levels of inflammatory cytokines, suggesting that cytokines play a role in CFS pathophysiology (12). By secretion of RANTES, FDOJ lesions may thus initiate inflammatory pathogenesis, leading to the development of chronic fatigue. Inflammatory cytokines are able to penetrate the blood-brain-barrier and bind to specific receptors, inducing behavioral

changes that are referred to as sickness behavior in animal models (13). At the same time, chemokines such as RANTES facilitate leukocyte trafficking across the blood-brain barrier, promoting central inflammatory processes (14). Immunohistochemical studies have shown that RANTES and other chemokines are present in several brain regions such as the hypothalamus, the limbic system and the hippocampus (15). Direct receptor binding of RANTES has been demonstrated for both neurons and astrocytes (16). In addition to their proinflammatory effects and chemotactic functions, chemokines have been implicated in various neurobiological processes potentially relevant to psychiatric disorders, such as neuromodulatory effects, neurotransmitter action and regulation of neurogenesis (17). It is intriguing to speculate that chemokines may constitute a third transmitter system in the brain, regulating brain function by interaction with both neurotransmitters and neuropeptides. Moreover, recent studies have suggested that RANTES modifies nociception by direct interaction with opioid receptors (OpR) (18), promoting receptor desensitization. As OpR signaling induces analgesia, RANTES-mediated desensitization leads to increased nociception (19).

Current *in vitro* data show that chemokine receptor activation is associated with the inactivation of OpR, supporting the theory of bidirectional heterologous desensitization (20, 21).

In conclusion, on the basis of known pathogenetic mechanisms of CFS, however, the above results point to an interaction between RANTES signaling and CNS functions. A comprehensive understanding of the complex networks outlined in this paper will require further research addressing the clinical relevance of chemokines such as RANTES to the development of CFS and psycho-immunological disorders. Surgical debridement of FDOJ areas is able to counteract the induction of RANTES signaling pathways and thus possibly reduce the progression of CFS disease and associated symptoms. The results submitted by the authors are able only to highlight the need for a large multi-center prospective study with clearly defined cohorts, to clarify causal backgrounds to the development of CFS.

REFERENCES

1. Oxenkrug, Tryptophan-kynurenine metabolism as a common mediator of genetic and environmental impacts in major depressive disorder: the serotonin hypothesis revisited 40 years later. *Isr J Psychiatry Relat Sci* 2010; 47(1):56-63.
2. Dantzer R. Cytokine-Induced Sickness Behavior: Where Do We Stand? *Brain Behav Immun* 2001; 15(1):7-24.
3. Ramesh G, MacLean AG, Philipp MT. Cytokines and chemokines at the crossroads of neuroinflammation, neurodegeneration, and neuropathic pain. *Mediators of Inflammation* 2013; Article ID 480739, 20 pages.
4. Bansal AS, Bradley AS, Bishop KN, Kiani-Alikhan S, Ford B. Chronic fatigue syndrome, the immune system and viral infection. *Brain Behav Immun* 2012; 26:24-31.
5. Lechner J, von Baehr V. RANTES and fibroblast growth factor 2 in jawbone cavitations: triggers for systemic disease? *Int J Gen Med* 2013; 6:277-90.
6. Bouquot JE, Roberts AM, Person P, Christian J. Neuralgia-inducing cavitation osteonecrosis (NICO). Osteomyelitis in 224 jawbone samples from patients with facial neuralgia. *Oral Surg Oral Med Oral Pathol* 1992; 73(3):307-19.
7. Wilke, I. Becker, S. Osteonekrose – Pathologische Grundlagen. *J MinerStoffwechs* 2007; 14(1):3-6.
8. Bouquot J, Shankland W, Margolis M. Through-transmission alveolar ultrasonography (TAU) B new technology for evaluation of bone density and desiccation. Comparison with radiology of 170 biopsied alveolar sites of osteoporitic and ischemic damage. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2002; 93:413-14.
9. Huber J, Kiefer FW, Zeyda M, et al. CC chemokine and CC chemokine receptor profiles in visceral and subcutaneous adipose tissue are altered in human obesity. *J Clin Endocrinol Metab* 2008; 93:3215-21.
10. Noris M, Gamba S, Bonazzola S, Remuzzi G. Interleukin-6 and RANTES in Takayasu Arteritis- A Guide for Therapeutic Decisions? *Circulation* 1999; 100:55-60.
11. Kiguchi N, Kobayashi Y, Kishioka S. Chemokines and cytokines in neuroinflammation leading to neuropathic pain. *Curr Opin Pharmacol* 2012; 12(1):55-61.
12. Stringer EA, Baker KS, Carroll IR, Montoya JG, Chu L, Maecker HT, Younger JW. Daily cytokine fluctuations, driven by leptin, are associated with fatigue severity in chronic fatigue syndrome: Evidence of inflammatory pathology. *J Transl Med* 2013; 11:93.
13. Rothwell NJ, Hopkins SJ. Inflammatory response: Pathway across the blood-brain barrier: How cytokines can induce these effects has been a mystery as they are unlikely to cross the blood-brain barrier. *Trends Neurosci* 1995; 18:130-36.
14. Ubogu E, Callahan M, Tucky B, Ransohoff R. Determinants of CCL5-driven mononuclear cell migration across the blood-brain barrier. Implications for therapeutically modulating neuroinflammation. *J Neuroimmunol* 2006; 179:132-44.
15. van der Meer P, Ulrich AM, Gonzalez-Scarano F, Lavi E. Immunohistochemical analysis of CCR2, CCR3, CCR5, and CXCR4 in the human brain: potential mechanisms for HIV dementia. *Exp Mol Pathol* 2000; 69:192-201.
16. Luo Y, Berman MA, Zhai Q, et al. RANTES stimulates inflammatory cascades and receptor modulation in murine astrocytes. *Glia* 2002; 39(1):19-30.

17. Stuart MJ, Baune BT. Chemokines and chemokine receptors in mood disorders, schizophrenia, and cognitive impairment: a systematic review of biomarker studies. *J. Neurobiol* 2014; 42:93-115.
18. Kiguchi N, Kobayashi Y, Kishioka S. Chemokines and cytokines in neuroinflammation leading to neuropathic pain. *Curr Opin Pharmacol* 2012; 12(1):55-61.
19. Szabo I, Chen XH, Xin L, et al. Heterologous desensitization of opioid receptors by chemokines inhibits chemotaxis and enhances the perception of pain. *Proc Natl Acad Sci U S A* 2002; 99(16):10276-81.
20. Steele AD, Szabo I, Bednar F, Rodgers TJ. Interactions between opioid and chemokine receptors: heterologous desensitization. *Cytokine Growth Factor Rev* 2002; 13(3):209-22.
21. Lee YK, Choi DY, Jung YY, et al. Decreased pain responses of C-C chemokine receptor 5 knockout mice to chemical or inflammatory stimuli. *Neuropharmacology* 2013; 67:57-65.

LETTER TO THE EDITOR

PROXIMAL FEMORAL NAIL ANTIROTATION INTERNAL FIXATION IN TREATING INTERTROCHANTERIC FEMORAL FRACTURES OF ELDERLY SUBJECTSL. JIA^{1,2}, K. ZHANG², ZG. WANG², L. WANG², SY. YANG² and YP. ZHENG¹¹*Orthopaedic Department, Qilu Hospital of Shandong University, Jinan, China;* ²*Orthopedic Trauma, Binzhou Medical University Hospital, Binzhou, China**Received October 24, 2016 – Accepted March 2, 2017*

This study compared the effect of dynamic hip screw (DHS) and proximal femoral nail anti-rotation (PFNA) in the treatment of intertrochanteric femoral fractures of elderly subjects and evaluated the effect of PFNA internal fixation. Two hundred and sixteen elderly patients with intertrochanteric femoral fracture who received treatment in Binzhou Medical University Hospital, Shandong, China were selected. They were divided into a PFNA group which adopted PFNA for internal fixation and a DHS group which adopted DHS for internal fixation, 108 cases in each group. The physical condition of the patients in the two groups were recorded on admittance. The fracture was typed according to Evans-Jensen classification criteria. Patients were followed up after surgery to evaluate the postoperative recovery and Harris score of hip joint function. Except for the length of hospital stay, the length of incision, intraoperative blood loss, volume of drainage and duration of operation of the PFNA group were all superior to those of the DHS group ($P < 0.05$); the incidence of postoperative complications of the PFNA group was lower than that of the DHS group ($P < 0.05$); the early Harris score of the treatment was superior to that of the DHS group, and there was no remarkable difference ($P > 0.05$). PFNA has more advantages than DHS in treating intertrochanteric femoral fracture of the elderly; hence it is worth wide application in clinical use.

To the Editor,

Incidence of intertrochanteric femoral fracture becomes increasingly higher with the acceleration of living pace and aggravation of aging. The incidence of hip fracture is 18% and 27%, respectively, among males and females aged 65 to 70 years and 30% and 40% among males and females aged 78 years (1, 2). Currently, surgery is the major therapy for intertrochanteric femoral fracture. Surgical treatment includes external fixation, internal fixation and artificial hip joint replacement; internal fixation can be categorised into intramedullary fixation and

extramedullary plate fixation. In clinical application, different types of internal fixation have different characteristics. In recent years, proximal femoral nail anti-rotation (PFNA) and dynamic hip screw (DHS) are the internal fixation tools most frequently used in treating intertrochanteric fracture. DHS internal fixation entails inserting screws into the head of the femur to fix the femur head and femur shaft. Conforming to the biomechanical characteristics of the upper segment of femur, it can effectively control rotation and bending and maintain fixation compression when the broken ends of fractured

*Key words: intertrochanteric femoral fracture, proximal femoral nail anti-rotation, dynamic hip screw, hip joint function**Mailing address:*

Dr Yanping Zheng,
Orthopaedic Department,
Qilu Hospital of Shandong University,
No. 107, Wenhua West Road, Jinan, Shandong, 250012, China
e-mail: zypzhengyp@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.

bone are compressed. As to unstable fractures, such as subtrochanteric oblique and trochanteric internal cortex comminuted, there is a high incidence of complications (3-5). Compared to DHS, which has limitations to clinical symptoms, PFNA internal fixation is more reliable, especially when the fracture is not stable (6). Experts from China and abroad continually dispute the effects of the two internal fixation methods (7, 8). To provide a reliable and scientific basis for the selection of the most suitable internal fixation, this study retrospectively analyzed and compared the effects of DHS and PFNA internal fixation in 216 cases.

MATERIALS AND METHODS

General data

Two hundred and sixteen elderly subjects with intertrochanteric femoral fracture who received treatment in Binzhou Medical University Hospital, Shandong, China from July 2011 to July 2013 were selected and divided into a PFNA group and a DHS group.

In the PFNA group, 108 patients (40 males and 68 females; ages ranging from 65~81 years: average 73 years), underwent PFNA internal fixation. In this group there were 45 cases of right fracture and 63 cases of left fracture; 19 cases were traffic accidents and 89 cases were fall damage; they were classified into category III fracture (63 cases), category IV fracture (43 cases) and category V fracture (2 case) according to Evans-Jensen classification criteria. All the patients underwent routine examination before surgery, and closed reduction in combination with PFNA internal fixation was performed between the second day and the tenth day after admission.

In the DHS group, 108 patients (72 females and 36 males; ages ranging from 62 to 82 years: average 72 years) underwent DHS internal fixation. Of these there were 42 cases of right fracture and 66 cases of left fracture; 11 cases were traffic accidents and 97 cases were fall damage; they were classified into category III (63 cases), category IV (45 cases) and no cases of category V. They underwent routine examination before surgery, and closed reduction in combination with DHS internal fixation was performed between the second day and the tenth day after admission.

Of all the 216 patients, 95 cases had cardiovascular and cerebrovascular diseases, 72 had category II diabetes, 27

had lung infection and 22 had urinary system infections. With regard to gender, age, height and fracture category of the two groups there were no statistical differences, hence the results were comparable.

Inclusion and exclusion criteria

Patients who were confirmed having intertrochanteric femoral fracture by physical examination and imaging (X-rays, computed tomography (CT) and Magnetic Resonance Imaging (MRI)) assisted examination, underwent either PFNA or DHS for internal fixation, had complete follow-up and clinical data, were examined to be normal by routine examination before operation, aged no less than 60 years, with recent fracture were included.

Patients who developed major vascular nerve injury or open fracture, could not undergo surgery due to organ dysfunction syndrome, hematological or mental diseases, were unable to walk because of hemiplegia and Parkinson's disease, refused to enter the research, failed to be followed-up or had Singh index below grade III were excluded.

All the included patients gave informed consent to participate in the study.

Therapeutic method

Skin traction, which was beneficial to relieve swelling, was performed after patients were admitted to hospital. Before surgery, routine examination was carried out to exclude complications. Patients were treated by surgery once the swelling of the injured limb disappeared (from day 4 to day 10 after admission). Patients in the DHS group were given general anesthesia or continuous epidural anesthesia; then manual reposition was performed on in supine position and maintained by a traction table; an incision was made on the outer side of hip joint after the reposition was confirmed as being satisfactory by C-arm fluoroscopy; then DHS (Shanghai Synthes Medical Co., Ltd, China) was inserted (Fig. 1). For instable fractures, one hollow screw was used to prevent the uncontrollable rotation of DHS; if patients had a greater trochanter fracture, a junction steel plate was used.

Patients in the PFNA group were treated by PFNA (Shanghai Synthes Medical Co., Ltd, China). Anesthesia, operative position and reduction operation were the same as for the DHS group. The patients lay in a supine position; fracture reduction was confirmed by C-arm fluoroscopy; a 6-cm incision was cut on the outer side above the femoral great trochanter to separate muscular fasciae and muscle and

expose the top of great trochanter; a guide pin was inserted into the marrow cavity and it penetrated the fracture end; marrow was expanded along the guide pin after it located in the middle of marrow cavity, as confirmed by anteroposterior fluoroscopy. An intramedullary needle was inserted into the marrow cavity; the screw blade was confirmed in the correct position by fluoroscopy, i.e., on the middle and lower boundary of the femoral neck as in anteroposterior view and in the middle in lateral view. A guide pin was inserted into femoral head and neck, and then a screw blade of an appropriate length, followed by remote lock and proximal nut; finally the placement of the intramedullary needle was again checked by fluoroscopy (Fig. 2).

Observation indexes

Hospitalization and surgery-related indexes, including duration of operation, length of incision, postoperative drainage volume, and length of stay, were recorded. Intraoperative blood loss was evaluated by detecting the level of hemoglobin. Harris scoring was used to evaluate the functional recovery of the hip joint; the total score was 100 points, including 47 points for function, 44 points for pain, 5 points for joint motion, and 4 points for deformity; a score of 90~100 points was evaluated as excellent, 80~90 points as good, 70~79 points as medium, and lower than 70 points as poor.

Information on healing of fracture, condition of internal fixation, early and late complications as well as Harris score of hip joint function was collected via telephone or by an outpatient visit.

Statistical analysis

SPSS19.0 was used for data processing. Measurement data were expressed as mean $\pm$ SD and processed by *t*-test. Enumeration data were processed by *chi*-squared test.

Difference was considered having statistical significance if $P < 0.05$.

RESULTS

Comparison of duration of operation, interval time between injury and operation, length of incision and intraoperative blood loss

The interval time between injury and operation of the PFNA and DHS groups was (2.1 $\pm$ 0.6) days and (2.9 $\pm$ 0.9) days, respectively, and the difference had no statistical significance ($P > 0.05$). The length of incision of the PFNA group and the DHS group was 3~5 cm (average 4 cm) and 10~15 cm (average 12.50 cm), respectively; and the difference had statistical significance ($P < 0.05$). PFNA internal fixation lasted for 45~90 min, average 56.34 $\pm$ 9.81 min, while DHS internal fixation lasted for 70~130 min, average 91.49 $\pm$ 15.12 min, and the difference had statistical significance ($P < 0.05$). Intraoperative blood loss of the PFNA group and the DHS group was 100~300 mL (average 142 $\pm$ 45 mL) and 200~500 mL (average 319 $\pm$ 74 mL) respectively, and was statistically significant ($P < 0.05$). Blood loss of the PFNA group was less than that of the DHS group.

Comparison of postoperative drainage volume and length of stay between groups

The postoperative drainage volume of the PFNA group was superior to that of the DHS group (115.38 $\pm$ 78.27 mL vs 194.67 $\pm$ 112.62 mL) ($P < 0.05$). The length of stay of the PFNA group and the DHS group was 14~26 days (average 16.48 $\pm$ 1.20 days) and 10~27 days (average 17.52 $\pm$ 2.30 days), respectively; no significant difference was observed ($P > 0.05$).

Table I. Comparison of Harris score of hip joint function between the two groups.

Group	N	1 month after operation	3 months after operation	6 months after operation	12 months after treatment
PFNA group	108	41.77 $\pm$ 4.54*	18.34 $\pm$ 2.13	9.50 $\pm$ 1.58	7.64 $\pm$ 0.82
DHS group	108	31.24 $\pm$ 3.86	15.94 $\pm$ 1.51	7.12 $\pm$ 0.85	5.12 $\pm$ 0.51

* $P < 0.05$, compared to the DHS group

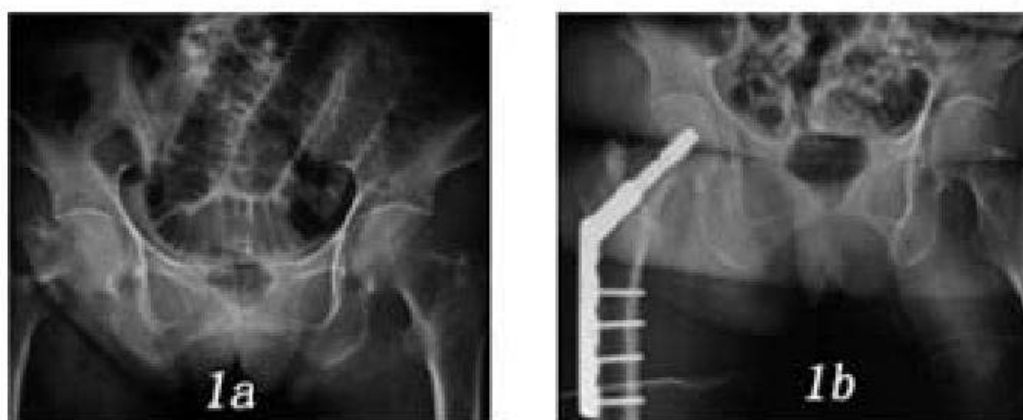


Fig. 1. X-rays of an 80-year-old female with intertrochanteric femoral fracture on the right side who underwent DHS internal fixation. **a)** On day of admission; **b)** anteroposterior X-ray after surgery.



Fig. 2. X-rays of a 73-year-old female with Evans-Jensen category III intertrochanteric femoral fracture on the right side who underwent PFNA. **a)** On day of admission; **b)** after surgery.

Comparison of postoperative complications between groups

No deaths occurred in either group. In the DHS group, the incidence of internal fixation-associated complications was 15.74%; there were 5 cases of coxa vara, 3 cases of femoral head and neck segmentation in different degrees, 9 cases of 2 cm higher femoral neck shrinkage induced by backward dropout of lag screws and collapse of fractured sites, 10 cases of lung infection and 5 cases of urinary system infection. The internal fixation associated complications of the PFNA group was 5.6%; there was 1 case of ipsilateral femoral shaft fractures induced by falling over, 5 cases of cortex cleavage on one side of the femoral

shaft, 4 cases of lung infection and 3 cases of urinary system infection. There was statistically significant difference between the two groups in the incidence of postoperative complications ($P < 0.05$).

Comparison of Harris score of hip joint function between two groups

The Harris score of hip joint function after one-month internal fixation in both groups suggested significant reduction compared to that prior to internal fixation ($P = 0.024, 0.030$). The early activity function recovery of the hip joint in the PFNA group was much superior to that in the DHS group; however, the difference between the two groups in

the long-term hip joint activity function recovery had no statistical significance ($P>0.05$) (Table I).

DISCUSSION

DHS, one of frequently used internal fixation systems, is featured by extramedullary fixation, long force arm and large bending moment (9). In the treatment of III, IV or V stable fractures, screw bending usually occurs because the pressure stress cannot be transferred by the femoral moment, and stress concentrates on the internal fixation (10). Abalo et al. (11) found that DHS was unable to effectively resist rotation even though it has a reliable structure and can produce dynamic and static pressure. PFNA is central fixation with a short force arm. In the application of PFNA, the load is transferred to the distant end of the fracture through an internal fixture, and moreover there is increased holding force and anti-rotation design which can effectively prevent rotation on the fracture end, achieve rigid internal fixation, and is beneficial to early functional exercise (12). In this study, PFNA and DHS suggested no remarkable difference in the long-term curative effect. Patients who underwent PFNA achieved weight-bearing walk immediately after surgery, which significantly reduced the incidence of complications during hospitalization. The clinical effect of PFNA was superior to that of DHS in the early stage. Compared to the DHS group, the assessment of various surgical indicators in the PFNA group was better, indicating that PFNA was featured by little trauma, simple surgery and short duration in treating unstable intertrochanteric femoral fractures.

PFNA with favorable biomechanical properties can provide reliable fixation for different categories of intertrochanteric femoral fracture and has better results in the early stage compared to DHS. It is the preferred choice of treatment in category Evans-Jensen III, IV and V intertrochanteric femoral fracture in combination with osteoporosis.

REFERENCES

1. Majernicek M, Dungal P, Kolman J, Malkus T, Vaculik J. Osteosynthesis of intracapsular femoral neck fractures by dynamic hip screw (DHS) fixation. *Acta Chir Orthop Traumatol Cech* 2009; 76(4):319-25.
2. Ho M, Garau G, Walley G, Oliva F, Panni AS, Longo UG, Maffulli N. Minimally invasive dynamic hip screw for fixation of hip fractures. *Int Orthop*. 2009; 33(2):555-60.
3. Buciuto R, Hammer R. RAB-plate versus sliding hip crew for unstable trochanteric hip fractures: stability of the fixation and modes of failure-radiographic analysis of 218 fractures. *J Trauma* 2001; 50(3):545-50.
4. Mohan R, Karthikeyan R, Sonanis SV. Dynamic hip screw: does side make a difference? Effects of clockwise torque on right and left DHS. *Injury* 2000; 31(9):697-99.
5. Strauss E, Frank J, Lee J, Kummer FJ, Tejwani N. Helical blade versus sliding hip screw for treatment of unstable intertrochanteric hip fractures: a biomechanical evaluation. *Injury* 2006; 37(10):984-89.
6. Rapp K, Becker C, Lamb SE, Icks A, Klenk J. Hip fractures in institutionalized elderly people: incidence rates and excess mortality. *J Bone Miner Res* 2008; 23(11):1825-31.
7. Windolf M, Braunstein V, Dutoit C, Schwieger K. Is a helical shaped implant a superior alternative to the Dynamic Hip Screw for unstable femoral neck fractures? A biomechanical investigation. *Clin Biomech (Bristol, Avon)* 2009; 24(1):59-64.
8. Wong TC, Chiu Y, Tsang WL, Leung WY, Yeung SH. A double-blind, prospective, randomised, controlled clinical trial of minimally invasive dynamic hip screw fixation of intertrochanteric fractures. *Injury* 2009; 40(4):422-27.
9. Nüchtern JV, Ruecker AH, Sellenschloh K, Rupprecht M, Püschel K, Rueger JM, Morlock MM, Lehmann W. Malpositioning of the lag screws by 1- or 2-screw nailing systems for pertrochanteric femoral fractures: a biomechanical comparison of gamma 3 and intertan. *J Orthop Trauma* 2014; 28(5):276-82.
10. Nordin S, Zulkifli O, Faisham WI. Mechanical failure of Dynamic Hip Screw (DHS) fixation in intertrochanteric fracture of the femur. *Med J Malaysia* 2001; 56 Suppl D:12-17.
11. Abalo A, Dossim A, Ouro Bangna AF, Tomta

- K, Assiobo A, Walla A. Dynamic hip screw and compression plate fixation of ipsilateral femoral neck and shaft fractures. *J Orthop Surg (Hong Kong)* 2008; 16(1):35-38.
12. Anwar R, Boorgula V, Nicholl JE. Intrapelvic penetration of the dynamic hip screw during removal: lesson learnt-always use a coupling screw. *J Trauma*. 2007; 63(5):E105-106.

LETTER TO THE EDITOR

FEMORAL NERVE REPAIR USING AN
H-TYPE NERVE REGENERATION CHAMBER IN RATSX-Y. HONG¹, X. HONG¹, W-W. GU², J. LIN¹ and W-T. YIN³

¹Department of Vascular Surgery, China-Japan Union Hospital of Jilin University, Changchun, China; ²Department of Hepatopancreatobiliary Surgery, China-Japan Union Hospital of Jilin University, Changchun, China; ³Department of Hand Surgery, China-Japan Union Hospital of Jilin University, Changchun, China

Received September 14, 2016 – Accepted March 2, 2017

Peripheral nerve injury in surgical trauma patients is very common. The femoral nerve can be divided into two branches: one regarding the quadriceps muscles, and one the skin. After nerve transection and suture, the motor axons have an equal opportunity to regenerate into the original muscle branch, or regenerate into the sensory skin nerves. These anatomical features of femoral nerve have made it important in nerve regeneration research. Thus, this study was designed to examine the effects of an H-type nerve regeneration chamber on motor nerve regeneration after femoral nerve injury. We performed femoral nerve injuries in adult rats and assessed nerve recovery over a 4-week post-operative period. Additionally, we evaluated nerve regeneration in the same animals anatomically, using several histological staining methods to provide structure analyses. We found that H-type nerve regeneration chamber provided enhanced improvement in nerve regeneration without nerve anastomosis, as compared with nerve anastomosis. Furthermore, incorrect nerve anastomosis reduced the nerve fiber diameter and thickness of myelin sheaths in regenerated nerve fibers. Finally, H-type nerve regeneration chamber provided enhanced functional recovery of nerve fibers, particularly for motor nerves. Together, our results suggest that direct nerve suture cannot effectively improve the functional recovery of damaged nerves, and nerve chemotaxis coupled with nerve regeneration chamber can effectively improve the effects of nerve regeneration, and enhance the prognosis of nerve injury repair.

To the Editor,

Peripheral nerve injury in surgical trauma patients is very common. Recent studies have shown that nerve chemotaxis can effectively improve the effect of nerve regeneration, and enhance the prognosis of nerve injury repair. The anatomical features of femoral nerve have made it highly important in nerve regeneration research (1-3). After nerve transection and suture, the motor axons have

an equal opportunity to regenerate into the original muscle branch or into the sensory skin nerves. Interestingly, it has been shown that more motor axons regenerate into the muscle as opposed to the skin branch of the femoral nerve in rats (1, 2, 4), suggesting nerve chemotaxis may play a differential role in regeneration of different branches of the femoral nerve. Thus, this study was designed to examine the effects of an H-type nerve regeneration

Key words: peripheral nerve injury, femoral nerve, regeneration, motor, sensory, nerve regeneration chamber

Mailing address:
Prof. Wei-Tian Yin,
Department of Hand Surgery,
China-Japan Union Hospital,
Jilin University,
Changchun 130031, China
e-mail: yinweitian@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.**

chamber on the regeneration of motor and sensory nerve branches after femoral nerve injury. To this end, we performed femoral nerve injuries in adult rats and assessed nerve recovery over a 4-week post-operative period, using several histological staining methods and an electrophysiological method.

MATERIALS AND METHODS

Animals

Female Wistar rats (n=60) weighting 200-250g at the start of the study were supplied from Shanghai Laboratory Animal Center. Upon arrival, rats were housed individually in a room with a 12-h light/dark cycle (light on 7:00-19:00) and controlled temperature ($23\pm1^{\circ}\text{C}$) and acclimated in the Animal Center. Rats were given food and water *ad libitum* except during the period of experiments. Animals were separated into 4 groups (n=15/group). All animal experiments were approved by the local Institutional Animal Care and Use Committee. The housing and treatment of the rats followed the guidelines of the "Guide for the Care and Use of Laboratory Rats" (Institute of Laboratory Animal Resources, Commission on Life Sciences 2011).

Surgery

All surgical procedures were conducted in a sterile environment as shown in Fig. 1. Unilateral (left) femoral nerve lesions were made under anesthesia with 1% ketamine (40 mg/kg) intraperitoneal injection. The trunk of the nerve was exposed under an operation microscope and injured at approximately 7 mm proximal to the bifurcation of the saphenous and quadriceps muscle branches. The sensory (i.e., S) and motor (i.e., M) nerves were transected and the proximal and distal ends of the nerves were inserted into a polyethylene H-type nerve regeneration chamber (10 mm length, 0.8 mm inner diameter) customized using polyethylene tubing (Becton Dickinson, Shanghai, China) and fixed with two 10-0 nylon stitches so that a 5-mm gap was left between the proximal and distal ends (3,5) in group A (i.e., S-S and M-M) and group B (i.e., S-M and M-S) rats. In separate groups of animals, the proximal and distal ends of the nerves were aligned using two 10-0 sutures (end-to-end anastomosis) in group C (i.e., S-S and M-M) and group D (i.e., S-M and M-S). Finally, 4-0 sutures were used to close the skin. Four weeks after

surgery, none of the rats showed any inflammation, ulcers, or other adverse conditions.

Histology

All rats were sacrificed 4 weeks after surgery. Nerve anastomoses in distal nerve tissue were conducted, and longitudinal sections were stained using standard H&E, Toluidine blue, Marsland-Luxolfastblue, and acetylcholinesterase.

Electrophysiological examination of nerves

Animals were anesthetized with 1% ketamine (40 mg/kg) intraperitoneal injection. The bilateral sciatic nerve was exposed, a core needle was inserted into the rat soleus muscle as the recording electrode (M). Super strong stimulation (50 mA) was used at proximal (P) and the distal (D) sciatic nerve branch, respectively, to induce two motor potentials. The motion potential amplitude (AMP) and latency were automatically displayed by the instrument. Conduction velocity = distance between two stimulus electrodes/action potential latency difference (conduction time). The right side was measure as normal control.

Statistical analysis

All data collection and analysis were carried out using STATA 12.0 (Lakeway Drive, USA) software, with the confidence level of 95%. When the data exhibited normal distribution, data were shown as mean $\pm$ standard deviation ($\bar{x}\pm s$) and analyzed with *t*-test. When the data exhibited skewed distribution, data were shown as the median (25% percentile-75% percentile), and analyzed using the Mann-Whitney U test. χ^2 test was used to compare differences of composition ratio. Student's *t*-test was used to compare difference of data with the mean $\pm$ standard deviation. For all comparisons, $p<0.05$ was considered statistically significant.

RESULTS

General observation

In group A, normal nerve growth was shown in the small gap. Regenerated nerve fibers were aligned orderly in the bridge of the gap without showing escaping phenomenon. Nerve bundles exhibited intact continuity and approximate state of maturity.

Visual observation found that the diameter of the regenerated nerve was close to the healthy nerve and the nerve regeneration site did not exhibit tumor growth or hyperplasia expansion. Furthermore, the bridging did not show adhesion, and could be removed intact after blunt dissection. Regenerated motor and sensory nerves of the femoral nerve bundle were successfully isolated from distal to proximal sites.

In group B, normal nerve growth was shown in the small gap. Regenerated nerve fibers were aligned orderly in the bridge of the gap without showing escaping phenomenon. However, nerve growth exhibited cross phenomenon. Visual observation found that the diameter of the regenerated nerve was close to the healthy nerve and the nerve regeneration site did not exhibit tumor growth or hyperplasia expansion. Furthermore, the bridging did not show adhesion, and can be removed intact after blunt dissection. Regenerated motor and sensory nerves of the femoral nerve bundle can be successfully isolated

from distal to proximal sites.

In group C, nerve anastomosis exhibited relative adhesion with the surrounding tissue. Regenerated nerve fibers at the anastomosis section were not aligned orderly in the bridge of the gap, and exhibited escaping phenomenon. Visual observation found that the diameter of the regenerated nerve was about 1/4 larger than the healthy nerve and the nerve regeneration site exhibited tumor growth or hyperplasia expansion. Furthermore, regenerated motor and sensory nerves of the femoral nerve bundle could not be successfully isolated from distal to proximal sites.

In group D, nerve anastomosis exhibited heavy adhesion with the surrounding tissue. Regenerated nerve fibers at the anastomosis section were not aligned orderly in the bridge of the gap, and exhibited enhanced escaping phenomenon. Visual observation found that the diameter of the regenerated nerve was about 1/2 larger than the healthy nerve and the nerve regeneration site exhibited tumor growth or

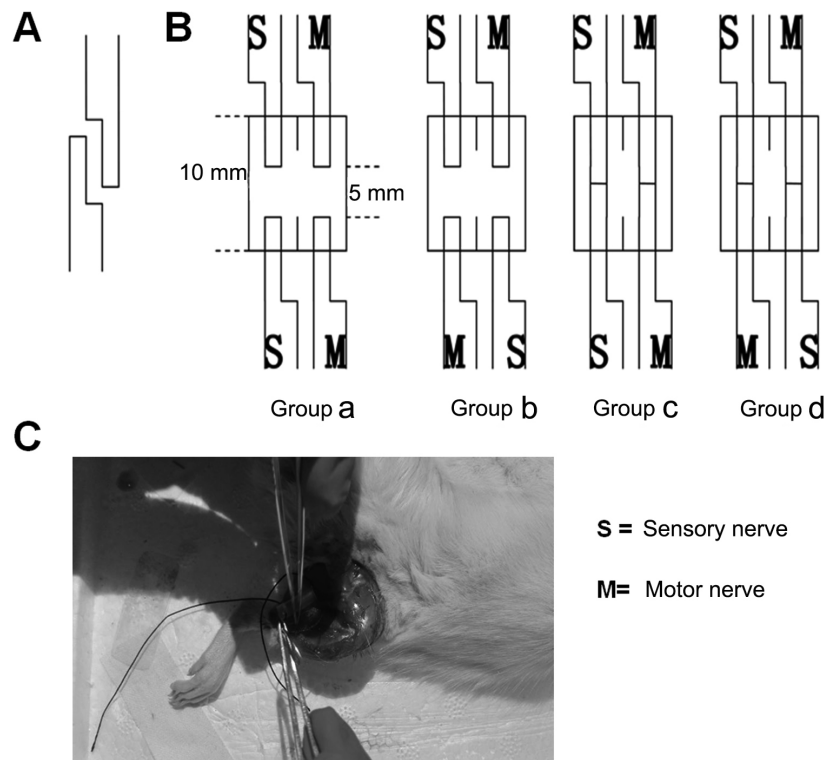


Fig. 1. Experimental design of surgery. All surgical procedures were conducted in a sterile environment. Unilateral (left) femoral nerve lesions were made under anesthesia.

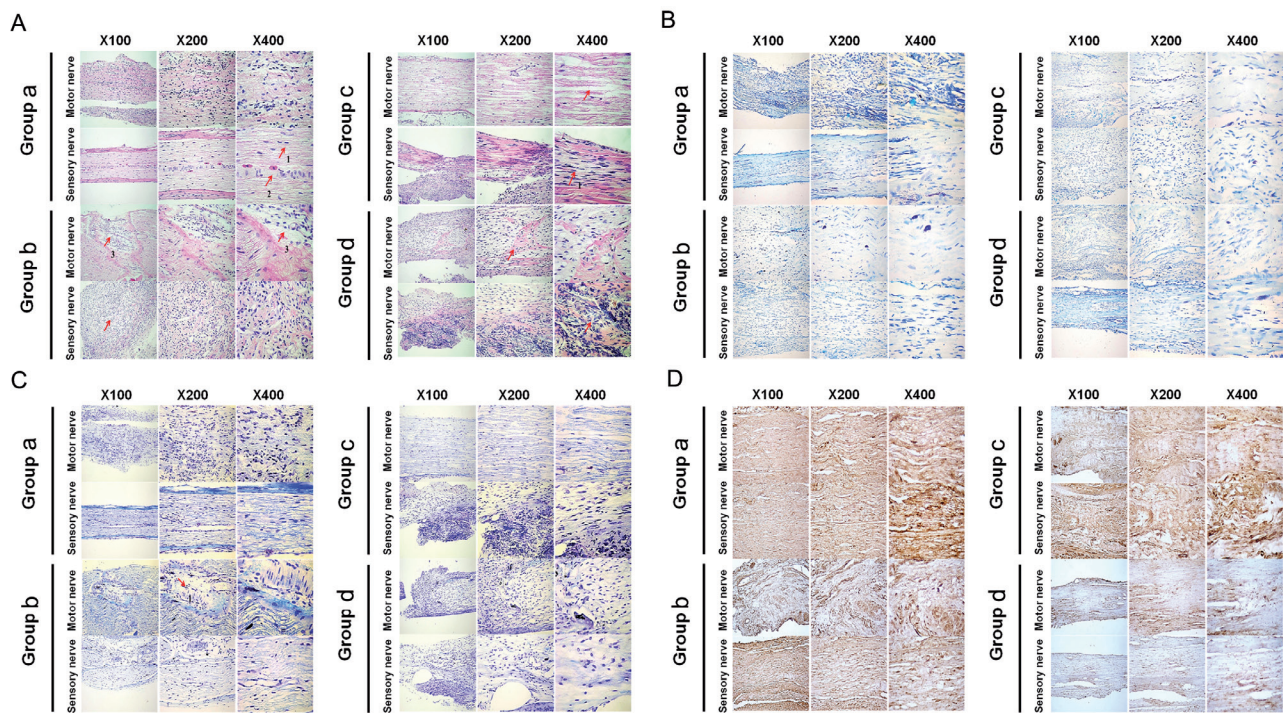


Fig. 2. Histological examination. *A)* H&E staining showed visible myelin sheath in pink, axonal in purple, and nuclei in blue. *B)* Toluidine blue staining showed visible myelin sheath in dark blue. *C)* Marsland-Luxolfastblue (LFB) dual staining showed nerve fibers in black and axons in blue. *D)* Acetylcholinesterase staining showed regeneration of nerve fibers.

hyperplasia expansion. Furthermore, regenerated motor and sensory nerves of the femoral nerve bundle could not be successfully isolated from distal to proximal sites.

Histology

H&E staining showed visible myelin sheath in pink, axonal in purple, and nuclei in blue (Fig. 2A). In groups A, B, and C, nerve fibers were seen in a parallel arrangement and Schwann cells were seen as scattered. In groups A and C, nerve fibers were aligned in an orderly way. In group B, nerve fibers were less orderly but continuous alignment and axons were scattered. However, proliferation of a large number of connective tissues around the nerve fibers was seen in group D.

Toluidine blue staining showed visible myelin sheath in dark blue (Fig. 2B). There are different degrees of myelin disintegration between each group. The highest degree of myelin disintegration was seen in group D. In groups A, B, and C, myelinated nerve fibers were seen and myelin

appeared complete and normal. However, nerve fibers were aligned much less orderly in group D.

Marsland-Luxolfastblue (LFB) dual staining showed nerve fibers in black and axons in blue (Fig. 2C). In groups A, B, and C, nerve fibers were seen in a parallel arrangement. However, the distribution of nerve fibers in group B was less uniform than in groups A and C. Nerve fibers were aligned much less orderly in group D.

Acetylcholinesterase staining showed that regeneration of nerve fibers was seen in orderly and wavy alignment in groups A, B, and C (Fig. 2D), and was most evident in group A. While the nerve fibers in group B were arranged less orderly than in group A, continuous nerve fibers scattered Ranvier knot were still present in group B. Furthermore, nerve fibers were aligned much less orderly in group D.

Nerve fiber diameter and thickness of myelin sheaths

The average diameters of myelinated nerve fibers in groups a, b, c, and d of rats 4 weeks after surgery

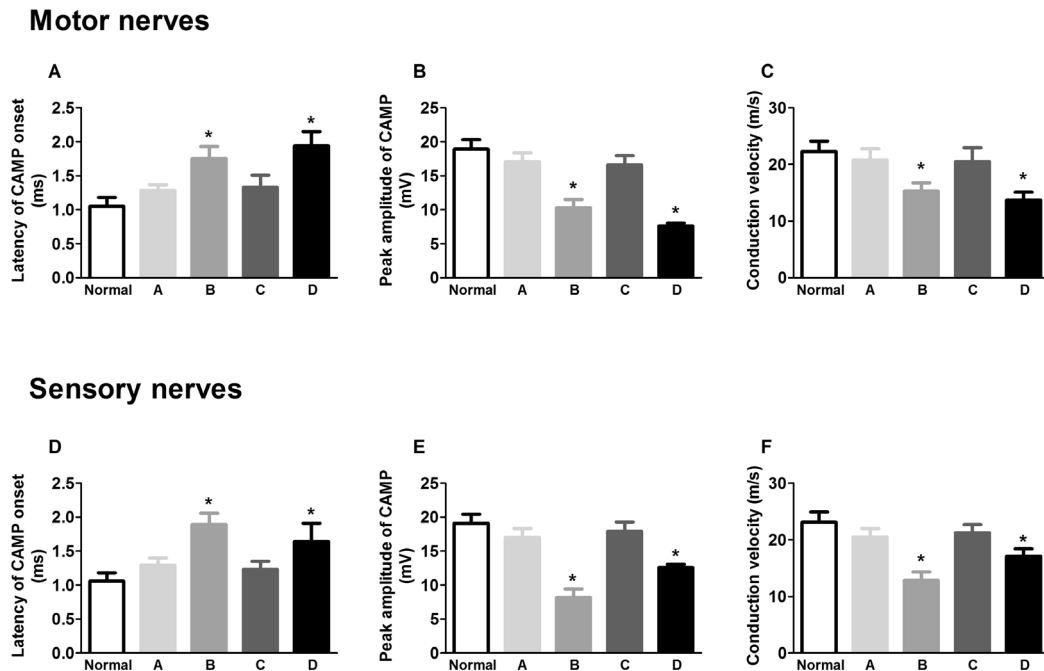


Fig. 3. Neurophysiological results of regenerated motor and sensory nerve fibers. **A, D)** Latency of CAMP onset (ms). **B, E)** Peak amplitude of CAMP (mV). **C, F)** Conduction velocity (m/s). *One-way ANOVA, $p < 0.05$.

and the thickness of myelin sheaths are shown in Table I. There was no significant difference in nerve fiber diameters among groups A, B, and C. However, the nerve fiber diameter was significantly smaller in group D than in groups A, B, and C ($p < 0.05$). Additionally, there was no significant difference in average thickness of the myelin sheath among groups A, B, and C. However, the average thickness of the myelin sheath of nerve fiber was significantly smaller in group D than those in groups A, B, and C ($p < 0.05$). Notably, nerve growth exhibited cross phenomenon in group B. However, the ratio of regenerated motor nerve fiber in distal motor nerves was $96.1 \pm 16\%$, while the ratio of motor nerve fibers in the distal sensory nerves was $5.9 \pm 2.1\%$, which was statistically significant ($p < 0.05$).

Neurophysiological results

We used electrophysiological experiments to further study the “H” type of nerve tube in the motor nerve and sensory nerve repair and the functional recovery. We first tested the recovery of

motor neurons. Each rat was used to calculate the peak amplitude of CAMP, the CAMP onset latency, and NCV. The neurological function of group A and group C was significantly better than that of group B and group D. In the motor nerve bundle, the recovery of group A was better than that of group C, and the recovery in group B was better than that in group D. It was proved that the small interval generated by nerve tube in motor nerve was beneficial to the recovery of nerve.

Similarly, the neurological function of sensory nerves in groups A and group C was significantly better than those of groups B and group D. In the sensory nerve bundle, the recovery of group C was better than that of group A, and recovery in group D was better than that in group B. It was proved that direct suturing was beneficial to sensory nerve recovery.

DISCUSSION

Peripheral nerve injury is a common injury in

Table I. Nerve fiber diameter and thickness of myelin sheaths in rats after four weeks.

Group	Average nerve fiber diameter (μm)	Average Thickness of myelin sheaths (μm)
A (n=10)	7.59±1.25	1.41±0.19
B (n=10)	6.25±0.95	1.25±0.16
C (n=10)	7.15±0.86	1.38±0.14
D (n=10)	2.1±0.13*	0.39±0.06*

*One-way ANOVA, $p < 0.05$

surgery (6). Evidence has shown that regenerated nerve often exhibit dysfunction, more or less regardless of precision of sutures (7). Furthermore, some studies found that no-gap nerve sutures prevented the nerve regeneration in chemotaxis environment (8), and prohibited chemotaxis to induce nerve regeneration (9). Thus, after incorrect nerve anastomosis, severe growth disorders and adhesions can lead to incorrect nerve regeneration, and result in diminished opportunity to restore nerve function (10). Recent studies have shown that chemotaxis-induced nerve regeneration is affected by many factors, including the gap of the nerve injury, volume and structure of distal nerve, and nerve regeneration environment (11). It has been speculated that the mechanisms underlying chemotaxis-induced nerve regeneration may involve various factors, such as neurotrophic factor interactions with the extracellular matrix proteins, recognition molecules on the axonal surface, and specific neuronal phenotypes (12). Recent studies have confirmed that small gap bridging technology of peripheral nerve regeneration can promote chemotaxis-induced nerve regeneration, and effectively repair damaged peripheral nerves (13). Our study used different connection methods after nerve injury to alter the target organs that receive sensory and motor nerve

fibers innervations so that we demonstrated that the structure of regenerated nerve was altered and the functional plasticity of sensory nerve fibers occur.

REFERENCES

1. Brushart TM. Preferential reinnervation of motor nerves by regenerating motor axons. *J Neurosci* 1988; 8:1026-31.
2. Madison RD, Robinson GA, Chadaram SR. The specificity of motor neurone regeneration (preferential reinnervation). *Acta Physiol (Oxf)* 2007; 189:201-6.
3. Irintchev A, Wu MM, Lee HJ, et al. Glycomimetic improves recovery after femoral injury in a non-human primate. *J Neurotrauma* 2011; 28:1295-306.
4. Madison RD, Archibald SJ, Brushart TM. Reinnervation accuracy of the rat femoral nerve by motor and sensory neurons. *J Neurosci* 1996; 16:5698-703.
5. Simova O, Irintchev A, Mehanna A, et al. Carbohydrate mimics promote functional recovery after peripheral nerve repair. *Ann Neurol* 2006; 60:430-37.
6. Zack-Williams SD, Butler PE, Kalaskar DM. Current progress in use of adipose derived stem cells in peripheral nerve regeneration. *World J Stem Cells* 2015; 7:51-64.
7. Hung HA, Sun G, Keles S, Svaren J. Dynamic regulation of Schwann cell enhancers after peripheral

- nerve injury. *J Biol Chem* 2015; 290:6937-50.
8. Terayama R, Yamamoto Y, Kishimoto N, Maruhama K, Mizutani M, Iida S, Sugimoto T. Peripheral nerve injury activates convergent nociceptive input to dorsal horn neurons from neighboring intact nerve. *Exp Brain Res* 2015; 233:1201-12.
 9. Feng B, Ma H, Hu H, et al. Effect of combination of nerve fragments with nerve growth factor in autologous epineurium small gap coaptation on peripheral nerve injury repair. *Cell Tissue Bank* 2015; 16: 497-502.
 10. Shah MB, Chang W, Yu X. Evaluating nerve guidance conduits for peripheral nerve injuries: a novel normalization method. *Neural Regen Res* 2014; 9:1959-60.
 11. Woo SL, Urbanchek MG, Leach MK, Moon JD, Cederna P, Langhals NB. Quantification of muscle-derived signal interference during monopolar needle electromyography of a peripheral nerve interface in the rat hind limb. *Conf Proc IEEE Eng Med Biol Soc* 2014; 2014:4382-85.
 12. Frost CM, Cederna PS, Martin DC, Shim BS, Urbanchek MG. Decellular biological scaffold polymerized with PEDOT for improving peripheral nerve interface charge transfer. *Conf Proc IEEE Eng Med Biol Soc* 2014; 2014:422-25.
 13. Trigo D, Smith KJ. Axonal morphological changes following impulse activity in mouse peripheral nerve in vivo: the return pathway for sodium ions. *J Physiol* 2015; 593:987-1002.

LETTER TO THE EDITOR

ORAL *CANDIDA* CARRIAGE AND SPECIES PREVALENCE IN DENTURE STOMATITIS PATIENTS WITH AND WITHOUT DIABETESF. JAVED¹, A.A. AL-KHERAIF², S.V. KELLESARIAN¹, F. VOHRA³ and G.E. ROMANOS^{4,5}¹Department of General Dentistry, Eastman Institute for Oral Health, University of Rochester, NY, USA;²Dental Health Department, College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia; ³Department of Prosthetic Dental Sciences, College of Dentistry, King Saud University, Riyadh, Saudi Arabia;⁴Department of Oral Surgery and Implant Dentistry, Dental School, Johann Wolfgang Goethe, University of Frankfurt, Frankfurt, Germany; ⁵Department of Periodontology, School of Dental Medicine, Stony Brook University, Stony Brook, NY, USA

Received February 14, 2017 – Accepted March 29, 2017

The objective of this study was to assess the oral *Candida* carriage and species prevalence in denture stomatitis (DS) patients with and without diabetes mellitus (DM). To address the focused question “In patients receiving dentures, how does having diabetes compared to no diabetes influences the carriage and species prevalence of oral *Candida*?” indexed databases were explored without time or language restrictions up to and including February 2017. Nine studies were included. In these studies, the number of diabetics ranged between 14 patients and 405 individuals with mean ages ranging between 33 years and 66.4 years. In five studies, the percentage of hyperglycemic patients in the diabetic group ranged from 14.3% to 100%. In one study, all diabetic patients were normoglycemic. Seven studies reported *Candida*-associated DS to be significantly higher among diabetics than non-diabetics. *Candida albicans* was the most prevalent species isolated among diabetics and non-diabetics with DS. Denture-wearers with DM are more susceptible to *Candida*-associated DS as compared to non-diabetics.

To the Editor,

Candida (C) *albicans* is the most common fungal specie found in the oral cavity and forms an integral part of the normal human oral flora (1). However, *Candida* species can become opportunistic pathogens in immunocompromised patients, such as those with poorly-controlled diabetes mellitus (DM) and acquired immune deficiency syndrome (2, 3). Denture stomatitis (DS) is a chronic erythematous inflammatory condition that occurs on the oral mucosal surfaces beneath the denture surfaces of partially and completely edentulous individuals (4).

DS is frequently asymptomatic; however, patients may complain of halitosis, burning sensation and/or altered taste (5). The prevalence of DS among denture-wearers ranges between 15% and 70% (5). It has been reported that poor glycemic control and therapeutic dentures in patients with DM predispose them to oral mucosal disorders including oral candidiasis (1). Bianchi et al. (6) also reported that counts of oral yeast colonies are elevated on the denture surfaces of diabetic patients as compared to non-diabetic (ND) individuals. It is therefore hypothesized that oral *Candida* colonization and

Key words: candida, candidiasis, denture stomatitis, diabetes, prevalence

Mailing address:

Dr. Fawad Javed,

Department of General Dentistry, Eastman Institute for Oral Health,
625 Elmwood Avenue, University of Rochester,
Rochester, NY 14620, USA

e-mail: fawad_javed@urmc.rochester.edu

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.

Table I. *Characteristics of studies included.*

Authors et al.	Study Groups	Age (mean years)	Gender F (%)	Type of Denture Stomatitis	Duration of denture wearing (years)	Glycemic status (%)	Smoking status (%)	Outcome
Chopde et al. (7)	100 DS 100 NDS	DS: 64.1 NDS: 65.8	DS: 50 NDS: 50	NA	NA	NA	NA	71.6% of diabetics had candida associated DS. Prevalence of DS was significantly higher in diabetics than non-diabetics.
Daniluk et al. (8)	32 diabetic 23 ND	DM: 66.4	55.8	Type I was more common among both diabetics and non-diabetics.	NA	NA	NA	Prevalence of DS was comparable among diabetics (71.4%) and non diabetics (75%)
Dorko et al. (9)	240 DS 350 NDS	NA	NA	NA	NA	Hyperglycemic**	NA	100% of diabetics had Candida-associated DS. Prevalence of DS was significantly higher in diabetics than non-diabetics.
Dorko et al. (10)	14 DSD 226 DSND	NA	55	NA	NA	NA	NA	100% diabetics had DS. Prevalence of DS was significantly higher in diabetics than non-diabetics.
Dorocka-Bobkowska et al. (11)	70 T2DM 58 ND	T2DM: 57 ND: 58	T2DM: 58.6 ND: 53.4	Type II was common among diabetics and type I among non-diabetics.	T2DM: 5.1 ND: 4.1	Hyperglycemic 14.3%	NA	Prevalence of DS was significantly higher in diabetics (87.8%) than non-diabetics (52.4%).
Dorocka-Bobkowska et al. (12)	110 T2DM 50 ND	T2DM: 63.2 ND: 66.9	T2DM: 57.3 ND: 42	Type-II was more common in diabetics than ND patients (57.3% vs 30%)	T2DM: 5.3 ND: 6.2	Hyperglycemic 56%	NA	Prevalence of DS was significantly higher in diabetics than non-diabetics.
Guggenheimer et al. (13)	405 T1DM 268 ND	T1DM: 33 ND: 31.8	T1DM: 49.6 ND: 59.7	NA	NA	Hyperglycemic	19.4% diabetics	Candida-associated DS was greater in diabetics than control group (15.1% vs 3% respectively).
Sanita et al. (14)	40 DSD 80 DSND 90 ND without DS	DSD: 62.4 DSND: 59.6 ND-NDS: 65.5	DSD: 87.5 DSND: 77.5 ND-NDS: 72.3	Mostly Type-II in all groups	DSD 13.7 DSND 15.1 ND-NDS 4.5	Normoglycemic†	12.5 15 13.3	Prevalence of DS was comparable among diabetics and non-diabetics
Vitkov et al. (15)	52 DSD 40 DSND 40 ND without DS	DSD: 65 DSND: 36 ND without DS: 59	DSD and DSND: NA ND without DS: 50	Type II was more common type among diabetics.	NA	Hyperglycemic 90%	NA	Prevalence of DS was significantly higher in diabetics (35%) than non-diabetics.

DS: Denture stomatitis; NDS: Without denture stomatitis; ND: Non-diabetic; T1DM: Type 1 diabetes mellitus; T2DM: Type 2 diabetes mellitus; DSD: Denture stomatitis with diabetes; DSND: Denture stomatitis without diabetes; NA: Not available

* Hemoglobin A1C > 8% and/or fasting blood glucose levels greater than 130 mg/dL

† Hemoglobin A1C < 7% and/or fasting blood glucose levels between 90-130 mg/dL

species prevalence varies among DS patients with and without DM. The aim of this study was to assess oral *Candida* carriage and species prevalence in DS patients with and without diabetes through a review of the literature.

MATERIALS AND METHODS

The addressed question was “In patients receiving dentures, how does having diabetes compared to no diabetes influence the carriage and species prevalence of oral *Candida*?” an electronic search without time or

language restrictions was conducted up to and including February 2017 in MEDLINE/PubMed, EMBASE, Scopus, ISI Web of knowledge, and Google-Scholar databases using the following medical subject headings (MeSH): “*Candida*”, “*Candida albicans*”, “*Candida tropicalis*”, “*Candida glabrata*”, “candidiasis”, “denture stomatitis”, “diabetes mellitus”, “colonization” and “prevalence”.

RESULTS

In total, 9 studies (7-15) were included and

processed for data extraction. In 7 studies (7, 9-13, 15) *Candida*-associated DS was significantly higher in diabetics as compared to ND individuals. Only one study (8) reported *Candida*-associated DS to be higher among the NDs in comparison to diabetics (75% vs 71.4%, respectively). *C. albicans* was the most common species isolated from the palatal and denture surfaces of denture-wearers with and without diabetes. Percentages of *C. albicans* carriage among diabetic and ND denture-wearers ranged between 51.6% - 100% and 64% - 93% respectively. In the study by Sanita et al. (14), *C. albicans* was isolated from 93% ND patients with DS. *C. tropicalis* and *C. glabrata* were reported as the second most prevalent oral yeast species (9, 11, 12, 14). Comparable percentages of *C. tropicalis* and *C. glabrata* were found in both diabetic and ND denture-wearers. Three studies (9, 11, 12) also reported other oral *Candida* species, such as *C. dublinensis*, *C. famata*, *C. guilliermondii*, *C. krusei* and *C. parapsilosis*, in denture-wearers with and without DM (Table I).

DISCUSSION

We reviewed indexed literature to assess *Candida*-associated DS in patients with and without DM. Results from nearly 78% studies (7, 9, 10, 12, 13, 15) showed that candida-associated DS was more commonly manifested in diabetic as compared to ND individuals. Most of the studies evaluated did not report the severity of DS; and none of the studies evaluated reported the SFR among the study groups. In this regard, it remains unclear whether or not the severity of DS and SFR have any association with oral *Candida* carriage among patients with and without DM. Furthermore, the duration of denture wearing has also been associated to a higher prevalence of DS. From the literature reviewed, the duration of denture wearing among diabetic and ND patients with DS ranged between 4 and 15 years. It is demanding to estimate a minimum duration of denture-wearing that might expose partially and/or completely edentulous individuals to DS. Hence, further studies with standardized parameters such as type of DS, denture-wearing age

and salivary flow rate of the included participants are warranted. It is emphasized that dentists and physicians should be aware of the fact that DM can influence the oral *Candida* carriage among patients with dentures. Therefore, diabetic patients should undergo periodical comprehensive oral evaluation, including reinforcement of oral/denture hygiene techniques, and denture evaluation, adjustment or replacement.

ACKNOWLEDGEMENTS

The Authors extend their appreciation to the International Scientific Partnership Program ISPP at King Saud University for funding this research work through ISPP#0075.

REFERENCES

1. Javed F, Klingspor L, Sundin U, Altamash M, Klinge B, Engstrom PE. Periodontal conditions, oral *Candida albicans* and salivary proteins in type 2 diabetic subjects with emphasis on gender. BMC Oral Health 2009; 9:12.
2. Nittayananta W, Jealae S, Winn T. Oral *Candida* in HIV-infected heterosexuals and intravenous drug users in Thailand. J Oral Pathol Med 2001; 30:347-54.
3. Patel PN, Sah P, Chandrashekar C, Vidyasagar S, Venkata Rao J, Tiwari M, Radhakrishnan R. Oral candidal speciation, virulence and antifungal susceptibility in type 2 diabetes mellitus. Diabetes Res Clin Pract 2017; 125:10-19.
4. Kurt A, Erkose-Genc G, Uzun M, Emrence Z, Ustek D, Isik-Ozkol G. The antifungal activity and cytotoxicity of silver containing denture base material. Niger J Clin Pract 2017; 20(3):290-95.
5. Gendreau L, Loewy ZG. Epidemiology and etiology of denture stomatitis. J Prosthodont 2011; 20:251-60.
6. Bianchi CM, Bianchi HA, Tadano T, Paula CR, Hoffmann-Santos HD, Leite Jr DP, Hahn RC. Factors related to oral candidiasis in elderly users and non-users of removable dental prostheses. Rev Inst Med Trop Sao Paulo 2016;58:17.
7. Chopde N, Jawale B, Pharande A, Chaudhari L, Hiremath V, Redasani R. Microbial colonization and their relation with potential cofactors in patients

- with denture stomatitis. *J Contemp Dent Pract* 2012; 13:456-59.
8. Daniluk T, Tokajuk G, Stokowska W, et al. Occurrence rate of oral *Candida albicans* in denture wearer patients. *Adv Med Sci* 2006; 51:77-80.
 9. Dorko E, Baranova Z, Jenca A, Kizek P, Pilipcinec E, Tkacikova L. Diabetes mellitus and candidiasis. *Folia Microbiol (Praha)* 2005; 50:255-61.
 10. Dorko E, Jenca A, Pilipcinec E, Danko J, Sviscky E, Tkacikova L. Candida-associated denture stomatitis. *Folia Microbiol (Praha)* 2001; 46:443-6.
 11. Dorocka-Bobkowska B, Budtz-Jorgensen E, Wloch S. Non-insulin-dependent diabetes mellitus as a risk factor for denture stomatitis. *J Oral Pathol Med* 1996; 25:411-15.
 12. Dorocka-Bobkowska B, Zozulinska-Ziolkiewicz D, Wierusz-Wysocka B, Hedzelek W, Szumala-Kakol A, Budtz-Jorgensen E. Candida-associated denture stomatitis in type 2 diabetes mellitus. *Diabetes Res Clin Pract* 2010; 90:81-86.
 13. Guggenheimer J, Moore PA, Rossie K, Myers D, Mongelluzzo MB, Block HM et al. Insulin-dependent diabetes mellitus and oral soft tissue pathologies: II. Prevalence and characteristics of Candida and Candidal lesions. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2000; 89:570-76.
 14. Sanita PV, Pavarina AC, Giampaolo ET, Silva MM, Mima EG, Ribeiro DG et al. Candida spp. prevalence in well controlled type 2 diabetic patients with denture stomatitis. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2011; 111:726-33.
 15. Vitkov L, Weitgasser R, Lugstein A, Noack MJ, Fuchs K, Krautgartner WD. Glycaemic disorders in denture stomatitis. *J Oral Pathol Med* 1999; 28:406-9.

LETTER TO THE EDITOR

COMBINED 3D-TOMOGRAPHY AND 3D-POWER DOPPLER FOR VASCULAR IMAGING OF THE LIVER

H-J. SHI, H-L. WANG, Y. WANG, H. ZHAO, N. XV, F. LIU and J-X. SUN

*The First Affiliated Hospital of Harbin Medical University, Harbin, Hei Longjiang Province, China**Received June 6, 2016 – Accepted February 28, 2017*

This study aimed to investigate the role of three-dimensional color tomographic ultrasound (3D-CTU) and three-dimensional color power Doppler (3D-CPD) imaging in diagnostics of liver lesions. Based on 3D-anatomical sectional structures of the liver, we used 3D-CTU and 3D-CPD techniques to reconstitute 3D-images of lobes, segments, and intra-segmental blood vessels, and lesions of the liver. With the use of this technique, we were able to generate 3D vascular patterns of liver lobes, segments and tumor lesions, and their spatial relationship. The observed vascular patterns of intra- and peri-tumor (including surrounding lobes and segments) and the spatial relation can be used for accurate positioning of liver lesions.

To the Editor,

Ultrasonographic methods are commonly used in the diagnostics of liver cancer. Currently, Doppler sonography is the major technique that can permit a direct visualization of intratumoral blood flow with non-invasiveness, since the characteristics of the vascular pattern of these tumors greatly contribute to a qualitative diagnosis (1-11). With the use of more recently developed power Doppler ultrasound, the acquired information from Doppler signals is not analyzed by frequency assessment but by using the amplitude of the signal (12, 13). As a result, three-dimensional color power Doppler (3D-CPD) is much more sensitive than the conventional Doppler systems.

Three-dimensional color tomographic ultrasound (3D-CTU) imaging is a newly developed technique based on surface and transparent imaging. It allows a concurrent display of organs and lesions in any two planes, thereby allowing the formation of a three-

dimensional image. 3D-CTU can be implemented on any axis (long, short, or oblique) in a manner of continuous layers to show the lump-shaped 3D-images of the lesions. This technique can provide very meaningful information for determining the spatial positioning of the lesions and evaluating their actual volume.

In this study, we sought to investigate vascularity in liver tumors with use of 3D-CPD, combined with 3D-CTU, for assistance in clinical diagnosis. The combined application of the two methods appears to be more suitable for *in vivo* morphology and clinical practice.

MATERIALS AND METHODS*Subjects*

From October 2004 to January 2006, a total of 56 patients with liver lesions, including 35 males and 21 females aged from 32 to 76 years, were enrolled in this

Key words: 3D-CTU, 3D-CPD, liver, imaging, blood vessels

Mailing address:

Prof. Jing-Xia Sun,
The First Affiliated Hospital
Harbin Medical University, 23 Youzheng Street,
Nangang District, Harbin, Hei Longjiang Province, China
Fax: +8645153641824
e-mail: sjingxia@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.

study at the First Affiliated Hospital of Harbin Medical University, China. All subjects gave informed, signed consent to participate in the study.

The color ultrasound Doppler (Voluson 730, GE), installed with post-3D-imaging software, was used in this study. The probe was of 3D-volume convex array, with frequency of 3.5 ~ 5.5 MHZ.

3D-CTU imaging

A regular pre-scan was performed to ensure the best quality of image acquisition prior to image collection of the organs or tissues intended for reconstitution. While collecting images, we positioned the probe on the center of the anticipated database. After the best sectional image was acquired, the probe was immobilized. In the meantime, the patient was instructed to hold breath in order to reduce any artifacts. The collection interval could be adjusted based on the size of sampling windows. As soon as it was completed, the reconstituted ultrasound images of sections A, B, and C would appear immediately on the screen. The process of acquisition lasted 5-10 s.

After the image acquisition was complete, 3D-reconstruction was performed. Firstly, sections A, B, or C were activated. A parallel movement could allow showing the structures of the region of interest. The point on this region was then positioned and the best direction selected for observation by rotating the X, Y, or Z axis. The 3D-reconstruction could proceed upon completion of this process.

The mixed mode of the maximum echo, the minimum echo, and the surface image formation were chosen for reconstruction. The mixing ratio could be adjusted by rotating the knob of the control panel to acquire the regular 3D-image of the region of interest. Subsequently, the sectional images were acquired from the long, short, or random oblique axis. The appropriate regions for imaging by moving to the left right side were also chosen. After the reconstructed images with a sectional line appeared in a full-screen, the images were finely rotated to display the fragments of 3D-sectional pictures. One side of this line was defined as the sectional diagram, and the other side is un-sectioned regular 3D-image. The reconstituted images were saved digitally in the hard drive.

3D-CPD imaging

At the status of grey-scale, scanned blood vessels be-

neath the chest ribs were firstly regular. After the images had become clearly visible, the condition of power diagram was turned to collect picture samples. At this moment, the probe was kept immobilized and the patient was instructed to hold his breath to reduce the chances of producing artifacts. After sampling, the system of 3D-reconstruction was initiated in order to display dynamic or static pictures for visualization of intrahepatic blood vessels and their spatial relationship. The data were then electronically saved.

The patient gave informed, signed consent to participate in this report.

RESULTS

3D-CPD imaging of liver lobes and segments

The liver is divided into two major sections: right and left lobes. The liver receives its blood supply from two sources: 80% is delivered by the portal vein (PV), and the remaining 20%, the oxygenated blood, is delivered by the hepatic artery. The portal vein is formed by the union of the splenic and the superior mesenteric veins with the inferior mesenteric vein draining into the splenic vein. The portal vein divides the liver into the upper and lower segments. The outflow of the liver is provided by the three hepatic veins. The right hepatic vein (RHV) divides the right lobe of the liver into anterior and posterior segments. The middle hepatic vein (MHV) divides the liver into the right and left lobes and runs in the same plane with the inferior vena cava (IVC) and the gallbladder fossa. The left hepatic vein (LHV) divides the left liver into medial and lateral segments.

In this study, 3D-CPD visualized RHV, MHV, IHV, and right PV between two hepatic veins. In addition, 3D-CPD was able to show various sections of PV, typically, cross-over relationship of PV branches and hepatic veins. Furthermore, the 3D-CPD could allow a 3D-visualization of spatial relation of IHV, right PV, RHV, and MHV, even tertiary branches of hepatic veins. Finally, 3D-CPD was able to show a complete picture of three hepatic veins converging to IHV, and the right PV traveling between RHV and MHV.

In a view of dynamic 360° of the liver, 3D-CPD

can allow us to fully recapitulate such 3D-relation of these vascular structures.

Comparative analysis of 3D-CTU and 3D-CPD imaging

We found that the results of 3D-CTU are consistent with those of 3D-CPD. In Fig. 1A, 3D-CTU of the right posterior lower segment shows MHV, the long axis of right PV traveling one the sectional plane, the short axis of PV in the segmental line, and cross-over relationship of right PV and MHV. 3D-CPD then demonstrated a clear spatial relationship of right PV and MHV. Similarly, 3D-CTU of the right anterior upper segment showed the long axis of right PV, the long axis of RHV in the sectional plane, and right PV and the short axis of RHV in the segmental line. The spatial relation of RHV and right PV was displayed in 3D-CDP (Fig. 1B). Furthermore, 3D-CTU of right

anterior lower segment allowed a visualization of branching patterns of right PV, and 3D-CPD then showed four branches of right PV between MHV and RHV (Fig. 1C). In 3D-CTU of the right posterior lobe, we showed that the right PV travels between the long axis of RHV and MHV in the segmental line. 3D-CPD demonstrated a cross-over relationship of RHV and MHV with right PV between them (Fig. 1D).

Finally, 3D-CTU imaging of the left interior lobe presented all the sections of the left PV and 3D-CPD showed an even clearer 3D-imaging of the left PV.

Diagnosis of liver lesions with the use of 3D-CTU

The “lump”-shaped sectional pictures allow us to detail the pathological characteristics of the lesions, the spatial relation of the lesions and surrounding tissues, thus providing the clinicians with more straightforward and meaningful information.

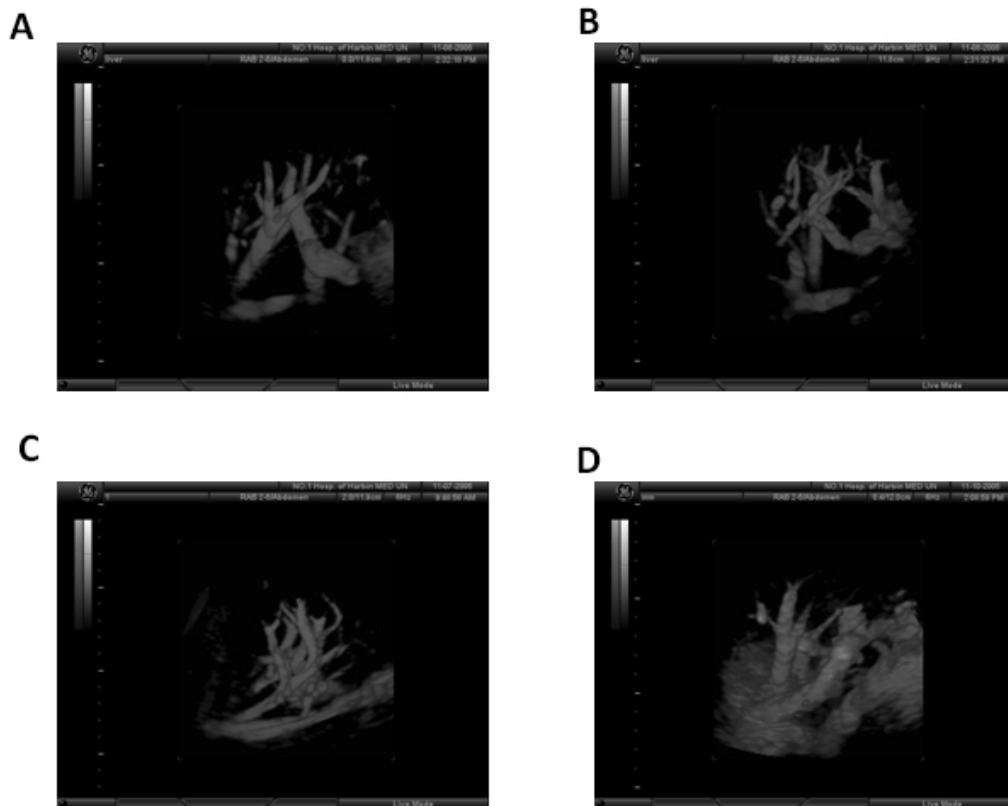


Fig. 1. Imaging of 3D-CTU and –CPD. A) Right posterior lower segment; (B) Right anterior upper segment; (C) Right anterior lower segment; (D) Right anterior lobe. Left panel, 3D-CTU imaging; Right panel, 3D-CPD imaging.

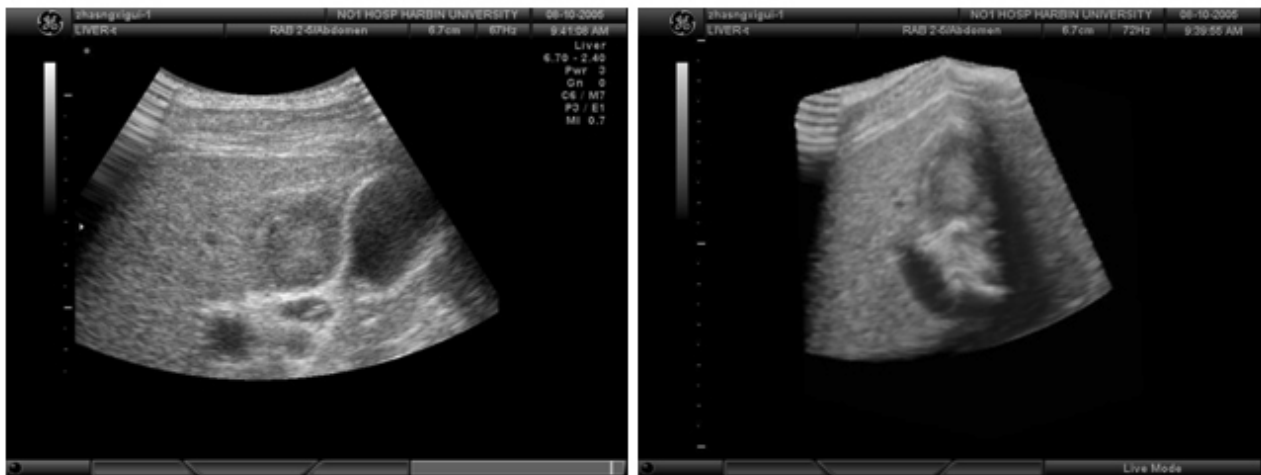


Fig. 2. 3D-CTU imaging of metastatic liver cancer. Left panel, 2D-ultrasound; right panel, 3D-CTU.



Fig. 3. 3D-CPD imaging of primary liver cancer. Left panel, 2D-CPD; right panel, 3D-CPD.

3D-CTU and 3D-CPD imaging were developed based on anatomical structures of liver cross-sections. With the use of intrahepatic vascular reconstruction, we can make a more convincing diagnosis of the liver lesions.

3D-CTU imaging would allow a definitive diagnosis of liver lesions based the vascular structures of the lesions and cross-section and their spatial relationship. As shown in Fig. 2, which is a case of lung cancer patients with liver metastasis, the two-dimensional ultrasound images show the tumor was located on the right side of the gallbladder. By cross-sectioning structure of the tumor, 3D-CTU

imaging showed that the right PV was located behind the tumor. According to anatomical diagram of liver sections, this lesion is in the upper segment of the right lobe. These data suggest that 3D-CTU is of great significance in choosing the appropriate therapeutic approaches.

Diagnosis of liver lesions by 3D-CPD

3D-CPD imaging could distinguish intrahepatic vascular structures and spatial relationship of liver lesions. Fig. 3 presents one case of primary liver cancer. In 3D-CPD imaging, irregular and rich blood signals can be seen in the space between MHV and

the right PV. According to the anatomical diagram of liver sections, this lesion is in the lower segment of the right lobe, which is distant from MHV and the PV. We concluded that the tumor could be surgically removed.

DISCUSSION

At present, with the rapid development of hepatic surgery, segmental, sub-segmental or wedge section has become a reality (14-16).

The combined use of 3D-CPD and 3D-CTU is capable of reconstructing ultrasound of the hepatic and portal veins by transverse and coronal, sagittal and coronal, or transverse and coronal layers. The methods can accurately display traveling of the hepatic and portal veins at will, to avoid overlap of blood vessels (17). In this study, using mixed mode of imaging for three-dimensional reconstruction of the intrahepatic blood vessels, we can display simultaneously the vena cava, hepatic vein, portal vein and their spatial relationship, which closely recapitulates actual normal anatomy.

The data generated from this study will provide a more detailed description of branching and traveling of intrahepatic duct systems for surgeons.

To sum up, the clinical value of three-dimensional imaging of hepatic blood vessels can be seen in these aspects: i) it can precisely position the liver lesions according to 3D vascular distribution and spatial relationship; ii) it can determine whether the tumor is surgically removable; iii) it can assist in choosing the most suitable surgical approaches; iv) it can be used for follow-up after non-surgical treatment; and v) it can be used for guided interventional therapy.

REFERENCES

1. Jang SI, Lee DK. Contrast-enhanced endoscopic ultrasonography: advance and current status. *Ultrasonography* 2014; 33:161-69.
2. Ehling J, Lammers T, Kiessling F. Non-invasive imaging for studying anti-angiogenic therapy effects. *Thromb Haemost* 2013; 109:375-90.
3. Camaggi V, Piscaglia F, Bolondi L. Recent advances in the imaging of hepatocellular carcinoma. From ultrasound to positron emission tomography scan. *Saudi Med J* 2007; 28:1007-14.
4. Chen RC, Chen WT, Liao LY, Cheng NY, Wang CK, Tu HY, Wang CS, Chen PH. Intravenous contrast-enhanced Doppler sonography and intra-arterial carbon dioxide-enhanced sonography in the assessment of hepatocellular carcinoma vascularity before and after treatment. *Acta Radiol* 2002; 43:411-14.
5. Chen RC, Chen WT, Tu HY, et al. Assessment of vascularity in hepatic tumors: comparison of power Doppler sonography and intraarterial CO(2)-enhanced sonography. *AJR Am J Roentgenol* 2002; 178:67-73.
6. Hsu KF, Su JM, Huang SC, et al. Three-dimensional power Doppler imaging of early-stage cervical cancer. *Ultrasound Obstet Gynecol* 2004; 24:664-71.
7. Cohen LS, Escobar PF, Scharm C, Glimco B, Fishman DA. Three-dimensional power Doppler ultrasound improves the diagnostic accuracy for ovarian cancer prediction. *Gynecol Oncol* 2001; 82:40-48.
8. Sauvain JL, Sauvain E, Rohmer P, et al. Value of transrectal power Doppler sonography in the detection of low-risk prostate cancers. *Diagn Interv Imaging* 2013; 94:60-67.
9. Huang YH, Chen JH, Chang YC, et al. Diagnosis of solid breast tumors using vessel analysis in three-dimensional power Doppler ultrasound images. *J Digit Imaging* 2013; 26:731-39.
10. Chiou HJ, Chou YH, Chen WM, Chen W, Wang HK, Chang CY. Soft-tissue tumor differentiation using 3D power Doppler ultrasonography with echo-contrast medium injection. *J Chin Med Assoc* 2010; 73:628-33.
11. Hsiao YH, Huang YL, Liang WM, Lin C, Kuo SJ, Chen DR. Characterization of benign and malignant solid breast masses: harmonic versus nonharmonic 3D power Doppler imaging. *Ultrasound Med Biol* 2009; 35:353-59.
12. Miyagi Y, Masaoka H, Akamatsu N, Sekiba K. Development of a three-dimensional color Doppler system. *Med Prog Technol* 1992; 18:201-8.
13. Downey DB, Fenster A. Vascular imaging with a three-dimensional power Doppler system. *AJR Am J Roentgenol* 1995; 165:665-68.
14. Kobayashi A, Imamura H, Miyagawa S, Shimada R, Makuuchi M, Kawasaki S. Extended right posterior segmentectomy for metastatic liver tumors. *Surgery* 1997; 121:698-703.

15. Takayama T, Nakatsuka T, Shimada K, et al. Function-preserving segmentectomy of the cirrhotic liver. *Hepatogastroenterology* 1995; 42:415-17.
16. Kitisin K, Packiam V, Bartlett DL, Tsung A. A current update on the evolution of robotic liver surgery. *Minerva Chir* 2011; 66:281-93.
17. Xu HX, Liu L, Lu MD, Li HP, Liu GJ, Li JP. Three-dimensional power Doppler imaging in depicting vascularity in hepatocellular carcinoma. *J Ultrasound Med* 2003; 22:1147-54.

LETTER TO THE EDITOR

THE NOVEL TECHNIQUE OF MICROWAVE HEATING OF INFANT FORMULAS AND HUMAN MILK WITH DIRECT TEMPERATURE MONITORING

A. LEVCHENKO¹, O. LUKOYANOVA², T. BOROVIK², M. LEVCHENKO³,
D. SEVOSTIANOV³ and P. SADCHIKOV²

¹*Quantum Radiophysics Division of P.N. Lebedev Physical Institute of Russian Academy of Sciences, Moscow, Russian Federation;* ²*Scientific Center of Children's Health, Moscow, Russian Federation;* ³*BabyField LLC, Moscow, Russian Federation*

Received January 20, 2017 – Accepted March 3, 2017

We discuss the physical mechanisms of microwave heating of milk and infant formulas, and possible causes of nutrient degradation and scalds of infants reported in a number of papers. We list the advantages of microwave heating, formulate criteria of safe heating, and discuss discrepancies between different papers. A novel technique of microwave heating which prevents scalds and saves nutrients is reported. It features an appropriate and stepless microwave power control with direct temperature monitoring under forced permanent mixing. The discussed heating method does not kill live lactobacilli, has no influence on pathogen growth and does not modify the content of nutrients. We conclude that guidelines, which prohibit milk heating in domestic microwave ovens, are quite correct. Nevertheless, specific techniques of microwave heating can be successfully used due to their usability, fast heating, guaranteed precise target temperature and overall automation.

To the Editor,

Parents and professionals started to use microwave treatment of human milk and infant formulas after widespread use of domestic microwave ovens in the 1980s, and a number of studies appeared after that (1) and references therein. Similar papers have been periodically published during the last 30 years. The interest is stimulated by high usability of microwave treatment, which is a “dry” technology (no hot water or steam), does not require any preparation, and is fast. Microwaving provides volumetric heating, although not uniform, while other methods heat only the surface of the container. Therefore, in theory, the probability of local overheating is less even under fast heating. It is also simple to monitor the temperature

of the content while microwaving in contrast to other heating techniques.

For a long time most authors were unanimous about purely thermal effect of microwaves; in 1996 Sieber et al. summarized their detailed review: «Microwave-treated milk does not differ very much from conventionally-heated milk...» (1). Most authors consider low-intensity microwaving to be quite safe (2, 3). The same is true for microwaving of donated blood (4, 5).

Nevertheless, both professionals and parents distrust microwave heating now. Most guidelines of human milk bank operation forbid using *domestic microwave ovens* for heating milk (6-9). Most such modern guidelines and papers refer merely to two

Key words: heating of human milk and infant formulas, microwave heating, microwave treatment, survivability of lactobacilli under microwaves

Mailing address:

Dr Alexey Levchenko,
P.N. Lebedev Physical Institute,
Russian Academy of Sciences, 53 Leninskiy Prospekt,
119991, Moscow, Russia
Tel.: +7 916 6196186
e-mail: levchenk@sci.lebedev.ru

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

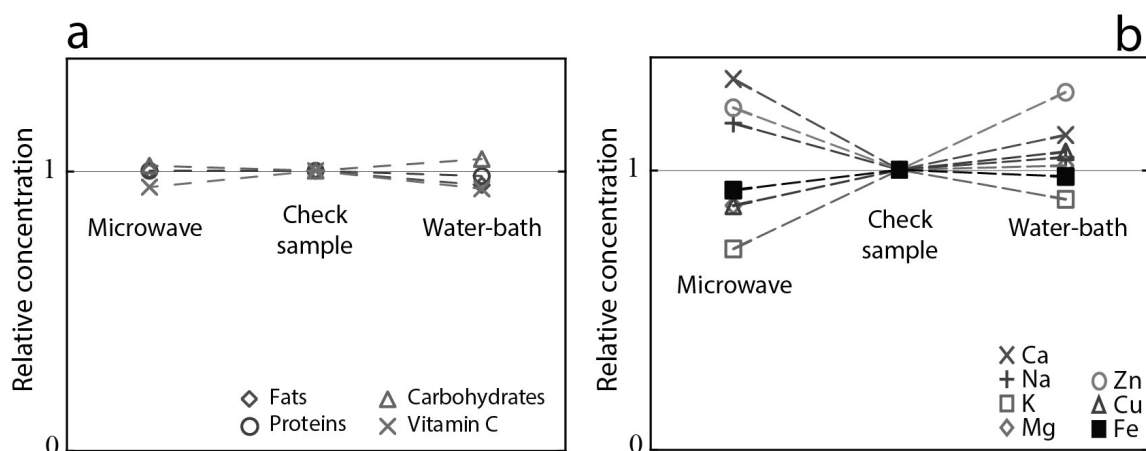


Fig. 1. Relative total contents of macronutrients and vitamin C (a) and dietary minerals (b) in infant formula for microwave and water-bath heating.

papers, which describe degradation of IgA and lysozyme, and *E.coli* growth acceleration in human milk after heating in a domestic microwave oven (10, 11). However, the methods used in these papers seem doubtful (see Discussion section). Furthermore, the conclusions contradict other papers, which reported no similar negative effects (1-3).

The other reason of prohibition of microwave heating is a number of scald injuries of infants caused by milk overheating in domestic microwave ovens (12, 13). In contrast to the former reason, the latter one is indisputable. Therefore, the new techniques must completely avoid any scalds.

Finally, we cite the summary of original paper by Quan et al.: «Further studies are needed to determine whether and how microwaving could safely be done» (10). Below we report such a safe technique.

MATERIALS AND METHODS

In the present study, we used a specially developed novel prototype of microwave warmer BabyField (BabyField LLC, Moscow, Russian Federation) which considerably differs from a domestic microwave oven in the following items:

- i) about 10 times lower peak power (60-100 W instead of 500-1100 W) with smooth stepless microwave power control;
- ii) forced mixing of content due to slow (4-5 rpm) rotation of the horizontal plastic drum ;

- iii) direct non-contact monitoring of the bottle temperature based on its heat emission; the operator can see the approximate temperature value during the heating process, and the heating is automatically stopped at $37 \pm 1^\circ\text{C}$.

Thanks to forced mixing and relatively slow volumetric microwave heating, the temperatures of the contents and surface of the bottle are almost equal during the heating process. Note that the technique is efficient for both glass and plastic bottles and even for glass jars with purees and yogurts. However, the BabyField prototype cannot operate with metallized cartons or pouch packages.

A polypropylene bottle (Avent) with 90 ml of an infant formula was placed inside the rotating drum. In several experiments we used human milk in three 2-ml Eppendorf tubes. The tube no. "1" floated on the surface, no. "2" was suspended in the middle, and no. "3" was at the bottom due to additional teflon weights. Hence, the human milk samples were placed in average in very similar physical conditions, and microwaves affect them as if the bottle were fully filled with milk. Then we put the bottle into the heater, pushed "start", and waited for the automatic stop. The final content temperature measured with a mercury thermometer was $36.5 \pm 0.5^\circ\text{C}$ in all experiments.

We used check samples, and heated those with water-bath warmer (Philips Avent SCF 355) for comparative analysis. For latter samples, we monitored their temperature by slowly moving an Hg thermometer within the sample during heating, and stopped heating at 37°C .

The contents of proteins, fat, carbohydrates, Ca, Na,

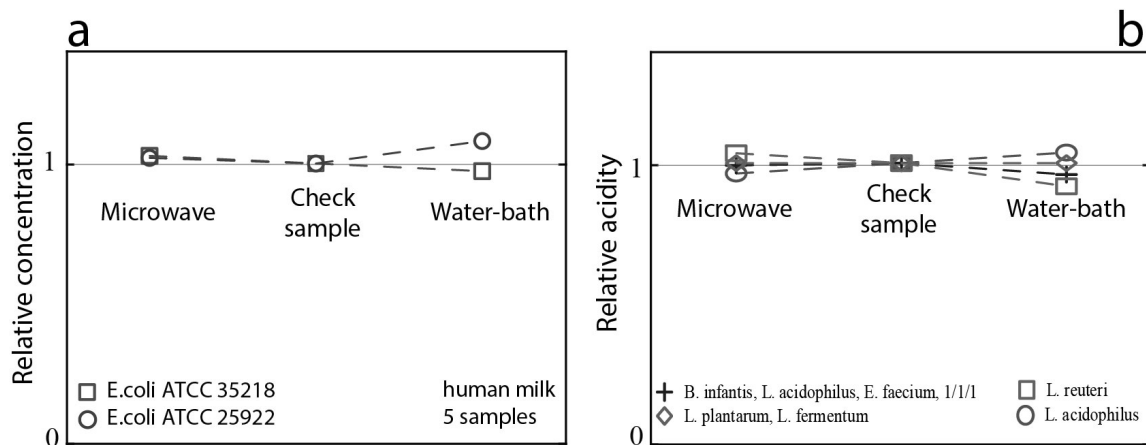


Fig. 2. Relative concentrations of *E. coli* (average values for 5 sets of samples) in human milk (a) and relative changes of acidity (average values) for samples of cow milk heated with lactobacilli (b) for microwave and water-bath heating.

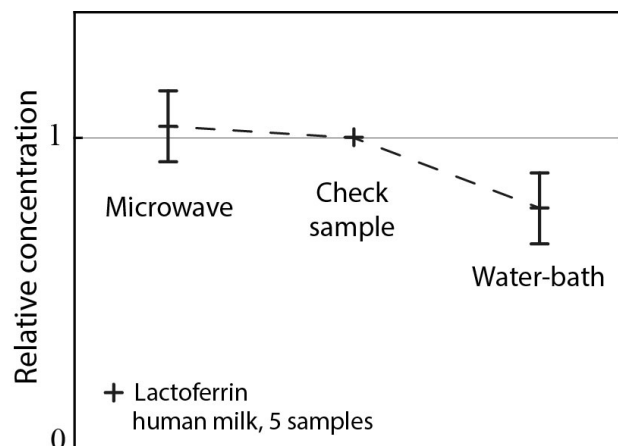


Fig. 3. Relative concentrations of lactoferrin (average values for 5 sets of samples) in human milk for microwave and water-bath heating.

K, Mg, Zn, Cu, Fe, and vitamin C were measured with routine methods in 90-ml samples of infant formula. The significant volume of each sample allows a quite representative result, so we used only one set of samples in these experiments.

Lactoferrin was measured in 5 sets of human milk samples with ELISA technique.

The growth of *E. coli* (ATCC 25922, 35218) with initial concentration 10^4 CFU/ml was studied in 5 sets of human milk samples by analogy with studies (10, 14).

Furthermore, we studied 4 sets of samples of cow milk with *L. acidophilus* (initial concentrations from 2×10^4 to 1.5×10^5 CFU/ml), 3 sets with mixture *L. acidophilus*/*B.*

infantis/*E. faecium* (1/1/1, total initial concentration 1.5×10^5 CFU/ml), 1 set with *L. plantarum* and *L. fermentum* (2.5×10^6 CFU/ml) and 3 sets of samples of infant formula with *L. Reuteri* (1.5×10^5 CFU/ml). The samples with live lactobacilli were heated up to 37°C in both microwave and water-bath warmers and then incubated at $35\text{--}40^\circ\text{C}$ for 12–40 h. The activity of lactobacilli was estimated by the acidity pH of the samples.

RESULTS

Avent SCF 355 heated the 90-ml samples from 20 to 37°C for 5 minutes on average; BabyField

for 70 seconds. Relative total contents (ratio of final content to initial one) of macronutrients, vitamin C and dietary minerals are shown in Fig. 1. Here and below the value for check samples corresponds to 1 on the diagrams.

Relative concentrations of *E.coli* and relative changes of acidity of samples heated with lactobacilli are depicted in Fig. 2. The initial acidity pH=6.5, while the final pH varied from 6.3 to 3 for different sets of samples, depending on the type of lactobacilli, their initial concentration, incubation time and temperature. For each set of samples, the listed parameters were fixed and identical for “microwave”, “check sample”, and “water-bath” samples. In Fig. 2.b the average values for each type of used lactobacilli are shown. Relative concentrations of lactoferrin (average values for 5 sets of samples of human milk) are depicted on Fig. 3.

DISCUSSION

Both microwave and water-bath heating did not affect the nutritive properties of milk or infant formulas and live lactobacilli appreciably in our experiments. Some decrease of lactoferrin after water-bath heating may be caused by surface overheating since measured temperature of water in the warmer reached 60°C. We did not observe any essential increase of *E.coli*, i.e. negative impact on the antibacterial properties of human milk, in contrast to (10) reported fivefold *E.coli* growth and decrease of activity of specific IgA after heating in a domestic microwave oven. Note that later studies (15) reported no changes of *E.coli* after microwaving.

In our opinion, the following physical aspects of experiments can lead to the mentioned discrepancy:

i) Intermittent control of microwave power in the majority of domestic microwave ovens. If the user sets “low” power level, the oven operates as follows: heating at full power 500-1000 W during 3-10 s, next it turns the heating off completely for 15-30 s, then turn it on again, and so on. Hence, the small 2-ml sample in the study by Quan et al. (10) could have been heated up to a rather high temperature for 3-10 s and then cooled again for 15-30 s many times.

ii) The distribution of the electric component of

the microwave field, which is responsible for heating, in all microwave ovens is not uniform and contains “cold” and “hot” areas with drastically differing fields. Nevertheless, in most studies, including that of Quan et al., the authors did not control the exact field value in the area of sample placing.

iii) The same microwave power heats samples of different volumes up to different temperatures. Moreover, the final temperature depends on the initial one, which could vary from near freezing point up to room temperature in different cases. Nevertheless, most domestic microwave ovens do not monitor the actual temperature of the contents; one may say they control the microwave power “blindfolded”. Thus, the temperature (especially of small samples) can reach high values even when the low power level is set. That is the cause of nutrient destruction and many cases of scalds.

Therefore, we can list the conditions necessary for safe heating, which must also be considered in future studies.

i) An appropriate accurate control of microwave power. Intermittent control, i.e. full-power heating for 1 s or longer leading to local overheating is inadmissible.

ii) The sample must be moved within the heating chamber in order to average the influence of “hot” and “cold” areas. Since microwaves penetrate liquids only to a limited depth, it is also necessary to mix the contents to guarantee uniformity of temperature (3).

iii) Direct monitoring of the content temperature is essential for safe heating up to an exact target temperature.

In conclusion, in the Authors’ opinion, the guidelines, which prohibit milk heating in domestic microwave ovens, are quite correct. However, the reason lies not in some “negative nature” of microwaves, but in features of operation of domestic microwave ovens, as they are not destined for such purposes. Specially developed techniques, which satisfy the items listed above, save all nutrients, anti-infective factors, and even live lactobacilli. Such techniques can be successfully used because of their advantages, such as faster heating (up to 4 times more than the water-bath in the present study), absence of hot water and steam (“dry” technology), overall

automation of the heating process and guaranteed exact target temperature.

ACKNOWLEDGEMENTS

This study was funded by BabyField LLC.

CONFLICT OF INTEREST: Dr M. Levchenko works for BabyField LLC, Moscow, Russian Federation, who funded this study.

REFERENCES

1. Sieber R, Eberhard P, Gallmann PU. Heat treatment of milk in domestic microwave ovens. *Int Dairy Journal* 1996; 6:231-36.
2. Ovesen L, Jakobsen J, Leth T, Reinholdt J. The effect of microwave heating on vitamins B1 and E, and linoleic and linolenic acids, and immunoglobulins in human milk. *Int J Food Sci Nutr* 1996; 47(5):427-36.
3. Sigman-Grant M, Bush G, Anantheswaran R. Microwave heating of infant formula: a dilemma resolved. *Pediatrics* 1992; 90(3):412-15.
4. Hirsch J, Menzebach A, Welters ID, Dietrich GV, Katz N, Hempelmann G. Indicators of erythrocyte damage after microwave heating. *Clin Chem* 2003; 49(5):792-99.
5. Hirsch J, Bach R, Menzebach A, Dietrich GV, Katz N, Hempelmann G. Temperature course and distribution during plasma heating with a microwave device. *Anaesthesia* 2003; 58(5):444-47.
6. Arslanoglu S, Bertino E, Tonetto P, et al. Guidelines for the establishment and operation of a donor human milk bank. *J Matern Fetal Neonatal Med* 2010; 23(S2):1-20.
7. Eglas A, Bunik M, Chantry CJ. ABM Clinical protocol #8: human milk storage information for home use for full-term infants. *Breastfeed Med* 2010; 5(3):127-4.
8. National Institute for Health and Clinical Excellence. Donor breast milk banks: the operation of donor breast milk bank services. London: National Institute for Health and Clinical Excellence 2010. Available from: www.nice.org.uk/guidance/CG93.
9. Baumer JH. Guideline review: Guidelines for the establishment and operation of human milk banks in the UK. *Arch Dis Child Educ Pract Ed* 2004; 89:ep27-ep28.
10. Quan R, Yang C, Rubinstein S, Lewiston NJ, Sunshine P, Stevenson DK, Kerner JA Jr. Effects of microwave radiation on anti-infective factors in human milk. *Pediatrics* 1992; 89(4):667-73.
11. Sigman M, Burke KI, Swarner OW, Shavlik GW. Effects of microwaving human milk: changes in IgA content and bacterial count. *J Am Diet Assoc* 1989; 89:690-93.
12. Sando WC, Gallaher KJ, Rodgers BM. Risk factors for microwave scald injuries in infants. *J Pediatr* 1984; 105(6):864-67.
13. Dixon JJ, Burd DA, Roberts DG. Severe burns resulting from an exploding teat on a bottle of infant formula milk heated in a microwave oven. *Burns* 1997; 23(3):268-69.
14. Hernandez J, Lemons P, Lemons J, Todd J. Effect of storage processes on the bacterial growth-inhibiting activity of human breast milk. *Pediatrics* 1979; 63(4):597-601.
15. Carbonare SB, Palmeira P, Silva ML, Carneiro-Sampaio MM. Effect of microwave radiation, pasteurization and lyophilization on the ability of human milk to inhibit *Escherichia coli* adherence to HEP-2 cells. *J Diarrhoeal Dis Res* 1996; 14(2):909-14.

LETTER TO THE EDITOR

EFFECT OF XIANLING GUBAO CAPSULES ON BONE MINERAL DENSITY IN
OSTEOPOROSIS PATIENTS

ZH. WU, X. ZHU, CK. XU, YJ. CHEN, L. ZHANG and CL. ZHANG

*The First People's Hospital of Shangqiu, Shangqiu, China**Received October 9, 2016 – Accepted March 13, 2017*

This paper aimed to analyze the clinical effects of Xianling Gubao capsules on the bone mineral density (BMD) of 94 patients with osteoporosis. After reviewing and analyzing the clinical information in our hospital from January 2015 to January 2016, the patients were divided into a control group and a treatment group with 47 cases in each group. Patients in the control group were treated with routine Western medicine, while the treatment group received Xianling Gubao capsules. Both groups were treated continuously for 3 courses (30 days each course) and had their BMD analyzed and compared. The effective rates of the treatment group and control group were 91.48% and 70.21%, respectively, with statistical significance ($P<0.05$). Compared with the same group before the treatment, the bone metabolic indexes (blood calcium, phosphorus, and alkaline phosphate) and the BMD of the femoral neck and lumbar vertebrae (L_{1-2} , L_{3-4} , L_{2-4}) of both groups were all improved with statistical significance ($P<0.05$) after the treatment. The above indexes of the treatment group were all significantly higher than those of the control group, with statistical significance ($P<0.05$). Compared with the same group before treatment ($P<0.05$), the osteocalcin (OC) levels of both groups were increased, and the Cross-linked N-telopeptide of type 1 collagen (CTX-1) levels were decreased after the treatment. The OC level in the treatment group was higher when compared with the control group, while the CTX-1 level was lower compared to the control group ($P<0.05$). Moreover, no significant side-effects or adverse events were observed during the treatment and observation period. The Xianling Gubao Capsule possesses a therapeutic effect for BMD in osteoporosis patients, which can effectively increase their BMD, improve their bone metabolism, and control the loss of bone mass, therefore, can be used in clinical promotion and application.

To the Editor,

With the quickly increasing age of the general population in China and other countries, the incidence rate of bone metabolism diseases, especially osteoporosis, is on the rise. These diseases have become a serious public health problem. Therefore, the prevention and curing of osteoporosis is now a priority. As the disease worsens, the BMD of the patients will decrease and their bones will become

fragile, making them more susceptible to lumbago and back pain. In particular, patients are very vulnerable to osteoporotic fractures under external force. Calcium supplements are often used in clinics, but these are not very effective and can cause various adverse reactions (1). However, traditional Chinese medicine (TCM) holds the opinion that osteoporosis is caused by poor kidney efficiency and is closely related to the spleen and stomach. TCM has a

*Key words: Xianling Gubao capsule, osteoporosis, bone mineral density**Mailing address:*

Dr. Zhihui Wu,
The First People's Hospital of Shangqiu,
No.292, Southern Kaixuan Road, Suiyang District,
Shangqiu City, 476100, Henan Province, China
Tel: +86 13393718970
e-mail: zhihuiwu2016@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.**

special advantage in improving the quality of life in osteoporosis patients. The Xianling Gubao Capsule nourishes the liver and kidneys, promotes blood circulation to remove meridian obstructions, and strengthens muscle and bone to fight osteoporosis. The Capsule is used in conjunction with teasel root, fructus psoraleae, rhizoma anemarrhenae, salvia miltiorrhiza, radix rehmanniae, and epimedium herb as a basic remedy for osteoporosis. In this study, the clinical data of the 94 osteoporosis patients who were admitted to our hospital from January 2015 to January 2016 were collected to explore the effects of the Xianling Gubao capsule on the BMD of patients.

MATERIALS AND METHODS

General information

Ninety-four osteoporosis patients admitted to the First People's Hospital of Shangqiu, China from January 2015 to January 2016 were selected for this study. According to their treatment time, they were divided into a control group and a treatment group with 47 patients in each. In the control group, there were 29 males and 18 females, aged 48-73 years (average of 53.15 ± 6.08 years), with a disease course of 3-10 years (average of 5.03 ± 3.06 years). According to the category of BMD level, 16 cases were mild, 23 were moderate, and 8 were severe. Correspondingly, in the treatment group, there were 28 males and 19 females, aged 58-78 years (average of 61.05 ± 7.53 years), with a disease course of 3-13 years (an average of 6.01 ± 4.34 years). Of these, 14 cases were mild, 26 were moderate, and 7 were severe. There was no statistical significance in the gender, age, disease course, and BMD levels between the two groups ($P > 0.05$).

The study received written approval from the First People's Hospital of Shangqiu Ethics Committee for Clinical Research. All of the patients participating in the study signed the informed consent form.

The diagnostic standards of Western medicine

Based on the Diagnostic Standard of Osteoporosis in China (3rd version), which was formulated by the Diagnostic Code Group of the Chinese Medical Association (2), the diagnostic standards of Western medicine for osteoporosis

are as follows: i) patients who had typical aches all over, mostly lumbago and back pain, and who were prone to fractures or kyphotic deformity of the spine after a minor injury; ii) the patients whose BMD value was less than 2 Standard Deviations below the mean.

The diagnostic standards of TCM

According to the differentiation standards of Chinese medicine in the Guiding Principles of Clinical Research on New Drugs of Traditional Chinese Medicine (3), the patients were diagnosed with spinal pain, weakness, unable to hold weight, giddiness, tinnitus, red or pale tongue, and a weak or delicate pulse.

Inclusion criteria

i) Conformation to the diagnostic criteria of osteoporosis according to the Diagnostic Standard of the Osteoporosis in China (3rd version) and Guiding Principles of Clinical Research on New Drugs of Traditional Chinese Medicine. ii) Aged 40-80 years. iii) Normal blood calcium level and renal function without severe fractures or other orthopedic disorders. iv) Not taken calcium supplemental drugs for at least 3 months before treatment.

Exclusion criteria

i) Severe mental illness, hypocalcemia, high blood calcium, renal insufficiency, thyroid disorder, chronic hepatic-nephrotic disease, or other metabolic diseases. ii) Under treatment with glucocorticoid or antipsychotic drugs for a long time. iii) Allergic to the drugs used in this study. 4) Taking anti-osteoporosis drugs.

Methods

The control group was treated with one pill of Caltrate D (Wyeth Pharmaceutical Co., Ltd., H10950029, Batch No.: 20142108) twice a day for three consecutive courses (30 days per course). The treatment group was treated with three Xianling Gubao capsules (Guizhou Tongjitang Pharmaceutical Co., Ltd., Z20025337, Batch No.: 20141109) three times a day for three consecutive courses (30 days per course).

Observational indexes

There were several indexes analyzed in this study: i) therapeutic effects; ii) the variations of metabolic indexes; iii) the improvement of the BMD of the femoral neck and

lumbar vertebrae (L1~2, L3~4, L2~4) after the treatment; iv) variations of OC and CTX-1 levels; v) clinical adverse reactions.

Efficacy determination

According to the Diagnostic Standard of Osteoporosis in China (3rd version) (4), there are three types of clinical effects: i) prominent effects, which meant that the patients had a significant increase in their BMD value, and their clinical symptoms and syndromes disappeared; ii) improved effects, which meant that the patients had an improved BMD value, and their clinical symptoms and syndromes were alleviated when compared with pretreatment levels; iii) invalid effects, which meant that the patient's BMD value did not improve and their clinical symptoms and syndromes were worsening.

The clinical total effective rate = [(the number of prominent effective cases + the number of improved effective cases) ÷ total number of cases in each group] × 100%

Laboratory inspection

BMD detection (5): a dual-energy X-ray analyzer (DEXA) (General Electric Co., USA, Instrument Type: DPX-MD) was used to test the BMD of the lumbar and femoral neck vertebrae with the mean value as the observation index.

Bone metabolism index (6): before and after treatment, 5 ml of fasting venous blood samples from the patients in both groups were extracted and placed into the anticoagulant vacuum vessels and centrifuged at 2400 rpm for 15 min to separate the serum and keep it under 80°C for testing. Then, the automatic biochemical analyzer (Roche Instrument Center AG, Switzerland, Instrument Type: c111) was used to detect the blood, phosphorus, and alkaline phosphatase levels.

Bone transformation index (7): Before and after treatment, 3 ml of fasting venous blood samples from patients in both groups were extracted and centrifuged at 2400 rpm for 15 min to separate the serum and keep it at 80°C for testing. Then, the OC and CTX-1 levels were detected. The OC level was detected by radioimmunoassay (Shanghai Biological Engineering Co., Ltd., China) while the CTX-1 level was measured by ELISA (Shanghai Biological Engineering Co., Ltd., China).

RESULTS

Comparison of the therapeutic effect between the control and treatment groups

The clinical effective rates of the treatment group and control group were 91.48% and 70.21%, respectively, with statistical significance ($P < 0.05$, Table I).

Comparison of bone metabolic indexes of the two groups before and after treatment

When compared to before treatment, the bone metabolic indexes of the two groups improved after treatment, with statistical significance ($P < 0.05$). The bone metabolic index of the treatment group was significantly higher when compared with the control group, with statistical significance ($P < 0.05$, Table II).

Comparison of BMD of femoral neck and lumbar vertebra of the groups before and after treatment

When compared with the same group before treatment, the BMD of the femoral neck and lumbar vertebrae (L_{1-2} , L_{3-4} , L_{2-4}) of the two groups increased markedly after treatment, with statistical significance ($P < 0.05$). The BMD of the femoral neck and lumbar vertebrae of the treatment group was significantly higher when compared with the control group, and the difference was statistically significant ($P < 0.05$, Tables III and IV).

Comparison of OC and CTX-1 levels between the two groups before and after treatment

When compared with the same group before treatment, the OC levels of the two groups increased after treatment and the CTX-1 levels decreased, both with statistical significance ($P < 0.05$). The degree of change in the treatment group was higher when compared with the control group, and the difference was statistically significant ($P < 0.05$, Table V).

Clinical adverse reactions

No obvious adverse reactions were observed in either of the two groups during the treatment and observation period.

Table I. Comparison of the therapeutic effect between control group and treatment group ($n=47$, %).

Grouping	Significant effective rate	Effective rate	Ineffective rate	Total effective rate
Control group	23 (48.73%)	10 (21.27%)	14 (29.78%)	33 (70.21%)
Treatment group	26 (55.31%)	17 (36.17%)	4 (8.51%)*	43 (91.48%)*

*compared with the control group, $P<0.05$.

Table II. Comparison of bone metabolic indexes of the two groups before and after treatment ($n=47$, $x\pm s$).

Grouping		Blood calcium (mmol/L)	Phosphorus (mmol/L)	Alkaline phosphatase (U/L)
Control group	Before treatment	2.31 $\pm$ 0.33	1.45 $\pm$ 0.24	84.76 $\pm$ 20.71
	After treatment	2.56 $\pm$ 0.38 ^Δ	1.55 $\pm$ 0.25 ^Δ	82.48 $\pm$ 22.33 ^Δ
Treatment group	Before treatment	2.31 $\pm$ 0.34	1.45 $\pm$ 0.23	84.24 $\pm$ 11.67
	After treatment	3.39 $\pm$ 0.65* ^Δ	2.44 $\pm$ 0.94* ^Δ	74.63 $\pm$ 13.09* ^Δ

Note: *compared with the control group, $P<0.05$; ^Δcompared with prior-treatment, $P<0.05$.

Table III. Comparison of BMD of femoral neck of the group before and after treatment ($n=47$, $x\pm s$).

Grouping		BMD of femoral neck (mg/c ²)
Control group	Before treatment	0.62 $\pm$ 0.05
	After treatment	0.65 $\pm$ 0.08 ^Δ
Treatment group	Before treatment	0.61 $\pm$ 0.04
	After treatment	0.72 $\pm$ 0.09* ^Δ

Note: *compared with the control group, $P<0.05$; ^Δcompared with prior-treatment, $P<0.05$.

Table IV. Comparison of BMD of lumbar vertebra before and after treatment of the two groups ($n=47$, $x\pm s$, mg/c²).

Grouping		L ₁₋₂ (mean value)	L ₃₋₄ (mean value)	L ₂₋₄ (mean value)
Control group	Before treatment	0.71 $\pm$ 0.03	0.58 $\pm$ 0.03	0.68 $\pm$ 0.09
	After treatment	0.82 $\pm$ 0.08 ^Δ	0.63 $\pm$ 0.02 ^Δ	0.74 $\pm$ 0.08 ^Δ
Treatment group	Before treatment	0.71 $\pm$ 0.04	0.58 $\pm$ 0.04	0.68 $\pm$ 1.01
	After treatment	0.93 $\pm$ 0.05* ^Δ	0.71 $\pm$ 0.03* ^Δ	0.82 $\pm$ 0.09* ^Δ

*compared with the control group, $P<0.05$; ^Δcompared with prior-treatment, $P<0.05$.

Table V. Comparison of OC and CTX-1 levels between the two groups before and after treatment.

Grouping		OC (mean value)	CTX-1 (mean value)
Control group	Before treatment	5.29±0.47	0.33±0.07
	After treatment	7.68±1.88 ^Δ	0.28±0.07 ^Δ
Treatment group	Before treatment	5.31±0.48	0.32±0.08
	After treatment	9.73±3.55 ^{*Δ}	0.21±0.07 ^{*Δ}

^{*}compared with the control group, $P < 0.05$; ^Δcompared with prior-treatment, $P < 0.05$.

DISCUSSION

As an important part of medicine, Chinese herbal medicine has been widely used at the clinical level with the advantage of having minimal side-effects. TCM holds the opinion that osteoporosis is caused by poor kidney function, which should be treated by improving kidney health in. In this study, the Xianling Gubao Capsules were used to treat patients with osteoporosis. As a result, the BMD value and the activity of osteocytes were improved, and the CTX-1 level was reduced, which indirectly reflects good bone resorption and bone mass (8). Thus, we concluded that the Xianling Gubao Capsules provided a reliable therapeutic effect, which has extensive clinical value and significance.

The Xianling Gubao Capsules are composed of *Epimedium leptorrhizum*, stearn, *Dipsacus asperoides*, *Psoralea corylifolia*, and Linn. The capsules proved very effective in preventing and treating osteoporosis by nourishing the liver and kidneys, joint the fractured bones, and strengthening the muscles (9). The results of this experiment showed that the treatment group had a success rate of 91.48%. When compared with the control group, the

level of the bone metabolic indexes in the treatment group was improved, and the BMD of the femoral neck and lumbar vertebrae increased significantly. Also, the OC level increased, and the CTX-1 level decreased, both with statistical significance ($P < 0.05$). Therefore, Xianling Gubao capsules can promote bone formation and metabolism and inhibit bone resorption without adverse reactions. It is safe and effective.

Xianling Gubao Capsules can play an active role in improving the BMD of patients with osteoporosis, helping with bone formation and inhibiting bone resorption.

REFERENCES

1. Eckert AW, Maurer P, Meyer L, et al. Bisphosphonate-related jaw necrosis--severe complication in maxillofacial surgery. *Cancer Treat Rev* 2007; 33(1):58-63.
2. Bernabei R, Martone AM, Ortolani E, Landi F, Marzetti E. Screening, diagnosis and treatment of osteoporosis: a brief review. *Clin Cases Miner Bone Metab* 2014; 11(3):201-7.
3. Zheng XY. Guiding principles for clinical research on new drugs of traditional Chinese medicine.

- Beijing: Chinese Medical Science and Technology Press 2002. Chinese.
4. Shi L, Ma JL, Yang L, Sheng L. Clinical study of Xianlinggubao Capsule on the patients with osteoporosis. *Mod J Integr Trad Chinese Western Med* 2013; 22(32):3552-53.
 5. Liao EY, Wu XP, Luo XH, Zhang H, Dai RC, Huang G, Wang WB. Establishment and evaluation of bone mineral density reference databases appropriate for diagnosis and evaluation of osteoporosis in Chinese women. *J Bone Miner Metab* 2003; 21(3):184-92.
 6. Azemati M, Shakerhosseini R, Hekmatdos A, et al. Comparison of the effects of canola oil versus sunflower oil on the biochemical markers of bone metabolism in osteoporosis. *J Res Med Sci* 2012; 17(12):1137-43.
 7. Dong H, Hutchins-Wiese H, Kleppinger A, et al. Effects of Omega-3 polyunsaturated fatty acid supplementation on bone turnover in older women. *Int J Vitam Nutr Res* 2014; 84(3-4):124-32.
 8. Wu JJ, Wen LP, Wu YG, Shen Q, Han Y. Effects of Xianling Gubao capsules for the treatment of bone loss induced by glucocorticoid. *Zhongguo Gu Shang* 2009; 22(3):193-95.
 9. Dai Y, Tu FJ, Yao ZH, Ding B, Xu W, Qiu XH, Yao XS. Rapid identification of chemical constituents in traditional Chinese medicine fufang preparation xianling gubao capsule by LC-linear ion trap/Orbitrap mass spectrometry. *Am J Chin Med* 2013; 41(5):1181-98.

LETTER TO THE EDITOR

THE ORIGIN, *IN VITRO* DIFFERENTIATION, AND STEMNESS SPECIFICITY OF PROGENITOR CELLS

W. KRANC¹, J. BUDNA², M. DUDEK¹, A. BRYJA¹, A. CHACHUŁA²,
S. CIESIÓŁKA², S. BORYS¹, M. DYSZKIEWICZ-KONWIŃSKA³, M. JESETA⁴,
L. POROWSKI¹, P. ANTOSIK⁵, D. BUKOWSKA⁵, K.P. BRÜSSOW¹,
M. BRUSKA¹, M. NOWICKI², M. ZABEL^{2,6} and B. KEMPISTY^{1,2}

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

Received November 2, 2016 – Accepted March 6, 2017

Since the successful collection of the first progenitor stem cells (SCs), there has been an increased interest in these cells as a model for undiscovered and unlimited potential of differentiation and development. Additionally, it was shown that SC populations display an ability to form pluripotent and/or totipotent cell populations. It was found that human ovarian granulosa cells (GCs) maintain a large capacity for differentiation into several other cell lineages, such as chondrogenic, osteogenic, neurogenic, and adipogenic, particularly during long-term, *in vitro* culture. In these cases, the specific media supplements that promote various pathways of differentiation, such as leukemia-inhibiting factor (LIF) and/or FSH, are well recognized. However, these are only some examples of the differentiation possibilities of human SCs *in vitro*, and other pathways still require further investigation. Many SC populations, which are directed to differentiate into specific cell types, are also successfully used in several human disease therapies, e.g. leukemia. Moreover, SCs are used for tissue scaffold construction in patients with respiratory and cardiovascular diseases. In this review, the most recent knowledge about the *in vitro* growth and differentiation capacity of SCs is presented. Furthermore, we discuss the possible worldwide application of SCs in advanced cell and tissue bioengineering. In conclusion, it is suggested that, in the future, SCs will be a basic strategy in human therapy, and their use will open new gates in regenerative and reconstructive medicine in the 21st century.

To the Editor,
The origin of stem cells

In recent years, considerable scientific interest has been generated by the phenomenon of progenitor

stem cell biology. Progenitor stem cells (SCs) are the precursors of various cell types and, therefore, are often described as undifferentiated cell populations that originate from different tissues and organs. Stem

Key words: progenitor stem cells, differentiation in vitro

Mailing address:
Bartosz Kempisty PhD,
Department of Histology and Embryology, Department of Anatomy,
Poznań University of Medical Sciences,
6 Świecickiego St., 60-781 Poznań, Poland
Tel./Fax: +48 61 8546567 / +48 61 8546568,
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.

cells, under appropriate physiological (*in vivo*) or laboratory (*in vitro*) conditions and in the presence of certain substances, begin to differentiate into fully mature cells of a specific type. The SCs may originate from embryonic and/or somatic tissue. They were first isolated from mouse embryos (embryonic stem cells, ESCs), and they have also been isolated from cord blood (CBSCs) and tissues derived from adult humans, i.e. bone marrow (1).

One of the most important features of stem cells is their ability to differentiate. Stem cells can be divided into different categories: (i) totipotent stem cells – which can differentiate into any cell type, including cells that constitute the placenta; (ii) pluripotent stem cells that are derived from inner mass of mammalian blastocyst and have ability to differentiate into cells of all three germ layers; (iii) multipotent stem cells that constitute the population of differentiated cells but maintain the ability to differentiate into different cell types (eg.: adipocytes into osteoblasts, myoblasts and chondroblasts and (iv) unipotent stem cells (precursor) - differentiate into only one cell type (2).

In recent years, an increased interest in stromal cells of the bone marrow has arisen in stem cell research. A characteristic feature of pluripotent mesenchymal stem cells (MSC) is their capability of differentiating into cells of various connective tissues. These cells also successfully differentiate into chondroblasts, osteoblasts, and adipocytes, similar to natural chondro-, osteo-, and adipogenesis (1).

The isolation of hES cells requires destruction of the entire embryo, which is considered unethical (3). Thus, researchers look for alternative methods for obtaining hES cells. Klimanskaya et al. obtained hES cells from single blastomeres isolated from 8-10 cell embryos. After this procedure, the embryos were capable of resuming normal growth. Another source of hES cells are embryos which cannot pass the following divisions as a result of chromosomal defects and cells derived from blastocysts developed *in vitro* from human unfertilized oocytes stimulated to the parthenogenesis (4).

According to Kossowska-Tomaszczuk et al., a population of human ovarian follicular cells, characterized by a huge stem cell potential, is also a

source of somatic stem cells. This unique population of granulosa cells (GCs) is cultured under certain *in vitro* conditions in media enriched with specific components such as transretinoic acid, ITS-1 (insulin, transferrin, selenium), transforming growth factor β 1, dexamethasone, and β -glycerophosphate, which allow these cells to differentiate into chondroblasts, osteoblasts, and neuroblasts (5).

Recovery, isolation, and in vitro culture of stem cells

Several studies demonstrated that progenitor stem cells can be successfully cultured and developed under *in vitro* conditions. The hES cells are derived from blastocysts, which are kept several days on a feeder layer of mouse embryonic fibroblasts (MEFs). The cells are usually collected with a pipette when a node-cultured embryonic blastocyst reaches the appropriate size. After collection, the cells are transferred to a new base and repeatedly passaged until they reach the morphology characteristic for ESCs. The second method of hES cell culturing is utilizing the embryonic node isolated from blastocysts. The embryonic node is devoid of surrounding trophoblast (6).

Another way to obtain pluripotent stem cells is through therapeutic cloning. The procedure involves *in vitro* preparation of cells that have the potential to development resembling the zygote. This procedure involves conversion of the haploid oocyte nucleus to the diploid nucleus from a mature somatic cell donor (7). Pluripotent stem cells can also be isolated from normal adult tissues. These stem cells possess two advantages. Firstly, contrary to the EMS, they do not form teratomas and may be taken from the patient's own body, so there is no risk of rejection. Secondly, obtaining these cells is not associated with the destruction of the embryo. The main sources of this cell type are the bone marrow, mobilized peripheral blood, and cord blood.

Nagamatsu et al. demonstrated that unipotent primordial germ cells (PGCs) can be transformed under certain conditions into pluripotent embryonic germ cells (EGCs) (8). The PGCs, under treatment with signaling inhibitors such as MAP2K (MEK), GSK3 β , and TGF β type I receptors, were transformed into pluripotent stem cells after 10 days of culture.

Transcriptomic analysis of PGCs transformed into EGCs showed expression of genes characteristic for pluripotent cells (Pou5f1 and Sox2).

Takahashi et al. presented cultured iPSCs derived from fibroblasts of adult human skin (human dermal fibroblast, HDF). Human iPSCs showed several similarities to embryonic stem cells: morphology, proliferation, surface antigens, gene expression, telomerase activity, and epigenetic status of pluripotent target-specific genes. The results of immunocytochemistry and long-term cell cultivation indicated that human iPSCs can differentiate *in vitro* into three germ layers. During the tests, the cells detected positive for β III-tubulin (ectoderm marker), glial fibrillary acidic protein (GFAP, ectoderm), α -smooth muscle actin (α -SMA, mesoderm), desmin (mesoderm), alpha-fetoprotein (AFP, endoderm), and vimentin (parietal mesoderm and endoderm). The RT-qPCR confirmed that these differentiated cells expressed forkhead box A2 (FOXA2, a marker of endoderm), AFP (endoderm), cytokeratin 8 and 18 (endoderm), SRY-box containing gene 17 (SOX17, endoderm), BRACHYURY (mesoderm), Msh homeobox 1 (MSX1, mesoderm), microtubule-associated protein 2 (MAP2, ectoderm), and paired box 6 (Pax6, ectoderm) (5).

The possible directions of stem cell differentiation

Stem cells are characterized by their multipotent properties. It is recognized that there are particular cells differentiated by internal, genetically programmed pathways. One of the most recent issues is the process of cellular transdifferentiation. This process involves the conversion of one particular cell type to another. During transdifferentiation, they run inactive pathways of cell differentiation, and, as a result, the cell is reprogrammed and acquires the properties of a different cell lineage. The conversion of one cell to another can also take place by dedifferentiation. Dedifferentiation is the conversion of the cells of the tissue to more primitive cells, characterized by specific activation mechanisms and inactive trails of differentiation. Consequently, cells with new biochemical and genetic properties are obtained. A good example of dedifferentiation is the regeneration of a tail in amphibians (9).

The process of transdifferentiation does not occur under physiological conditions in mammals. However, there are studies showing that in places such as the human body, there are cells that can be subjected to dedifferentiation. During dedifferentiation, specific cells gain plasticity and specificity, and they can develop into different cell types. Granulosa cells, under the influence of certain factors, can be converted into another type of cell. Research conducted by Brevini et al. showed that GCs, under epigenetic factor modifiers, such as 5-azacytidine (5-aza-CR) and VEGF, differentiate into muscle cells (10). Some scientists suggest that GCs express stem cell markers such as OCT-4, NANOG, and SOX2 and present high telomerase activity (11).

A recent study by Kossowska-Tomaszczuk et al. demonstrated an increased ability of GCs to differentiate into several cell types (5). These experiments relied on long-term cultivation of GCs in the presence of LIF. *In vitro* culture under such conditions showed that GCs exhibit mesenchymal stem cell markers. The results indicated that long-term, cultured GCs, in the presence of specific factors, can differentiate into chondroblasts, osteoblasts, and neuroblasts.

Recent neuroscience research showed a high ability of iPSCs to differentiate into neural cells. In these experiments, iPSCs were cultured for two weeks in a PA6 feeder layer. The cells proliferated quickly, and some of them had structure typical for nerve cells. RT-qPCR analysis showed expression of aromatic L-amino acid decarboxylase (AADC), member 3 (DAT), choline acetyltransferase (ChAT), and LIM homeobox transcription factor 1 beta (LMX1B). These markers are dopaminergic neuron markers (6). Human iPSCs can also differentiate into cardiac cells. Differentiation is possible by using activin A and bone morphogenetic protein 4 (BMP4) in the culture (12). Takahashi et al. cultured cells with selected growth factors, and, after 12 days of culture, cells showed expression of the following cardiomyocyte markers: troponin T type 2 cardiac (TnTc); myocyte enhancer factor 2C (MEF2C); myosin, light polypeptide 7 regulators (Myl2); myosin, heavy polypeptide 7, cardiac muscle, beta (MYHCB); and NK2 transcription factor-related,

locus 5 (nkx2.5) markers (6).

Recent studies showed that MSCs from human bone marrow have the capacity to differentiate into cardiomyocytes in the presence of 5-azacytidine in the culture medium. The resulting cells had a fusiform shape with irregular appendages. Myogenic cells were positive for beta-myosin heavy chain (beta-MHC), desmin, and alpha-cardiac actin. In addition, myofilament-like structures could be observed in these cells. Expression of beta-MHC, desmin, alpha cardiac actin, and cardiac troponin T mRNA was substantially increased. Culture of cardiomyocytes from bone marrow is a promising source of cells for the repair of myocardial tissue, i.e. after myocardial infarction (12).

The application of stem cells in biomedicine

The use of stem cells is a possibility for regenerative medicine, which deals with the treatment of damaged tissue. Application of specific stem cells may result in the reconstruction/regeneration of damaged organs. Stem cells have been successfully used for several decades in the treatment of diseases associated with the hematopoietic system (including leukemia). Recently, multipotent mesenchymal stem cells were isolated from Wharton's jelly collected from the umbilical cord (2). Additionally, several properties of iPSCs bring hope for their application in medicine and biomedical tissue engineering. The main advantages are their pluripotent properties and the lack of immunological reactions since they represent reprogrammed somatic cells of the patient being treated (13).

For ethical reasons, as well as technical problems, it is very difficult to obtain an adequate amount of stem cells from various sources: skeletal muscle, cardiac muscle, liver, pancreas, or the central nervous system (13).

The procedure involves the mobilization of hematopoietic cells from the bone marrow into the blood, and then converting them to cells containing the CD34 antigen on their surface. If the quantity of cells is adequate, they are harvested and stored in liquid nitrogen until transplant. The treatment with autologous hematopoietic cell transplantation is preceded by chemotherapy in order to destroy all mature immune system cells, including an autoreactive clone. As a result of stem cell

transplantation, the patient may temporarily become independent of insulin. However, there is a risk of relapse due to any genetic predisposition of patients. Another method of diabetes treatment is the use of stem cells from umbilical cord blood. The recent study by Huang et al., demonstrated that stem cells from umbilical cord blood can differentiate into insulin-producing cells in healthy men (14).

In vivo in humans, the use of human iPSCs is quite dangerous since the implanted dedifferentiated cells can give rise to teratomas (6).

Hematopoietic stem cells have been used for several decades to treat blood diseases and cancers in children and adults. Recently, several studies presented the possibility of using stem cells for the treatment of pulmonary disorders (15).

Bronchopulmonary dysplasia is one lung diseases that can be treated with MSCs. It is a chronic lung disease occurring in premature infants. These children require mechanical ventilation and oxygen therapy due to respiratory system failure. A study by van Haaften et al., demonstrated MSCs may intratracheally protect blood vessels of the lungs and alveolar structure from pulmonary hypertension (15).

Human MSCs are widely used in the treatment of heart diseases such as cardiomyopathy. The therapy based on MSCs reduces fibrosis, angiogenesis stimulation, and improves the structure and function of the ventricles (16). Many authors have also expressed hope for the use of GCs in regenerative medicine and cell therapy. Follicular fluid, which is discarded in the standard method for the *in vitro* treatment of infertility, would be an excellent source of stem cells. Under the appropriate culture conditions, GCs could be differentiated into chondroblasts, osteoblasts, neuroblasts, or muscle cells (11). Taking into account this data, it can be concluded that stem cells therapy has improved in the past few years; however, full recognition of the biomedical properties of these cells, as well as their application in many alternative human therapies, still requires further investigation.

ACKNOWLEDGEMENTS

Publication of this article was made possible by

grant number 2014/13/D/NZ9/04798 “SONATA” from the Polish National Centre of Science.

REFERENCES

1. Crisan M, Yap S, Casteilla L, et al. A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 2008; 3:301-13.
2. Fiorina P, Voltarelli J, Zavazava N. Immunological applications of stem cells in type 1 diabetes. *Endocr Rev* 2011; 32:725-54.
3. Vazin T, William JF. Human embryonic stem cells: Derivation, culture, and differentiation: A review. *Restor Neurol Neurosci* 2010; 28:589-603.
4. Klimanskaya I, Chung Y, Becker S, Lu SJ, Lanza R. Human embryonic stem cell lines derived from single blastomeres. *Nature* 2006; 444:481-85.
5. Kossowska-Tomaszczuk K, De Geyter C, De Geyter M, Martin I, Holzgreve W, Scherberich A, Zhang H. The multipotency of luteinizing granulosa cells collected from mature ovarian follicles. *Stem Cells* 2009; 27:210-19.
6. Csobonyeiova M, Krajciova L, Nicodemou A, Polak S, Danosovic L. Induction of pluripotency in long-term cryopreserved human neonatal fibroblasts in feeder-free condition. *Cell Tissue Bank* 2016; 21:1-8.
7. Markoulaki S, Meissner A, Jaenisch R. Somatic cell nuclear transfer and derivation of embryonic stem cells in the mouse. *Methods* 2008; 45:101-14.
8. Nagamatsu G, Kosaka T, Saito S, et al. Tracing the conversion process from primordial germ cells to pluripotent stem cells in mice. *Biol Reprod* 2012; 86:182.
9. Brockes JP, Kumar A. Plasticity and reprogramming of differentiated cells in amphibian regeneration. *Nat Rev Mol Cell Biol* 2002; 3:566-74.
10. Brevini TA, Pennarossa G, Rahman MM, et al. Morphological and molecular changes of human granulosa cells exposed to 5-azacytidine and addressed toward muscular differentiation. *Stem Cell Rev* 2014; 10:633-42.
11. Dzafic E, Stimpfel M, Virant-Klun I. Plasticity of granulosa cells: on the crossroad of stemness and transdifferentiation potential. *J Assist Reprod Genet* 2013; 30:1255-61.
12. Laflamme MA, Chen KY, Naumova AV, et al. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nat Biotechnol* 2007; 25:1015-24.
13. Wilson KD, Wu JC. Induced Pluripotent Stem Cells. *JAMA* 2015; 16:1613-1614.
14. Huang CJ, Butler AE, Moran A, et al. A low frequency of pancreatic islet insulin-expressing cells derived from cord blood stem cell allografts in humans. *Diabetologia* 2011; 54:1066-74.
15. Van Haaften T, Byrne R, Bonnet S, et al. Airway delivery of mesenchymal stem cells prevents arrested alveolar growth in neonatal lung injury in rats. *Am J Respir Crit Care Med* 2009; 180:1131-42.
16. Golpanian S, Wolf A, Hatzistergos KE, Hare JM. Rebuilding the damaged heart: mesenchymal stem cells, cell-based therapy, and engineered heart tissue. *Physiol Rev* 2016; 96:1127-68.

LETTER TO THE EDITOR

RESPIRATORY TRAINING DURING REHABILITATION OF ACUTE ORGANIC FLUORINE-POISONED PATIENTS TREATED BY NON-INVASIVE POSITIVE PRESSURE VENTILATION

L. LIU, DZ. LIU, QP. WANG, ZL. ZHU, HM. LI and XY. LU

*Xinxiang Central Hospital, Xinxiang City, China**Received October 24, 2016 – Accepted March 13, 2017*

This paper aimed to analyze the effects of respiratory training on pulmonary function during the rehabilitation period for acute organic fluorine-poisoned patients treated by non-invasive positive pressure ventilation (NIPPV). Sixty-two acute organic fluorine-poisoned patients admitted to the Xinxiang Central Hospital, Xinxiang City, China, from May 2012 to March 2016 were selected and randomly divided into an observation group and a control group, with 31 cases in each. Both groups received NIPPV. The patients in the control group exercised daily, while the patients in the observation group received contracting lips-abdominal breathing training. The therapeutic effects, pulmonary ventilation function, serum levels of α -antitrypsin1 (α -AT₁), surfactant protein D (SP-D), neutrophil elastase (NE), transforming growth factor beta 1 (TGF- β ₁), and quality of life were analyzed and compared between the two groups both before and after the administration of treatment. The total effective rate of the observation group was 93.55%, which was significantly higher when compared with the control group (74.19%) ($P < 0.05$). The levels of forced expiratory volume in one second (FEV₁), FEV₁/FVC ratio, vital capacity (VC), carbon monoxide diffusion capacity (DLco), and maximal voluntary ventilation (MVV) of the observation group were better when compared with the control group and had statistical significance ($P < 0.05$). Before treatment, the serum levels of α -AT₁, SP-D, NE, and TGF- β ₁ and quality of life had no statistical significance in either group ($P > 0.05$); after treatment, these indexes and the quality of life for the observation group were significantly higher when compared with the control group, with statistical significance ($P < 0.05$). The respiratory training in acute organic fluorine-poisoned patients treated by NIPPV can improve the serum indexes, dilute toxicity, and recover pulmonary function, which play key roles in improving the therapeutic effects and quality of life of patients, and is worthy of clinical promotion.

To the Editor,

Acute organic fluorine poison generally refers to the acute intoxication caused by residual liquid gas, organic fluorine monomer, cracking gas, and fluorine polymer, which can cause substantial damage to lung function (1). With the development of industrialization, the incidence rate of acute fluorine

poisoning induced by industrial emission and improper management has continuously increased, seriously impacting the quality of life and health of patients (2). Acute organic fluorine-poisoned patients will have impaired pulmonary function after treatment. Therefore, restoring their pulmonary function, which improves therapeutic effects and

Key words: respiratory training, noninvasive positive pressure ventilation, acute organic fluorine poison, pulmonary

Mailing address:

Dr. Liu DZ,
Xinxiang Central Hospital, No. 56, Jinsui Avenue,
Weibin District, Xinxiang City 453099,
Henan Province, People's Republic of China
Tel.: +8613203721139
e-mail: dezhiлию6@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.

quality of life, has become a hot topic of study. In order to explore the effects of respiratory training on pulmonary function during the rehabilitation period for acute organic fluorine-poisoned patients treated by non-invasive positive pressure ventilation (NIPPV), this study recruited 62 patients who received different recovery training methods.

MATERIALS AND METHODS

General information

Sixty-two patients with acute organic fluorine poisoning admitted to the Xinxiang Central Hospital, Xinxiang City, China, between May 2012 and March 2016 were enrolled in this study and randomly divided into an observation and a control group group, each with 31 cases. The observation group included 18 males and 13 females, with ages ranging from 24-59 years (average 41.49 ± 3.28 years). The mean acute physiology and chronic health evaluation II (APACH II) score was (13.5 ± 2.4). The control group included 19 males and 12 females, ranging in age from 25-58 years (average 41.50 ± 3.30 years). The mean APACH II score was (13.4 ± 2.6). The differences in gender, APACH II scores, age, etc. were not statistically significant ($P > 0.05$), therefore, the two groups were comparable.

This study was approved by the Xinxiang Central Hospital Ethics Committee for Clinical Research. All the patients who participated in the study signed an informed consent.

Treatment methods

Noninvasive positive pressure ventilation

NIPPV was delivered to the patients of both groups by a face mask (Respironics, Phillip, USA) connected to a ventilator set in bi-level positive airway pressure S/T mode with an initial supportive pressure of 6.0-8.0 cm H₂O and an end-expiratory positive pressure of 5.0-6.0 cm H₂O. Also, the supportive pressure and end-expiratory positive pressure were regulated accordingly, maintaining about 90% SpO₂.

Respiratory training

The patients of the control group exercised daily (e.g. jogging, shadowboxing etc.), while the observation group underwent respiratory training including: i) contracting

lips-abdominal breathing, in which the patients are in either a standing, semi-reclining, or recumbent position with one hand on the chest and the other on the abdomen, breathing deeply and slowly, inhaling through the nasal cavity and raising the abdomen upwards. The lips are contracted to exhale very slowly, while simultaneously curling the abdomen with the help of two hands for 10 min twice a day with a respiratory frequency of 10-12 breaths/min; ii) Artificial breathing resistance training in which a 900-1000 ml balloon was utilized. The patients had to blow up the balloon fully after deeply inhaling. This was repeated for 5-10 min, 3-4 times a day.

Observation indexes and evaluation standards

Several indexes required observing in this study, including the therapeutic effects, variation of pulmonary ventilation function, α -antitrypsin (α -AT1), surfactant protein D (SP-D), neutrophil elastase (NE), transforming growth factor beta 1 (TGF - β 1), and quality of life before and after treatment. The evaluation standards (3) for the therapeutic effects are divided into three grades: Excellent refers to the patients after treatment who experienced great improvement or complete remission of clinical symptoms, with normal breathing and serum indicators; Good refers to the patients whose clinical symptoms and serum levels improved and breathing almost normalized; Invalid meant no improvement of clinical symptoms and difficulty breathing. The total effective rate = (Excellent + Good)/100%. The pulmonary ventilation function includes forced expiratory volume in one second (FEV₁), FEV₁/FVC ratio, vital capacity (VC), carbon monoxide diffusion capacity (DLco), and maximal voluntary ventilation (MVV). The 36-item Short-Form Health Survey Questionnaire (SF-36) was used to evaluate patient quality of life (e.g. physical function, social function, mental state, health condition, etc.), a higher score indicated a better quality of life (4).

Statistical analysis

SPSS19.0 software was used to analyze the data in this study. The measurement data were checked by *t*-test and expressed as standard deviation ($\bar{x} \pm s$). The enumeration data were checked by χ^2 and expressed as percentage (%) and number (N). In addition, $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of therapeutic effects of the two groups

The results in this study showed that the total effective rate of the observation group was 93.55%, which was significantly higher when compared with the control group ($P < 0.05$), as shown in Table I.

Comparison of the pulmonary ventilation function between the two groups

The results showed that the FEV_1 , FEV_1/FVC ratio, VC, DLco, and MVV of the observation group were vastly superior when compared with the control group, and the differences were statistically significant ($P < 0.05$). The results are shown in Table II.

Comparison of serum levels of α -AT1, SP-D, NE, TGF- β_1 between the two groups before and after treatment

Before treatment, the differences in the serum levels of α -AT1, SP-D, NE, and TGF- β_1 between the two groups lacked statistical significance ($P > 0.05$). After treatment, the indexes of the observation group were greater when compared with the control group. The differences were statistically significant ($P < 0.05$). The results are shown in Table III.

Comparison of quality of life of the two groups before and after treatment

Before treatment, various indexes of quality of life between the two groups had no statistically significant difference ($P > 0.05$). After treatment, however, the indexes of the observation group were much higher when compared with the control group ($P < 0.05$). The results are shown in Table IV.

DISCUSSION

It takes about 7 to 12 hours (latent period) to cause noticeable symptoms in patients invaded by organic fluorine. When the invasion spreads throughout the body, patients show symptoms such as coughing, and chest distress. In addition, organic fluorine has a strong capacity for pneumotropism, badly impairing lung tissues and function. The pulmonary diseases caused by acute organic fluorine poison mainly include

pulmonary edema and chemical pneumonitis, and can even lead to respiratory failure, multiple visceral damage, or death (5). Organic fluorine in the human body will cause diffuse injury to the pulmonary alveoli and mesenchyme, elevated permeability of pulmonary capillaries, and decreased active materials on the cytomembrane. This can result in the collapse of the diseased regions, imbalance of the ventilation-perfusion ratio, and acute respiratory distress syndrome (6). Therefore, mechanical ventilation is required for preventing the continuously decreasing oxygenation index in patients with acute organic fluorine poisoning.

When compared with the traditional invasive mechanical ventilation therapy, NIPPV can effectively lower the incidence rate of complications, as the operation is quite simple and does not require an artificial airway. Also, in the bilevel positive airway pressure model, the power comes from the spontaneous breathing of the patient. Therefore, exhalation and inhalation are alternatively low pressure and high pressure, and the patient's own airways can be kept on the positive pressure state, which can reduce energy consumption, collapse, as well as the increased tidal volume (TV) or oxygenation index. Clinical data have shown that respiratory training contributes to pulmonary function restoration in acute organic fluorine-poisoned patients treated with NIPPV (7). Contracting lips-abdominal breathing training can increase inner pressure in the air tract by elevating outside resistance.

The breathing training in this study can increase the diaphragmatic muscle activity, range, and contractility, thereby improving both breathing efficiency and pulmonary function. Contracting lips-abdominal breathing training can stabilize the inner pressure by slowing the expiratory flow rate, avoiding early closure of the surrounding small airways, reducing functional residual capacity, and improving the pulmonary ventilation volume and SpO₂ levels. The artificial resistive respiratory function training can significantly strengthen the breathing muscles by stimulating thorax activity, forcing complete pulmonary expansion to increase the vital capacity and facilitate the blood-gas

Table I. Comparison of therapeutic effect of the two groups (n, %).

Groups	Excellent	Good	Invalid	Total effect rate
Observation group (n=31)	16 (51.61)	13 (41.94)	2 (6.454)*	29 (93.55)*
Control group (n=31)	12 (38.71)	11 (35.48)	8 (25.81)	23 (74.19)
χ^2	1.042	0.272	4.292	4.292
P	>0.05	>0.05	<0.05	<0.05

*compared with the control group, $P<0.05$

Table II. Comparison of the pulmonary ventilation function between the two groups ($\bar{x}\pm s$, %).

Groups	FEV ₁	FEV ₁ /FVC	VC	DLco	MVV
Observation group (n=31)	67.13±11.84*	69.49±6.48*	51.21±10.03*	46.71±9.04*	57.62±10.86*
Control group(n=31)	56.14±11.66	72.59±3.05	63.44±13.79	51.81±10.21	71.49±14.14
t	3.682	2.410	3.993	2.082	4.331
P	<0.05	<0.05	<0.05	<0.05	<0.05

*compared with the control group, $P<0.05$

Table III. Comparison of serum levels of α -AT1, SP-D, NE, TGF- β 1 between the two groups before and after treatment ($\bar{x}\pm s$, ng/ml).

Groups	Periods	α -AT1	SP-D	NE	TGF- β ₁
Observation group (n=31)	Before treatment	420.71 $\pm$ 13.44	17.31 $\pm$ 3.89	90.11 $\pm$ 7.39	188.64 $\pm$ 7.13
	After treatment	400.07 $\pm$ 11.09*	13.64 $\pm$ 3.30*	80.63 $\pm$ 6.31*	160.14 $\pm$ 6.72*
Control group (n=31)	Before treatment	420.72 $\pm$ 13.46	17.33 $\pm$ 3.86	90.13 $\pm$ 7.41	189.63 $\pm$ 7.11
	After treatment	414.45 $\pm$ 11.55	16.26 $\pm$ 4.87	88.65 $\pm$ 6.18	175.21 $\pm$ 7.25

*compared with control group, $P<0.05$ **Table IV.** Comparison of quality of life of the two groups before and after treatment ($\bar{x}\pm s$, scores).

Groups	Periods	Physiological function	Social function	Mental state	Sate of health
Observation group (n=31)	Before treatment	50.13 $\pm$ 2.06	51.89 $\pm$ 3.20	60.28 $\pm$ 2.70	53.41 $\pm$ 3.01
	After treatment	62.85 $\pm$ 3.68*	65.85 $\pm$ 4.63*	77.34 $\pm$ 3.81*	74.48 $\pm$ 3.28*
Control group (n=31)	Before treatment	50.11 $\pm$ 2.04	51.91 $\pm$ 3.16	60.30 $\pm$ 2.67	53.37 $\pm$ 2.97
	After treatment	56.34 $\pm$ 4.31	58.55 $\pm$ 5.11	69.54 $\pm$ 4.67	62.11 $\pm$ 4.09

*compared with control group, $P<0.05$

exchange, and effectively prevent the closure of the small airways and bronchus (8). In this study, the total therapeutic effective rate of the observation group was 93.55%, which is significantly higher than that of the control group (74.19%). In addition, the levels of FEV₁, FEV₁/FVC ratio, VC, DLco, and MVV were much higher when compared with the control group, and had statistical significance ($P < 0.05$). After treatment, the various indexes and quality of life in the observation group were far superior to the control group, with statistical significance ($P < 0.05$). The results indicate that breathing training can improve serum indexes, pulmonary function, and quality of life by diluting the poison gas level in fluorosis patients.

Respiratory training has a positive effect on patients with fluorosis by improving serum indexes and pulmonary function indicators, is of significant importance to improve treatments and the quality of life of fluorosis patients, and merits further study.

REFERENCES

1. Valic F, Skuric Z, Bantic Z, Rudar M, Hecej M. Effects of fluorocarbon propellants on respiratory flow and ECG. *Br J Ind Med* 1977; 34(2):130-36.
2. Borak J, Diller WF. Phosgene exposure: mechanisms of injury and treatment strategies. *J Occup Environ Med* 2001; 43(2):110-19.
3. Gueli N, Verrusio W, Linguanti A, et al. Montelukast therapy and psychological distress in chronic obstructive pulmonary disease (COPD): a preliminary report. *Arch Gerontol Geriatr* 2011; 52(1):e36-39.
4. McHorney CA, Ware JE Jr, Raczek AE. The MOS 36-Item Short-Form Health Survey (SF-36): II. Psychometric and clinical tests of validity in measuring physical and mental health constructs. *Med Care* 1993; 31(3):247-63.
5. Koreeda A, Yonemitsu K, Mimasaka S, Ohtsu Y, Tsunenari S. An accidental death due to Freon 22 (monochlorodifluoromethane) inhalation in a fishing vessel. *Forensic Sci Int* 2007; 168(2-3):208-11.
6. Perina DG. Noncardiogenic pulmonary edema. *Emerg Med Clin North Am* 2003; 21(2):385-93.
7. Bhatt SP, Luqman-Arafath TK, Gupta AK, et al. Volitional pursed lips breathing in patients with stable chronic obstructive pulmonary disease improves exercise capacity. *Chron Respir Dis* 2013; 10(1):5-10.
8. Petrovic M, Reiter M, Zipko H, Pohl W, Wanke T. Effects of inspiratory muscle training on dynamic hyperinflation in patients with COPD. *Int J Chron Obstruct Pulmon Dis* 2012; 7:797-805.

LETTER TO THE EDITOR

IMPACT OF LOW-ENERGY SHOCK IMPULSE ON NITRIC OXIDE SERUM LEVELS IN HEALTHY RABBITSW. ŻURAWIŃSKI¹, K. SOSADA¹, M. MUC-WIERZGOŃ² and T. KOKOT²

¹*Department of Emergency Medicine, School of Medicine in Katowice, Silesian Medical University in Katowice, Katowice, Poland;* ²*Department of Internal Medicine, School of Public Health, Silesian Medical University in Katowice, Bytom, Poland*

Received December 5, 2016 – Accepted March 10, 2017

Nitric oxide (NO) has emerged as a potent effector molecule for a post-resuscitation disease as a result of the low-energy defibrillation. In this article, the authors analysed the changes of endogenous nitric oxide levels in serum of 35 healthy rabbits after the transthoracic application of low-energy two-phase shock impulse. The animals were randomised into four groups according to the electrical energy dose to be applied during the experiment. Life parameters of the animals were monitored using the BeneViev T5 patient monitor. The nitric oxide concentration in the groups was measured before, 15 and 360 minutes after applying the low-energy two-phase linear electrical impulse. From 15 to 360 minutes the decrease in nitric oxide concentration was observed. The effect was dependent on single impulse and accumulated energy volumes. The highest decrease of NO concentration was observed in 15 minutes following the application of two electrical impulses of accumulated energy of 8 J/kg/bm. After 360 minutes following the application, a significant increase in nitric oxide levels was recorded. The changes in the nitric oxide levels in the blood serum 15 minutes after the defibrillation may initiate the vasomotor disorders as a result of the low-energy influence of the shock impulse.

To the Editor,

Low-energy defibrillation induces development of ultra-structural myocardial disorders that can be revealed only in electron microscopy. These disorders affect mostly the muscle and endothelial cells and are usually of functional and reversible nature. Low-energy defibrillation can independently trigger or exacerbate the same pathomechanisms that are observed in post-resuscitation disease development and are mainly related to ischaemia-reperfusion injury. Its effects included deteriorated oxidative myocardial metabolism, increased production of reactive oxygen species with concomitant mitochondrial and free radical damage

as well as impaired Ca²⁺ ion homeostasis combined with sarcoplasmic reticulum dysfunction (1-3). Administration of nitric oxide (NO) or NO donors prior to ischemia attenuates the consequences of myocardial ischemia/reperfusion (4). High nitric oxide concentration during shock causes vascular dilation, decreased vascular resistance and reduced myocardial contractility (5, 6). Under normal, basal conditions in blood vessels, NO is produced from the amino acid L-arginine by the enzymatic action of nitric oxide synthase (NOS). Inter cellular effect of NO is mainly correlated to guanylate cyclase activation and increased cGMP synthesis (5). The physiological actions of NO include the regulation

Key words: low-energy two-phase shock impulse, transthoracic application, defibrillation, nitric oxide, healthy rabbits

Mailing address:

Małgorzata Muc-Wierzgoń MD PhD
Dept. of Internal Medicine,
Silesian Medical University, 41-902 Bytom,
Żeromskiego 7 st. Poland
Fax/tel.: +48/32/2812122
e mail: mwierzgon@sum.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.

of vascular tone and blood pressure, prevention of platelet aggregation, inhibition leukocyte adhesion to vascular endothelium and inhibition of vascular smooth muscle proliferation (5-7). Breathing nitric oxide reduces ischemia/reperfusion injury in animal models and in patients (7) and inhaled nitric oxide during therapeutic hypothermia remained beneficial and further improved outcomes after cardiac arrest and cardiopulmonary resuscitation (8). The changes of the concentration of endogenous nitric oxide on the development of the post-resuscitation disease remained unclear.

The purpose of the present study was to assess the changes of endogenous nitric oxide concentration in blood serum of healthy rabbits after low-energy defibrillation.

MATERIALS AND METHODS

Animals

The research was conducted on 35 healthy European mixed breed rabbits of body mass ranging between 320 and 415 grams. During the experiment, the animals were kept in standard laboratory conditions (air temperature $20\pm 3^{\circ}\text{C}$, humidity range between 50% and 70%) in 12-hour light-dark cycles (light phase: 7 a.m. - 7 p.m., dark phase: 7 p.m. - 7 a.m.). Feeding the animals was ceased 12 hours before the experiment, with maintained access to water *ad libitum*.

Research protocol

Research performance was subject to the consent of the Local Ethics Committee for Experiments on Animals in Katowice – No. 34/2010 of 14 June 2010.

The animals were randomised into four groups against the electrical energy dose to be applied during the experiment and premedicated with atropine sulphate mixture at a dose of 0.05 mg/kg/bm, droperidol at a dose of 0.1 mg/kg/bm and morphine sulphate at a dose of 10 mg/kg/bm. After preparing the animals for further laboratory procedures, they were anaesthetized intramuscularly with ketamine (Bioketan) at a dose of 50 mg/kg/bm. Anaesthesia was sustained until the experiment was completed by fractionated intramuscular administration of ketamine every 30-50 min.

After anaesthesia, the animals were connected to

equipment for monitoring life functions followed by right atrial catheter insertion and intracarotid cannulation into the ascending aorta. The first 10 ml (7 ml of venous blood and 3 ml of arterial blood) blood samples were taken, followed by application of a single transthoracic two-phase linear shock impulse in the studied B2J and B4J groups or of dual shock impulse within 2-min interval in the studied B8J group in energy doses converted to kg/bm (Table I). Energy volume provided during the electrical discharge complied with the 2015 European Resuscitation Committee guidelines for electrical defibrillation. During the experiment, the following life parameters of the animals were monitored with the use of BeneViev T5 patient monitor with PMP module: 5-lead ECG, heart rate, respiratory rate, internal body temperature (MR 402B sensor), arterial blood pressure in the ascending aorta, blood pressure in the right atrium and arterial blood saturation (518 A sensor). 0.9% NaCl solution was transfused intravenously in the volume of 15 ml/kg/bm in slow drip infusion. Passive oxygen therapy with 100% oxygen of 2.0 l/min flow was applied for arterial blood saturation levels below 94% maintained for more than 1 min. After 15 min following the application of shock impulse, the second blood sample in the volume of 3 ml (venous blood), and after 360 min the third blood sample in the volume of 10 ml (7 ml of venous blood and 3 ml of arterial blood) were collected. The animals were sacrificed afterwards, which was directly followed by sternotomy and cardiac excision. Cardiac muscle and large blood vessels were separated at the level of ascending aorta and pulmonary trunk. After blood extraction, the heart was weighed net of pericardial sac. Cardiac tissue sections from the central part of the left ventricular wall myocardium were collected with biopsy forceps for biochemical tests. In the animal control group (K0), all the procedures were performed in compliance with protocol for the studied group except for shock impulse application.

Coronary flow pressure measurement

In order to measure and monitor the coronary perfusion pressure, the right cardiac atrium and ascending aorta were cannulated.

The external jugular vein and internal carotid artery were dissected. A pediatric 5 Fr Arrow CS-14502 double-lumen catheter was inserted into the external jugular vein directly in the suprasternal area using the Seldinger

method. The insertion depth of 45 mm was applied to obtain access to right atrial lumen. The internal carotid artery was cannulated directly in the supramanubrial area with the use of 1.0/32 mm or 1.2/32 mm Becton Dickinson helplock.

Pressure in the right atrium and ascending aorta was measured continuously with the use of BeneView T5 patient monitor equipped with PMP module and one-off IBP transducers connected with pressure line to the cannula inserted in the carotid and jugular vessels of the rabbit. Systolic blood pressure (BPs), diastolic blood pressure (BPD) and mean ascending aortic pressure (BPM) and mean right atrium pressure (RAP) measurements were performed. Coronary flow pressure (CPP) was calculated as the difference between diastolic aortic pressure and right atrium pressure.

Application of low-energy two-phase shock impulse

The transthoracic electrical impulse was applied with the use of standard paediatric spoons and Zoll M Series defibrillator generating low-energy two-phase linear impulse (Zoll Rectilinear Biphasic).

The Zoll M-series defibrillator has the function of active body impedance compensation ensuring high efficiency of the procedure regardless of the animal body mass. Defibrillator spoons were placed onto shaven and gel-covered skin in the right subclavian and cardiac apex areas. Electrical impulse was applied synchronously with ECG R wave. The Zoll M-series defibrillator has programmed defibrillation energy levels which imposes technical limitations when choosing the adequate energy dose per animal body mass. In defibrillation within the range between 1 and 10 J, it is possible to change the energy in 1 J increments. Defibrillation with energy above 10 J is possible only at the following programmed levels: 15 J - 20 J - 30 J - 50 J - 75 J - 100 J - 120 J - 150 J - 200 J.

Determination of nitric oxide in blood serum using the ELISA method

Determination of nitric oxide was conducted with the ELISA method using a Diaclone kit (France). The ELISA tests were performed according to the manufacturer's instructions. During the procedure, a Rayto RT-2600C washer and BioTek WWW ELx800 reader were used. Absorbance levels were read with the use of μ Qaunt (Bio Tek, USA) microplate reader and the results were

processed with KCJunior (Bio Tek, USA) software. The inaccuracy of the methods for NO is equal to 6.5%.

Statistical analysis

Statistical analyses were performed with the SAS statistical package, version 9.4 (SAS Institute Inc., Gary, NC, USA). Frequency distributions of two qualitative variables were compared with the Fisher's exact test. The student's *t*-test was used to compare the quantitative variables divided into two groups with distribution in both groups consistent with the normal distribution. Otherwise, the Mann-Whitney U-test was applied. Where quantitative variable was divided into more than two groups, the analysis of variance (ANOVA) was used. Differences between the repeated measurement results were assessed with the use of the following: the student's *t*-test for repeated measurements or Wilcoxon's signed-rank test, depending on the normality of the distribution of differences. Furthermore, the expected values of the studied parameters were assessed with 95% confidence interval (95 % PU), followed by multi-factor analysis of variance. Correlation strength was presented using the Spearman's rank correlation coefficient. The results of continuous measurements were subject to multi-dimensional analysis based on linear models with consideration to autoregressive structure of covariance.

The parameter measurement results in the period before the electrical impulse application were averaged and introduced to the model as a base value.

RESULTS

Within the examined biochemical parameters and monitored life functions, no disorders triggering ischemia and myocardial hypoxia that could affect the nitric oxide concentration in blood serum regardless of shock impact effect of the shock impulse were detected in the rabbits.

The effect of transthoracic application of low-energy two-phase shock impulse was a decrease in nitric oxide concentration in the blood serum of the rabbits, dependent on single impulse and accumulated energy volumes (Table II).

The largest decrease in nitric oxide concentration observed in 15 min following the application of the shock impulse was the effect of impact of

Table I. Total energy applied to the rabbits in the control and studied groups depending on body mass.

Group	Body mass of the experimental animals (g)	Energy during the single impulse (J)	Number of impulses	Accumulated energy (J)
K0 (0 J/kg of body mass)	x	0	0	0
B2J (2 J/kg of body mass)	2750-3249	6	1	6
	3250-3749	7	1	7
	3750-4249	8	1	8
B4J (4 J/kg of body mass)	2500-3124	10	1	10
	3125-4374	15	1	15
	4375-6250	20	1	20
B8J (2 x 4 J/kg of body mass)	2500-3124	10	2 within an interval of two minutes	20
	3125-4374	15	2 within an interval of two minutes	30
	4375-6250	20	2 within an interval of two minutes	40

Table II. Measurement results and analysis of nitric oxide concentration in the K0 control group and B2J, B4J and B8J studied group before the application (P1) and 15 minutes (P2) and 360 minutes after the application (P3) of low-energy two-phase linear electrical impulse.

Concentration of the nitric oxide in the blood serum (μmol/L)								
Group	N	Variable	Mean value	Minimum	Lower quartile	Median	Upper quartile	Maximum
K0	5	P1- NO	36.934	36.340	36.890	36.990	37.090	37.360
		P2- NO	36.918	36.360	36.900	36.930	37.100	37.300
		P3- NO	36.930	36.370	36.910	36.910	37.120	37.340
B2J	10	P1- NO	36.993	36.340	36.860	37.075	37.320	37.360
		P2- NO	27.427	22.100	23.210	24.890	31.150	35.900
		P3- NO	28.379	23.330	23.770	25.250	33.270	36.330
B4J	10	P1- NO	37.057	36.320	36.890	37.070	37.360	37.390
		P2- NO	18.348	11.920	12.900	19.845	21.370	23.310
		P3- NO	20.373	13.460	13.590	23.045	23.690	23.810
B8J	10	P1- NO	36.869	36.310	36.340	37.035	37.110	37.360
		P2- NO	13.124	8.970	9.890	11.040	16.730	19.180
		P3- NO	16.111	11.640	12.180	13.150	21.430	21.870

accumulated energy of 8 J/kg/bm in the B8J group. After 360 min following the application of the shock impulse, a significant increase in nitric oxide concentration was recorded, however its value in all animals continued to be significantly lower compared to the output values.

A very strong correlation between the decrease in nitric oxide concentration in blood serum of rabbits with electrical impulse energy volume (correlation coefficient $\rho_s = 0.0091$, $p < 0.0001$) was observed.

DISCUSSION

Nitric oxide displays cardioprotective function by participating in the process of ischemic cardiac preconditioning. This function is attributed to reduced oxygen free radical production in effect of temporary and reversible inhibiting of complex I of the respiratory chain (9). Electric shocks increased reactive oxygen species (ROS) generation and are one of the major causes of myocardial injury after cardiac arrest (10, 11).

In our study, a decrease in nitric oxide concentration in blood serum of rabbits in effect of transthoracic application of low-energy two-phase shock impulse was confirmed. Decrease in concentration depends on single impulse and accumulated energy volumes. The highest decrease of NO concentration in blood serum was observed in 15 minutes following the application of two electrical impulses of accumulated energy of 8 J/kg/bm. After 360 minutes a significant increase in nitric oxide levels was recorded. Decrease in nitric oxide concentration in blood serum should be attributable to progressing free radical damage mediated by peroxynitrite anion formed in reaction with superoxide anion radical. In addition, an increased nitrotyrosine amount in tissues observed in electrical impulse application confirms progressing damage mediated by reactive nitrogen species. Reactive nitrogen species-mediated damage is also suggested by reduced myocardial injury caused by non-specific inhibitors of nitric oxide synthases (NOS) administered in the period preceding the electrical discharge (13-15). The impact of low-energy two-phase impulse on nitric oxide concentration in

blood serum was studied in healthy, non-arrested animals. This enables assessing the importance of current induced cardiac injury during electrical defibrillation, regardless of the other factors (e.g. age, co-morbidities, medication). In conclusion, these results may be applied to evaluate endothelial function after the low-energy defibrillation and may open new diagnostic and therapeutic possibilities of post-resuscitation disease.

REFERENCES

1. Tsai MS, Huang CH, Tsai CY et al. Ascorbic acid mitigates the myocardial injury after cardiac arrest and electrical shock. *Intensive Care Med* 2011; 37:2033-40.
2. Schneider A, Albertsmeier M, Bottiger BW, Teschendorf P. Post-resuscitation syndrome. The role of inflammation in cardiac arrest. *Anaesthesist* 2012; 61:424-36.
3. Kruzliak P, Pechanova O, Kara T. New perspectives of nitric oxide donors in cardiac arrest and cardiopulmonary resuscitation treatment. *Heart Fail Rev* 2014; 19(3):383-90.
4. Schulz R, Kelm M, Heusch G. Nitric oxide in myocardial ischemia/reperfusion injury. *Cardiovascular Res* 2004; 61:402-13.
5. Bian K, Murad F. What is next in nitric oxide research? From cardiovascular system to cancer biology. *Nitric Oxide* 2014; 43:3-7.
6. Huang TH, Sun Ch, Chen Y-L, Wang CJ, Yin TC, Lee MS, Yip HK. Shock wave therapy angiogenesis through VEGFR2 activation and recycling. *Mol Med* 2016; 22:850-62.
7. Minamishima S, Kida K, Tokuda K, et al. Inhaled nitric oxide improves outcomes after successful cardiopulmonary resuscitation in mice. *Circulation* 2011; 124(15):1645-53.
8. Kida K, Shirozu K, Yu B, et al. Beneficial effects of nitric oxide on outcomes after cardiac arrest and cardiopulmonary resuscitation in hypothermia-treated mice. *Anesthesiology* 2014; 120(4): 880-89.
9. Chen Q, Camara AKS, Stowe D, Hoppel CL, Lesnefsky EJ. Modulation of electron transport protects cardiac mitochondria and decreases myocardial injury during ischemia and reperfusion.

- Am J Physiol Cell Physiol 2007; 292:C137-C147.
10. Tsai MS, Huang CH, Tsai Ch, et al. Combination of intravenous ascorbic acid administration and hypothermia after resuscitation improves myocardial function and survival in a ventricular fibrillation cardiac arrest model in the rat. *Ac Emergency Med* 2014; 21:257-65.
 11. Carlson DL. The role of reactive oxygen species in the heart after electrical stimulation. *Crit Care Med* 2009; 36(12):3274-75.
 12. Gladwin MT, Raat NJ, Shiva S, DeZfulian C, Hogg N, Kim-Shapiro DB, Patel RP. Nitrite as a vascular endocrine nitric oxide reservoir that contributes to hypoxic signaling, cytoprotection, and vasodilation. *Am J Physiol Heart Circ Physiol* 2006; 291:H2026-H2035.
 13. Duda M, Konior A, Klemenska E, Beresewicz A. Preconditioning protects endothelium by preventing ET-1-induced activation of NADPH oxidase and xanthine oxidase in post-ischemic heart. *J Mol Cell Cardiol* 2007; 42:400-410.
 14. Zhang Y, Boddicker KA, Rhee BJ, Davies LR, Kerber RE. Effect of nitric oxide synthase modulation on resuscitation success in a swine ventricular fibrillation cardiac arrest model. *Resuscitation* 2005; 67:127-34.
 15. Rochette L, Lorin J, Zeller M, Guillard JC, Lorgis L, Cottin Y, Vergely C. Nitric oxide synthase inhibition and oxidative stress in cardiovascular diseases: Possible therapeutic targets? *Pharmacol Ther* 2013; 3:239-57.

LETTER TO THE EDITOR

APPLICATION VALUES OF DETECTION OF SERUM CA199, CA242 AND CA50 IN THE DIAGNOSIS OF PANCREATIC CANCER

XF. LEI^{1,2}, SZ. JIA², J. YE³, YL. QIAO², GM. ZHAO², XH. LI² and H. CHANG¹

¹Department of Hepatobiliary Surgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, China; ²Department of Hepatobiliary Surgery, Liaocheng People's Hospital, Liaocheng, China; ³Department of Pathology, Liaocheng People's Hospital, Liaocheng, China

Received October 28, 2016 – Accepted March 13, 2017

The first two Authors contributed equally to this work

Pancreatic cancer is characterized by rapid onset, high malignancy and high mortality, thus its early diagnosis is effective for improving the survival rate of patients. To discuss the values of detection of carbohydrate antigen (CA) 50, CA242 and CA199 in the diagnosis of pancreatic cancer, this study randomly selected 112 patients who were confirmed as having stage Ia~IIb pancreatic ductal adenocarcinoma (PDAC) in the Shandong Provincial Hospital, China, from May 2012 to May 2013 as a malignant group. One hundred patients with benign pancreatic lesions and 90 healthy people were selected in the same period as a benign group and a healthy control group, respectively. The levels of serum CA199, CA242 and CA50 were detected using electrochemiluminescence. Results demonstrated that the levels of serum CA199, CA242 and CA50 of the malignant group were significantly higher than those of the benign group and the healthy control group ($P=0.001$; $P=0.003$; $P=0.000$). The positive rate of the tumor markers of the malignant group was higher than that of the benign group and the healthy control group, and the differences had statistical significance ($P=0.006$; $P=0.004$; $P=0.005$). In the malignant group, sensitivity of CA199 was the highest (81.42%) as was the specificity of CA242 (80.14%). The detection of two or more markers could improve sensitivity (joint detection based on parallel tests) and specificity (joint detection based on serial tests). Thus the levels of serum tumor markers including CA199, CA242 and CA50 could be used as the assisted indicators for the diagnosis of early-stage PDAC. Joint detection of the three tumor markers is of great significance to improve the diagnostic sensitivity and accuracy of early PDAC.

To the Editor,

Pancreatic cancer is a tumor with high malignancy and poor prognosis (1). Like most tumors, a highly efficient and completely applicable comprehensive treatment scheme has not been elaborated for pancreatic cancer. The current comprehensive treatment is still dominated by surgery supplemented

by radiotherapy and chemotherapy (2). Pancreatic cancer patients show no obvious symptoms in the early stage, therefore the best treatment time is usually missed it is often diagnosed in the late stage, thus, early diagnosis and treatment are the keys for lowering the death rate.

Currently, the diagnosis of pancreatic cancer

Key words: pancreatic cancer, tumor marker, clinical diagnosis

Mailing address:

Dr Hong Chang,
Department of Hepatobiliary Surgery,
Shandong Provincial Hospital,
Affiliated to Shandong University,
Jinan, 250021, Shandong, China
e-mail: lxfbz123@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.

mainly relies on serum tumor markers and imaging examinations (3). Although the development of imaging has improved the diagnostic level of PDAC, early screening is still limited. Tumor markers have been applied in clinics for more than 160 years. They can be found either in embryonic tissue rather than normal tissue, or they are highly expressed in tumor tissues. Their existence or quantitative change can indicate the property of tumors, and can be used for understanding cell differentiation, cell functions and assist in the diagnosis, classification, prognosis determination and treatment guidance of tumors (4-6). Carbohydrate antigens (CA), such as CA199, CA50 and CA242, are markers for pancreatic cancer which have been frequently used (7). In recent years, it has been proposed that the joint application of different tumor markers can significantly improve the diagnosis rate of malignant tumors in the early stage (8). To evaluate the values of joint detection of the three kinds of tumor markers in the clinical diagnosis of pancreatic cancer, this study compared and analyzed the levels of serum CA199, CA50 and CA242 of healthy controls and patients with benign or malignant pancreatic cancer who received treatment in the Shandong Provincial Hospital Affiliated to Shandong University, Shandong, China.

MATERIALS AND METHODS

General data

Three hundred and two subjects were selected from the Shandong Provincial Hospital Affiliated to Shandong University, Shandong, China between May 2012 and May 2013, including 112 patients with pancreatic cancer, 100 patients with pancreatitis, and 92 subjects with eligible physical conditions. All enrolled patients and healthy subjects signed informed consent. The levels of serum CA199, CA50 and CA242 of the subjects were detected.

The inclusive criteria for patients with pancreatic cancer were as follows: aged 36-61 years; with symptoms such as emaciation, weakness, anorexia, dyspepsia, loss of weight, abdominal discomfort or pain, jaundice; diagnosed by tumor marker detection, X-ray examination, computed tomography (CT), magnetic resonance imaging (MRI), ultrasonic examination, and fine needle aspiration cytology. Patients with gastrointestinal cancer,

renal carcinoma, lung cancer or other organs and tissues associated malignant tumors were excluded.

The inclusive criteria for patients with pancreatitis were as follows: clinical symptoms such as upper abdominal pain and with or without emaciation and diarrhea; with increased amylase in blood and urine during acute attack; with calcification points in the pancreas area by abdominal plain films, pancreatic calcification, pancreatic duct dilation by B ultrasound or CT, or limited or diffuse pancreatic increase or atrophy. Patients with pancreatic cancer were excluded.

The inclusive criteria for the healthy control group were: subjects who were confirmed with no diseases in the heart, liver, lung and kidney by complete examination, had normal hepatic and renal functions, had levels of tumor markers within normal scopes, and had no family history of cancer.

Methods

4.0 mL of fasting venous blood was collected from each subject in the morning and centrifuged at 3000 r/min at a diameter of 10 cm for 5 min. Then the serum was separated. The levels of CA199, CA242 and CA50 were measured by solid-phase double-site sandwich immunoradiometric assay using a fully automatic chemiluminescence immunity analyzer (Uni Cel Dx I 800, Beckman Coulter Inc., USA). All the operations were fulfilled by two experienced professionals according to the manufacturer's instructions (Shanghai Chengtian Medical Instrument Co., Ltd., China). The normal scopes of CA199, CA242 and CA50 were ≤ 39 U/mL, ≤ 15 U/mL and ≤ 25 U/mL. Results which exceeded the upper limit were considered as positive, while results which did not exceed the upper limit were determined as negative.

Flow test cytometry

Flow cytometry was carried out strictly following the procedures shown in Fig. 1.

Statistical analysis

Data were analyzed using SPSS ver. 20.0. Independent sample *t*-test was performed. Enumeration data were expressed as percentage. The comparison between the three groups was performed using analysis of variance. Difference was considered as statistically significant if $P < 0.05$.

RESULTS

Comparison of general data

In the malignant group, there were 62 males and 50 females, aged 38-76 years (average 57 ± 3 years). Ninety healthy people who underwent physical examination in our hospital were selected: 53 males and 37 females, aged 37-78 years (average 57 ± 3 years). One hundred patients, aged 36-77 years (average 54 ± 8 years), who were diagnosed with benign pancreatic disease and hospitalized were selected and set as the benign group. The difference of age and gender between the three groups suggested no statistical significance; therefore, the results were comparable.

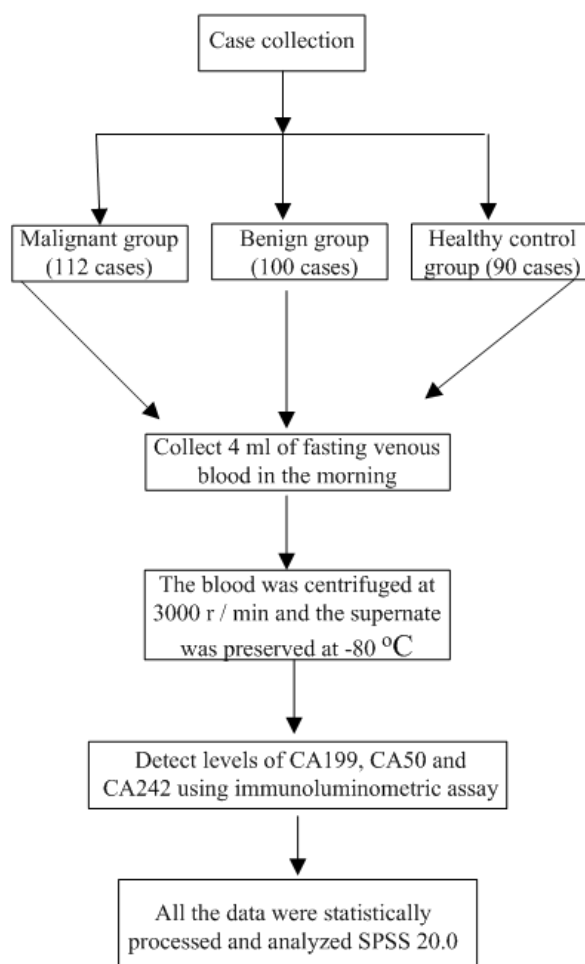


Fig. 1. Test flow chart.

Results of serum CA199, CA50 and CA242 levels of the three groups

The levels of serum CA199, CA50 and CA242 of the malignant group were significantly higher than those of the benign group ($t=15.353$, $P=0.001$; $t=13.252$, $P=0.003$; $t=16.501$, $P=0.000$) and the healthy control group ($t=16.251$, $P=0.000$; $t=14.582$, $P=0.002$; $t=17.239$, $P=0.000$). The differences of CA199, CA242 and CA125 between the benign group and the healthy control group were not statistically significant ($t=1.461$, $P=0.085$; $t=1.674$, $P=0.078$; $t=1.737$, $P=0.076$) (Fig. 2).

Positive expression of CA199, CA242 and CA50 in the three groups

The positive rates of CA199, CA242 and CA50 of the malignant group were higher than those of the benign group ($\chi^2=9.552$, $P=0.006$; $\chi^2=11.217$, $P=0.004$; $\chi^2=10.238$, $P=0.005$) and the healthy control group ($\chi^2=12.547$, $P=0.003$; $\chi^2=15.493$, $P=0.000$; $\chi^2=13.502$, $P=0.000$). The differences of the positive rates of CA199, CA242 and CA50 between the benign group and the healthy control group suggested no statistical significance ($\chi^2=1.963$, $P=0.079$; $\chi^2=1.822$, $P=0.082$; $\chi^2=1.839$, $P=0.081$) (Table I).

Evaluation of single or joint detection of CA199, CA242 and CA50 in the diagnosis of PDAC

The sensitivity of CA199 in the diagnosis of pancreatic cancer was the highest (81.42%) and the specificity of CA242 was the highest (80.14%). In comparison, the sensitivity and specificity of CA50 were low (62.72% and 67.82%). When the three kinds of tumor markers were jointly detected using parallel tests, if the level of any of the three tumor markers was higher than or equal to the reference scope, then it was determined as positive, indicating higher sensitivity (93.24%) and negative predicted value (negative predicted value=true negative/true negative+false negative; 95.20%). When the three tumor markers were detected using serial tests, if the levels of the three tumor markers were higher than or equal to the reference scope, then it was determined as positive, indicating higher specificity (98.53%)

Table I. *Positive expression of CA199, CA50 and CA242 in the three groups.*

	The malignant group (n=112)	The benign group (n=100)	The healthy control group (n=90)
CA199	87(77.68%)	21(21%)	6(6.67%)
CA242	73(65.12)	4(4%)	2(2.22%)
CA50	50(44.64)	5(5%)	3(3.33%)
(n, %)			

Table II. *Efficacy evaluation of single or joint detection of CA199, CA242 and CA50 in the diagnosis of pancreatic cancer.*

	Sensitivity (%)	Specificity (%)	Positive predicted value (%)	Negative predicted value (%)
CA199	81.42%	74.63	57.84	89.87
CA242	74.56	80.14%	69.71	88.62
CA50	62.72%	67.82%	46.26	81.23
CA199+CA242+CA50 (parallel)	93.24%	57.26	43.99	95.2%
CA199+CA242+CA50 (serial)	44.07	98.53%	93.03%	80.46

and positive predicted value (positive predicted value=true positive/(true positive+false positive; 93.03%) (Table II).

DISCUSSION

The detection of tumor markers can be used in the early diagnosis of pancreatic carcinoma. It has been extensively acknowledged that glucolipid, glycoprotein, mucin and cell metabolism secretions

are in close correlation with the occurrence of tumors. The commonly used tumor markers include CA199, CA242 and CA50, which are tumor markers existing on the surface of tumor cell membrane and partially distributed in the tumor markers of normal tissues. CA199 is a Sialyl Lewis antigen and a tumor marker which has been acknowledged universally. CA199 shows a high level in patients with multiple tumors and has the highest level in patients with pancreatic cancer (9, 10). CA242 is a blood group

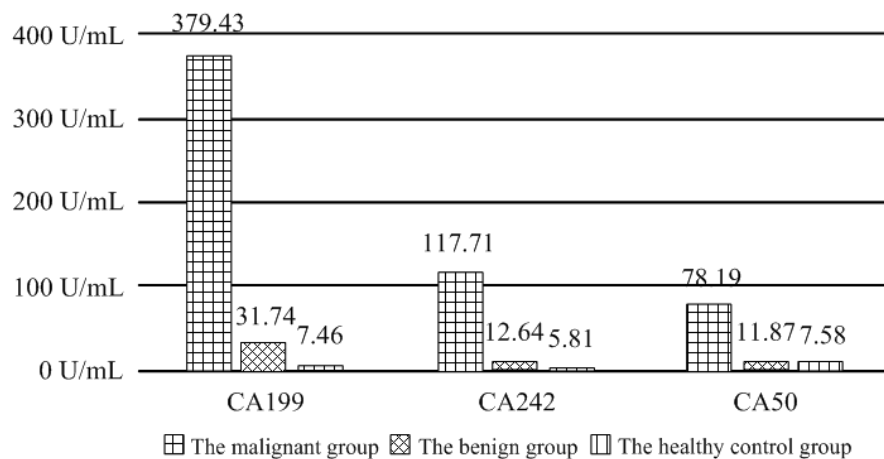


Fig. 2. Comparison of serum CA199, CA242 and CA50 levels between the three groups.

Lewis a antigenic determinant. It has been found that, CA242 had higher specificity compared to CA199 in the diagnosis of pancreatic cancer and it seldom increased in patients with some benign diseases such as pancreatitis, biliary calculi, chronic hepatitis and liver cirrhosis (11). CA50, a broad-spectrum tumor marker, has poor specificity, but indicates a high application value in the diagnosis of pancreatic cancer, and its level alternation can provide a reference for the prediction of the metastasis and recurrence of pancreatic cancer (12).

This study found that the levels of CA199, CA242 and CA50 in the serum of patients with pancreatic cancer were much higher than those of patients who were diagnosed with benign pancreatic cancer. Parallel tests based on joint detection can improve the sensitivity and negative predicted value; because of its low specificity and positive predicted value, it is suitable for the screening of patients with no specific symptoms, and parallel tests based on joint detection can enhance the specificity and positive predicted value, but with low specificity and negative predicted value, which provides an important basis for the confirmed diagnosis of pancreatic cancer.

In conclusion, the joint detection of CA199, CA242 and CA50 can enhance detection efficacy and is effective and valuable to the diagnosis, monitoring and prognosis of pancreatic cancer.

REFERENCES

1. Jemal A, Siegel R, Want E, et al. Deletion of the expression of CD44v6 gene in patients with pancreatic cancer by duplex real time reverse transcription chain assay. *Int J Lab Med* 2011; 32(18):2051.
2. Siegel R, Ma JM, Zou ZH, et al. Cancer statistics, 2014. *CA Cancer J Clin* 2014; 64(1):9-29.
3. Long J, Luo GP, Xiao ZW, et al. Cancer statistics: current diagnosis and treatment of pancreatic cancer in Shanghai, China. *Cancer Lett* 2014; 346(2):273-77.
4. Bünger S, Laubert T, Roblick UJ, Habermann JK. Serum biomarkers for improved diagnostic of pancreatic cancer: a current overview. *J Cancer Res Clin Oncol* 2011; 137(3):375-89.
5. Chu D, Kohlmann W, Adler DG. Identification and screening of individuals at increased risk for pancreatic cancer with emphasis on known environmental and genetic factors and hereditary syndromes. *JOP* 2010; 11(3):203-12.
6. EGTM. Tumor markers in gastrointestinal cancers-EGTM recommendation European group on tumor markers. *Anti-cancer Res* 2009; 19:2811-15.
7. Duffy MJ, Sturgeon C, Lamerz R, et al. Tumor markers in pancreatic cancer: a European Group on Tumor Markers (EGTM) status report. *Ann Oncol* 2010; 21(3):441-47.
8. Chen T, Zhang MG, Xu HX, Wang WQ, Liu L, Yu XJ. Preoperative serum CA125 levels predict the prognosis

- in hyperbilirubinemia patients with resectable pancreatic ductal adenocarcinoma. *Medi (Baltimore)* 2015; 94(19):e751.
9. Szajda SD, Waszkiewica N, Chojnowska S, Zwierz K. Carbohydrate markers of pancreatic cancer. *Biochem Soc Trans* 2011; 39(1):340-43.
 10. Xu HX, Liu L, Xiang JF, et al. Postoperative serum CEA and CA125 levels are supplementary to perioperative CA19-9 levels in predicting operative outcomes of pancreatic ductal adenocarcinoma. *Surgery* 2017; 161(2):373-84.
 11. Ozkan H, Kava M, Cengiz A. Comparison of tumor marker CA242 with CA199 and carcinoembryonic antigen (CEA) in pancreatic cancer. *Hepatogastroenterol* 2003; 50(53):1669-74.
 12. Yang SZ, Ji SQ. Serum and pleural fluid levels of CA50 in malignant and tuberculous effusions. *Respir Med* 1994; 88(1):27-29.

LETTER TO THE EDITOR

CONTINUOUS BLOOD PURIFICATION IN CHILDREN WITH SEVERE SEPSIS

XH. GUO¹, YF. SUN², SZ. HAN¹, J. MIAO¹, M. CUI¹ and JB. WANG¹

¹Department of Pediatrics, Binzhou People's Hospital, Bingzhou, China; ²Department of Oncology, Binzhou People's Hospital, Bingzhou, China

Received November 3, 2016 – Accepted March 27, 2017

This study aims to analyse the clinical effects of continuous blood purification (CBP) in children with severe sepsis to form a basis for CBP application in this context. One hundred and twenty children with severe sepsis treated in Binzhou People's Hospital, Shandong, China, from June 2013 to June 2014 were divided into two groups, a treatment and a control group, depending on parental preference. The control group was treated conventionally, and the treatment group underwent CBP in addition to conventional therapy. Cardiovascular and respiratory parameters were measured on admission and after 72 h. This study also assayed interleukin (IL)-8 and tumour necrosis factor (TNF)- α levels, and monitored clinical outcomes and prognosis. The cardiovascular and respiratory parameters of the treatment group improved to a significantly greater extent than did those of the control group (all $P < 0.05$). After treatment, the levels of IL-8, IL-6, IL-10, and TNF- α declined in both groups, but more so in the treatment group (all $P < 0.05$). The white blood cell count and C-reactive protein level fell more in the treatment than control group, with statistical significance (both $P < 0.05$). CBP remarkably improved the cardiovascular and respiratory functions of children with severe sepsis, probably by eliminating factors mediating inflammation.

To the Editor,

Sepsis is a common childhood disease, and severe sepsis has a high fatality rate, ranging from about 25% to 80% in children with septic shock (1). Sepsis-induced tissue hypoperfusion triggers hypotension, a rise in lactic acid levels, and/or oliguria (2, 3). Severe sepsis is usually associated with changes in cardiovascular and respiratory functions. Currently, conventional clinical treatments include early goal-directed therapy, sepsis bundle therapy, and antibiotic treatment (4). In patients with sepsis, various inflammatory mediators, including tumour necrosis factor (TNF)- α , interleukin (IL)-1, IL-6, IL-8, platelet chemokines, myocardial depressant factor, the high mobility group box-1 protein, and antibodies

against receptors for the anti-inflammatory cytokines IL-10, IL-4, IL-13, and IL-1, may trigger organ dysfunction (5, 6).

The therapeutic indications for continuous blood purification (CBP) have expanded in recent years, and the use of CBP to treat children with severe sepsis has become an important clinical issue (7). CBP was formerly used to treat adult renal failure, intoxication, and electrolyte abnormalities. Over the past few years, CBP has often been reported to be valuable for the prevention and treatment of severe sepsis, reducing the concentrations of inflammatory factors and downregulating the inflammatory response throughout the entire body (8). This study evaluated conventional treatment and CBP in 120

Key words: sepsis, inflammatory factors, continuous blood purification, respiratory function

Mailing address:

Dr Xiaohui Guo, Department of Pediatrics,
Binzhou People's Hospital,
No. 515, Huanghe 7th Road, Binzhou,
Shandong, 256610, China
Tel.: +86 0543 3323481
e-mail: gxhguoxh@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.

children with severe sepsis who were admitted to Binzhou People's Hospital, Shandong, China, and explored the clinical utility of CBP. Thus, whether CBP was curative in children with severe sepsis was determined, in an effort to guide future clinical treatment.

MATERIALS AND METHODS

Subjects

A total of 120 children with severe sepsis, admitted to the paediatric intensive care unit of Binzhou People's Hospital, Shandong, China, from June 2013 to June 2014 were randomly enrolled. Their symptoms met the international sepsis diagnostic criteria (2005) (9). All parents gave written informed consent. The study was approved by the medical ethics committee of Binzhou People's Hospital. According to parental preferences, 54 children constituted the CBP treatment group. In total, 22 children had acute haemorrhagic necrotic enteritis, 18 had bronchopneumonia, 9 had acute peritonitis, and 5 had suppurative meningitis. Furthermore, 17 children exhibited dysfunction of two organs, 23 showed dysfunction of three organs, 9 had dysfunction of four organs, and 5 had dysfunction of five organs. The other children received conventional treatment only. Of the 66 children in the control group, 29 had bronchopneumonia, 17 had acute haemorrhagic necrotic enteritis, 12 had acute peritonitis, 6 had suppurative meningitis, and 2 had urinary tract infections. Finally, 22 children exhibited dysfunction of two organs, 30 showed dysfunction of three organs, 10 had dysfunction of four organs, and 4 had dysfunction of five organs. The demographic characteristics of the two groups did not differ significantly (all $P > 0.05$) (Table I).

Conventional treatment

All children received anti-shock treatment, anti-infection treatment, drugs inhibiting inflammation, mechanical ventilation, and interventions designed to maintain a stable internal environment, in line with sepsis treatment guidelines.

Blood purification

Children in the treatment group underwent CBP; 10 underwent continuous vena-venous hemofiltration (CVVH); 8 underwent CVVH in combination with blood

perfusion; and 5 underwent plasma exchange. Single-lumen needles and double-lumen catheters were implanted into the femoral vein and low-molecular-weight heparin was used as an anticoagulant. Children with bleeding tendencies underwent heparin-free haemodialysis and children in the control group did not receive CBP.

Observations

The mean values of various indicators, prior to and 72 h after treatment, were recorded. Heart rate, respiratory frequency, and blood pressure were all recorded electrocardiographically and the nurses wrote hourly reports. Mean arterial pressure (MAP) was calculated using the following formula: $\text{MAP} = \text{diastolic blood pressure (DBP)} + (\text{systolic blood pressure [SBP]} - \text{DBP})/3$. Arterial oxygen pressure (PaO_2) and arterial oxygen saturation (SaO_2) were also recorded.

The IL-8, TNF- α , IL-6, and IL-10 levels of the two groups were measured by enzyme-linked immunosorbent assays (ELISAs) before and after treatment.

Infection was monitored by measuring the white blood cell (WBC) and C-reactive protein (CRP) levels in peripheral blood.

Statistical analysis

All data were analysed using SPSS software (ver. 20.0; SPSS Inc., Chicago, IL, USA), and are expressed as means $\pm$ standard deviation (SD). Continuous data were compared using the paired samples *t*-test, and categorical data with the *chi*-squared test. A *P* value < 0.05 was taken to reflect statistical significance.

RESULTS

Improvement in cardiovascular function in the two groups

Heart rate, MAP, and blood lactate concentration did not differ significantly between the two groups before treatment ($P > 0.05$). After treatment, the improvements in the treatment group were superior to those in the control group, with statistical significance ($P < 0.05$; Table II).

Improvement in respiratory function

The respiratory rate, PaO_2 , and SaO_2 did not differ significantly between the two groups

Table I. Comparison of general data.

Group	Treatment group	Control group	χ^2/t	P
N	54	66		
Gender	Male	32 (59.3)	1.793	>0.05
	Female	22(40.7)		
Age	≤1 year-old	14(25.9)	0.85	>0.05
	>1 year-old	40(74.1)		
Respirator assisted ventilation	25(46.3)	34(51.5)	3.299	>0.05
Pediatric critical illness score (mean±SD)	75.96±3.50	76.19±2.88	-0.259	>0.05
Number of patients with infectious shock	22(40.7)	32(48.5)	1.949	>0.05

N(%)

Table II. Comparison of the improvement of various cardiovascular function indicators.

Group		Treatment group	Control group	t	P
Heart rate (beat/min)	Before	139.5±29.4	136.7±26.7	0.134	0.716
	After	101.6±18.3	113.3±23.3	3.182	0.003
Mean arterial pressure (mmHg)	Before	74.2±13.2	75.6±11.8	1.322	0.256
	After	84.7±12.5	77.8±21.6	8.321	0.000
Concentration of lactate in venous blood (mmol/L)	Before	4.48±1.37	4.51±1.26	0.156	0.314
	After	2.68±0.86	3.87±0.73	2.311	0.044

(mean±SD)

Table III. Comparison of various respiratory function indicators.

Group		Treatment group	Control group	t	P
Respiration (time/min)	Before	39.5±10.1	38.3±9.2	0.159	0.518
	After	27.6±5.7	33.3±4.3	2.177	0.036
PaO ₂ (mmHg)	Before	44.9±5.2	41.4±3.4	0.167	0.672
	After	92.7±7.5	77.8±11.6	7.998	0.000
SaO ₂ (%)	Before	51.1±11.2	53.2±13.8	0.386	1.414
	After	75.6±7.3	64.3±1.3	3.729	0.001

(mean±SD)

Table IV. Comparison of the changes of levels of IL-8, TNF- α , IL-6 and IL-10.

Group		Treatment group	Control group	t	P
IL-8 (ng/ mL)	Before	2.93±0.44	2.57±0.23	1.367	0.124
	After	1.42±0.2	2.43±0.12	5.121	0.000
NF- α (ng/ mL)	Before	3.29±0.27	3.25±0.25	0.987	0.176
	After	1.14±0.38	2.17±0.11	4.710	0.000
IL-6 (pg/ mL)	Before	119.62±7.68	114.64±10.52	1.582	0.107
	After	80.52±27.91	120.76±7.61	9.978	0.000
IL-10 (pg/mL)	Before	67.82±12.49	68.71±13.17	0.874	0.247
	After	60.22±12.07	64.76±13.99	2.642	0.012

(mean±SD)

before treatment ($P>0.05$). After treatment, the improvements in the treatment group were superior to those in the control group, with statistical significance ($P<0.05$; Table III).

Changes in the IL-8, TNF- α , IL-6, and IL-10 levels of the two groups after treatment

Before treatment, none of the IL-8, TNF- α , IL-6, or IL-10 levels differed significantly between the two groups (all $P>0.05$). After treatment, all levels fell. The improvements in the treatment group were superior to those in the control group, with statistical significance ($P<0.05$; Table IV).

Improvements in WBC and CRP levels

Before treatment, the WBC and CRP levels of the treatment and control groups were 11.93 ± 3.62 ($\times 10^9/L$) vs 12.04 ± 3.73 ($\times 10^9/L$) and 100.83 ± 24.51 (mg/L) vs 101.13 ± 23.92 (mg/L), respectively; the differences were not significant (both $P>0.05$). After treatment, the WBC and CRP levels of the treatment group were 7.11 ± 1.58 ($\times 10^9/L$) and 56.74 ± 13.08 (mg/L), respectively, i.e. there were significantly reduced. These reductions were greater than those in the control group, the respective figures for which were 8.02 ± 1.43 ($\times 10^9/L$) and 74.43 ± 15.87 (mg/L); the differences were statistically significant ($t=2.379$, $P=0.012$; $t=5.432$, $P=0.000$, respectively).

DISCUSSION

CBP improves oxygenation and oxygen transportation, and facilitates oxygen metabolism by eliminating excess water and reducing pulmonary oedema. Joanners-Boyau et al. (10) described 24 septic shock patients with impairment of more than two organs. These patients underwent CBP for 96 h; the ultrafiltration rate was 40–60 mL/(kg·h). The MAP, the cardiac index, and systemic venous resistance index (SVRI) were all improved. Also, after CBP, various indicators of cardiovascular and respiratory function, including heart rate, MAP, and the blood lactate acid level, improved to a much greater extent than in the control group, similar to our findings. Yu et al. (11) treated 16 patients with both sepsis and severe pancreatitis with high-volume

haemofiltration (HVHF) for 72 h and found that the MAP, and the acute physiology and chronic health evaluation scores, improved significantly. Also, the levels of TNF- α , IL-6, and IL-10 decreased significantly. CBP eliminates blood inflammatory mediators; relieves inflammatory reactions; improves capillary permeability, pulmonary respiratory function, pulmonary venous pressure, and pulmonary capillary wedge pressure; and reduces oxygen consumption and gas exchange via HVHF and ultrafiltration. Our results suggest that the levels of inflammatory factors were more reduced in the treatment than in the control group, indicating that CAP continuously filtered or absorbed such mediators, thus mitigating organ injury (12).

In conclusion, CBP is an effective treatment for sepsis, significantly improving the oxygenation capacity, correcting acidosis, and removing inflammatory cytokines. Our work provides the basis for clinical treatment of sepsis; CBP is very useful in this context.

REFERENCES

- Galley HF. Oxidative stress and mitochondrial dysfunction in sepsis. *Br J Anaesth* 2011; 107(1):57-64.
- Tsiotou AG, Sakorafas GH, Anagnostopoulos G, Bramis J. Septic shock; current pathogenetic concepts from a clinical perspective. *Med Sci Monit* 2005; 11(3):RA76-85.
- Goldstein SL, Somex MJ, Baum MA, et al. Pediatric patients with MODS receiving continuous renal replacement therapy. *Kidney Int* 2005; 67(2):653-58.
- Ono J, Yokota K, Fukushima T. Continuous renal replacement therapy for multiple organ failure and multiple organ dysfunction syndrome. *Nihon Rinsho* 2004; 62(Suppl 5):446-49.
- Huang SY, Tang RB, Chen SJ, Chung RL. Serum interleukin-6 level as a diagnostic test in children with sepsis. *J Chin Med Assoc* 2003; 66(9):526-27.
- Latifi SQ, O'Riordan MA, Levine AD, Stallion A. Persistent elevation of serum interleukin-6 in intraabdominal sepsis identifies those with prolonged length of stay. *J Pediatr Surg* 2004; 39(10):1548-52.
- Kornecki A, Tauman R, Lubetzky R, Sivan Y. Continuous renal replacement therapy for non-renal

- indications: experience in children. *Isr Med Assoc J* 2002; 4(5):345-48.
8. Rodrigo E, de-Francisco AL, Escallada R, Ruiz JC, Fresnedo GF, Piñera C, Arias M. Measurement of renal function in pre-ESRD patients. *Kidney Int Suppl* 2002; 80:11-17.
 9. Goldstein B, Giroir B, Randolph A; International Consensus Conference on Pediatric Sepsis. International pediatric sepsis consensus conference: definitions for sepsis and organ dysfunction in pediatrics. *Pediatr Crit Care Med* 2005; 6(1):2-8.
 10. Joannes-Boyau O, Rapaport S, Bazin R, Fleureau C, Janvier G. Impact of high volume hemofiltration on hemodynamic disturbance and outcome during septic shock. *ASAIO J* 2004; 50(1):102-9.
 11. Yu C, Liu ZH, Chen ZH, Gong DH, Ji DX, Li LS. Improvement of monocyte function and immune homeostasis by high volume continuous venovenous hemofiltration in patients with severe acute pancreatitis. *Int J Artif Organs* 2008; 31(10):882-90.
 12. Peng Y, Yuan Z, Li H. Removal of inflammatory cytokines and endotoxin by veno-venous continuous renal replacement therapy for burned patients with sepsis. *Burns* 2005; 31(5):623-28.

LETTER TO THE EDITOR

URINARY ALBUMIN, IMMUNOGLOBULIN G AND BETA-MICROGLOBULIN IN THE DIAGNOSIS OF DIABETES IN THE ELDERLY

J. LI, LM. ZHANG and X. HUANG

¹*Department of Endocrinology, Central Hospital of Zibo, Zibo, China**Received November 24, 2016 – Accepted March 2, 2017*

Diabetes shows no obvious clinical symptoms in the early stage, which makes it difficult to be detected; however, as a common and frequently occurring disease, its morbidity increases with the improvement in the standard of living. Therefore, early detection, diagnosis and treatment of diabetes are of great importance. This study investigated the application values of urinary albumin (Alb), immunoglobulin G (IgG) and Beta-microglobulin (β_2 mG) in the diagnosis of diabetes. Seventy-four elderly patients with confirmed diabetes who were admitted to the Central Hospital of Zibo from May 2014 to May 2015 were taken as an observation group, and fifty normal healthy people were taken as a control group. Levels and positive detection rates of Alb, IgG and β_2 mG in the two groups were compared. The results showed that the levels of Alb, IgG and β_2 mG of the observation group were higher than those of the control group, and the positive detection rates of Alb, IgG and β_2 mG were 74.3%, 62.2% and 67.6%, respectively, which were higher than 6%, 16% and 12% in the control group; the differences were all statistically significant ($P < 0.05$). The difference of the content of Alb, IgG and β_2 mG between patients with different disease courses was statistically significant ($P < 0.05$). Therefore, Alb, IgG and β_2 mG levels are closely related to severity of diabetes, which provides a medical reference to the diagnosis and examination of diabetes and has important clinical values.

To the Editor,

Diabetes, a metabolic disease characterized by hyperglycemia, is caused by insulin deficiency and abnormal secretion and is featured by complicated conditions, long therapeutic course and high morbidity (1, 2). Diabetes frequently occurs among the elderly, and is often associated with high blood pressure, arteriosclerosis, and dyslipidemia (3). The complications, such as fatigue, obesity, polyphagia, polydipsia, polyuria and emaciation, can lower the quality of life of the patients (4). Currently, the pathogenesis of diabetes has not been clarified, and its pathogenic factors include inheritance, age, race and the lifestyle of patients (5, 6). With the aging

of patients and the weakening of immunity, protein, carbohydrate and fat metabolism disorders occur, which severely damage the nerves, blood vessels, heart, liver and kidneys, weakens the functions of organs, induces lesions or failure, thus endangering life (7, 8). Therefore, early and effective diagnosis, and treatment are of great importance in the control of diabetes to can prevent the rising of blood glucose level. A recent study (9) showed that patients with diabetes demonstrated a rising trend of Alb, IgG and β_2 mG levels. To demonstrate the above conclusions, this study detected these levels of elderly patients with diabetes and analyzed their significance in clinical diagnosis.

*Key words: urinary albumin, immunoglobulin G, Beta-microglobulin**Mailing address:*

Dr Jin Li,
Department of Endocrinology,
Central Hospital of Zibo, No. 54, Gongqingtuan West Road,
Zhangdian District, Zibo, Shandong, 255036, China
Tel.: +86 18654350090
e-mail: lijinqra@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.

MATERIALS AND METHODS

General data

Seventy-four elderly patients with confirmed diabetes who were admitted to the Central Hospital of Zibo, Shandong, China from May 2014 to May 2015 were enrolled as an observation group, among whom there were 40 males and 34 females, with ages varying from 58 to 96 years (mean 74.89 ± 7.14 years). Fifty healthy subjects were enrolled as a control group, among whom there were 28 males and 22 females, with ages varying from 54 to 94 years (mean 73.42 ± 7.65 years). All the patients had no history of diabetic complications or drug allergy, and signed informed consent. The difference between the two groups in the general data such as age and gender was not statistically significant ($P > 0.05$).

Specimen collection

All research subjects drank 300 mL of water after urination in the morning; 10 mL of urine was collected from each subject after 1 hour and then in the following 24 hours. Each 100 mL of urine was mixed with 0.5 mL of methylbenzene; the urine collected in the 24 h was mixed, and 2 mL was taken and transferred to a test tube. The upper layer of methylbenzene was abandoned. Then it was centrifuged for 10 min. The supernate was taken as the sample for detection and preserved at -20°C until use.

Detection methods

The detection was performed at room temperature using a Japanese Olympus 400 automatic biochemical analyzer and reagents provided by the China Institute of Atomic Energy with radioimmunoassay, according to the instructions.

Observational indexes and evaluation criteria

Alb, IgG and $\beta_2\text{mG}$ levels were detected and compared between the two groups. The normal scopes of urinary indexes were: Alb < 14.8 mg/L, IgG < 5.45 g/L and $\beta_2\text{mG} < 154$ $\mu\text{g/L}$. If the clinical testing index value was over the normal range, it was determined as positive.

Statistical analysis

Data were analyzed by SPSS ver. 20.0. Measurement data were expressed as mean $\pm$ SD and processed by *t*-test;

enumeration data were expressed as *n* (%) and processed by *Chi*-squared test; $P < 0.05$ meant that the difference was statistically significant.

RESULTS

Comparison of detection rates of diabetes based on Alb, IgG and $\beta_2\text{mG}$ between the two groups

The results showed that the detection rates of diabetes based on the Alb, IgG and $\beta_2\text{mG}$ levels were 74.3%, 62.2% and 67.6%, respectively, in the observation group, which were higher than those of the control group (6.0%, 16.0% and 12.0%); the differences were statistically significant ($P < 0.05$; Table I).

Comparison of Alb, IgG and $\beta_2\text{mG}$ levels between the two groups

The results showed that the Alb, IgG and $\beta_2\text{mG}$ levels of the control group were lower than those of the observation group ($P < 0.05$; Table II).

Relationship between the course of diabetes and detection results of lAb, IgG and $\beta_2\text{mG}$

The seventy-four patients were divided into two groups: disease course less than 10 years ($n = 43$) and no less than 10 years ($n = 31$). With the extension of disease course, the lAb, IgG and $\beta_2\text{mG}$ levels showed a rising tendency, and the positive detection rates of diabetes based on the three indexes increased; the differences between the two groups were statistically significant ($P < 0.05$; Table III).

DISCUSSION

Urine albumin molecules are low in weight, and only a few molecules can penetrate the glomerular filtration membrane normally (10). The molecular weight of IgG is larger than that of albumin; therefore, it is seldom seen in the urine of normal people. When glomerular injury is serious, the level of IgG increases in urine, resulting in nonselective proteinuria (11). $\beta_2\text{mG}$ with small molecular weight can freely penetrate the capillary walls of glomerulus and then enter sacculus cavity to be one component of crude urine; however, $\beta_2\text{mG}$ in filtrate is almost reabsorbed by proximal tubule cells and

Table I. Comparison of detection rates of diabetes based on Alb, IgG and β_2 mG between the two groups[n (%)].

Group	Alb		β_2 mG		IgG	
	Negative	Positive	Negative	Positive	Negative	Positive
Observation group(n=74)	19 (25.7%)	55 (74.3%)*	28 (37.8%)	4 (62.2%)*	24 (32.4%)	50 (67.6%)*
Control group (n=50)	47 (94.0%)	3 (6.0%)	42 (84.0%)	8 (16.0%)	44 (88.0%)	6 (12.0%)

* $P < 0.05$ **Table II.** Comparison of Alb, IgG and β_2 mG levels between the two groups.

Group	Alb (mg/L)	β_2 mG (μ g/L)	IgG (g/L)
Observation group	16.25 $\pm$ 2.57*	212.25 $\pm$ 12.26*	14.24 $\pm$ 3.97*
Control group	7.28 $\pm$ 1.27	107.35 $\pm$ 9.34	5.17 $\pm$ 1.98

* $P < 0.05$ compared to the control group**Table III.** Comparison of the urine protein examination results of patients with different disease courses.

Course of disease	n	Alb (mg/24h)	IgG (mg/24h)	β_2 mG (mg/24h)
<10	43	52.39 (0.10~829.20) [#]	10.19 (0.07~82.08) [#]	1.24 (0.05~6.60) [#]
≥ 10	31	534.13 (4.79~1953.7)	38.14 (3.11~134.87)	2.02 (0.19~8.70)

Note: The values of Alb, IgG, β_2 mG levels were medians, and the values in the bracket refers to the actually measured ranges. [#] indicates $P < 0.05$ compared to the ≥ 10 group.

split into amino acids in the lysosome of the cells, leading to low content in the urine of healthy people. When the brush border membrane of proximal tubule cells is distracted in the process of protein intake or the catabolism of lysosomal proteins reduces, the discharge of β_2 mG in urine increases. Therefore, β_2 mG is a sensitivity index in the detection of early kidney tubules injury (12).

The results of the present study showed that the detection rates of diabetes based on Alb, IgG and β_2 mG levels of the control group were lower than those of the observation group; the differences were statistically significant ($P < 0.05$). It was found that, the Alb, IgG and β_2 mG levels were closely related to diabetes, and the diagnosis based on them suggested high specificity and accuracy, which can provide a

reference to the diagnosis and detection of diabetes.

Furthermore, this study also found that the course of diabetes was connected with the occurrence, development and severity of renal damage. As the disease developed, the disorder of Alb, IgG and β_2 mG levels was increasingly obvious, which indicated that the kidney was gradually damaged. These findings suggest that detection of Alb, IgG and β_2 mG levels through radioimmunoassay is an easy and efficient way to predict the extent of renal damage of patients with diabetics, which is beneficial to the early and accurate determination of renal damage.

REFERENCES

1. RYozai K, Shikata KM, Tone A, et al. Methotrexate prevents renal injury experimental diabetic rats via anti-inflammatory actions. *J Am Soc Nephrol* 2005; 16(11):3326-38.
2. Wu H, Walker J, Damhuis RA, Brewster DH, Wild SH; Scottish Diabetes Research Network Epidemiology Group. Metformin and survival of people with type 2 diabetes and pleural mesothelioma: A population-based retrospective cohort study. *Lung Cancer* 2016; 99:194-99.
3. Zhang SQ, Wang JL, Song CX, Zhu LL, Yu YR. Lower prevalence of proliferative diabetic retinopathy in elderly onset patients with diabetes. *Diabetes Res Clin Pract*. Available online 21 September 2016.
4. Rajpathak SN, Fu CM, Brodovicz K, Engel SS, Heaton PC. Sulfonylurea monotherapy and emergency room utilization among elderly patients with type 2 diabetes. *Diabet Res Clin Pract* 2015; 109(3):507-12.
5. Chien MN, Lee CC, Liu SC, Chen WC, Leung CH, Wang CH. Basal insulin initiation in elderly patients with type 2 diabetes in Taiwan: a comparison with younger patients. *Int J Gerontol* 2015; 9(3):142-45.
6. Chiba Y, Kimbara YY, Kodera R, et al. Risk factors associated with falls in elderly patients with type 2 diabetes. *J Diabet Complications* 2015; 29(7):898-902.
7. Jiang HJ, Guan GJ, Zhang R, Liu G, Liu HY, Hou XH, Cheng J. Increased urinary excretion of orosomucoid is a risk predictor of diabetic nephropathy. *Nephrol* 2009; 14(3):332-37.
8. Shih CJ, Chen HT, Kuo SC, Ou SM, Chen YT. Cardiovascular outcomes of dipeptidyl peptidase-4 inhibitors in elderly patients with type 2 diabetes: a nationwide study. *J Am Med Direct Assoc* 2016; 17(1):59-64.
9. Narita T, Hosoba M, Kakci M, et al. Increased urinary excretions of immunoglobulin, ceruloplasmin, and transferrin predict development of microalbuminuria in patients with type 2 diabetes. *Diabet Care* 2012; 29(1):142-44.
10. Upala S, Wijarnpreecha K, Congrete S, Rattanawon P, Sanguankeo A. Bariatric surgery reduces urinary albumin excretion in diabetic nephropathy: a systematic review and meta-analysis. *Surg Obes Relat Dis* 2016; 12(5):1037-44.
11. Lopes-Virella MF, Hunt KJ, Baker NL, Virella G. High levels of AGE-LDL, and of IgG antibodies reacting with MDA-lysine epitopes expressed by oxLDL and MDA-LDL in circulating immune complexes predict macroalbuminuria in patients with type 2 diabetes. *J Diabet Complications* 2016; 30(4):693-99.
12. Monteiro MB, Thieme K, Santos-Bezerra DP, et al. Beta-2-microglobulin (B2M) expression in the urinary sediment correlates with clinical markers of kidney disease in patients with type 1 diabetes. *Metabolism* 2016; 65(6):816-24.

LETTER TO THE EDITOR

THE RELATIONSHIP BETWEEN PREPTIN, FORKHEAD BOX PROTEIN O1 AND MECHANISTIC TARGET OF RAPAMYCIN LEVELS IN PREDIABETIC PATIENTS

H. ISMAYILNAJADTEYMURABADI and D. KONUKOGLU

*Department of Biochemistry, Istanbul University, Cerrahpasa Medical School, Istanbul, Turkey**Received January 8, 2017 – Accepted April 11, 2017*

Prediabetes is a state of high risk for developing some metabolic disorders. Previous studies have shown that components of some mediators involved in glucose metabolism regulation may have a profound effect during developing prediabetes state. This study investigates the effect of some novel prediabetic-related factors in prediabetes individuals for the first time. Sixty prediabetes (American Diabetes Association criteria) and 25 healthy control subjects were enrolled in the study. Systemic and chronic inflammatory diseases, coronary heart disease, and malignant disease patients were excluded. Anthropometric measurements and fasting glucose, insulin, homeostasis model assessment of insulin resistance (HOMA-IR), lipid profile, preptin, and serum and leukocyte levels of FOXO-1 and mTOR were determined. The findings showed an elevated level of leukocyte mTOR in the Impaired Glucose Tolerance (IGT) group and leukocyte FOXO-1 in the Impaired Fasting Glucose (IFG) and IGT groups compared to the control group. Moreover, higher levels of serum, and leukocyte FOXO-1 in the control group, and leukocyte mTOR level in the IFG group were detected in females compared to males. There was a positive correlation between all of the studied serum parameters, and a positive correlation between basal glucose concentration and leukocyte mTOR and FOXO-1. According to our results, elevated serum and cellular levels of mTOR in the IGT group and FOXO-1 in IFG and IGT groups may be triggered by increased glucose concentration. Indeed, mTOR-mediated variations in cellular level from female patients and FOXO-1-mediated variations of male patients indicated that these factors might play a critical role in glucose intolerance.

To the Editor,

Prediabetes is a serious health problem and a high-risk state associated with diabetes development and cardiovascular complications (1, 2). Prediabetes is a condition of fasting hyperglycemia, IFG and IGT where glycemic parameters are higher than normal but do not reach the diabetes threshold (3, 4). Preptin is a hormone with 34 amino acids derived from proinsulin-like growth factor II (pro IGF-II) which is secreted from pancreatic islet beta-cells and leads to increases in insulin secretion. Preptin also inhibits

liver-mediated glucose production and triggers glucose-dependent insulin secretion. Moreover, Preptin has an anabolic effect on bone metabolism, mimicking the effects of insulin, reducing osteoclast apoptosis, increasing cell differentiation and cell activity in osteoblasts and osteoclasts (5, 6). There seems to be an agreement that there may be an association between obesity, hyperinsulinemia, and insulin resistance with preptin (6, 7). Mechanistic Target of Rapamycin (mTOR) is a cytoplasmic kinase which regulates cell growth and metabolism against

*Key words: prediabetic state, preptin, forkhead box protein 01**Mailing address:*

Dr Hojjat Ismayilnajadteymurabadi,
Cerrahpasa Medical School,
Department of Biochemistry,
Istanbul University, Fatih, 34452 - Istanbul, Turkey
Tel.: +90 (536) 642 0319
e-mail: ismayilnajadh@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.

mitogens, and hormones such as insulin and cytokines (8, 9). Based on previous studies, both glucose and insulin activate mTOR. Thus, high glucose levels activate mTOR, thus hyperinsulinemia itself may lead to insulin resistance. On the other hand, insulin itself activates the mTOR pathway (10). Indeed, glucose-dependent insulin resistance is stimulated by the activation of mTOR. However, in some studies, inhibition of mTOR pathway by rapamycin resulted in an increased risk of diabetes, glucose intolerance and hyperglycemia, and consequently increased insulin resistance (11, 12). To our knowledge, a direct comparison between mTOR and glycemic status in prediabetes patients could be a remarkable result in managing of prediabetic individuals. Outcomes in the context of serum and cellular levels of mTOR have not yet been reported. Forkhead Transcription Factors (FOXO) play an important role in the activity of insulin. This effect can occur particularly by altering gluconeogenesis and glycolytic gene expression in the liver (13). It has been proposed that high levels of reactive oxygen species (ROS) and succeeding oxidative stress are major elements in the development of diabetic complications. The FOXO family of forkhead transcription factors including FOXO-1 has an effective function in regulation of cellular oxidative stress responses. On the other hand, FOXO transcription factors play an important role in the activity of insulin. FOXO1, as an important transcriptional regulator of gluconeogenesis, is also regulated by insulin and has effects on liver lipid metabolism (13).

The aim of the present study was to compare serum levels of preptin and serum and cellular mTOR and Forkhead box protein O1 (FOXO-1) levels, lipid profiles and glycemic control parameters between healthy and prediabetes subjects. To the best of our knowledge, relationships between the three above-mentioned metabolic parameters with insulin may be present or, at least, these parameters may take part in diabetes pathogenesis.

MATERIALS AND METHODS

Sixty patients who were observed for pre-diabetes, at the Cerraphasa medical laboratory, Turkey, from June

2008 to February 2013 and 25 age-matched healthy controls were included in our study. The prediabetic patients were diagnosed according to the American Diabetes Association (ADA, 2008) criteria, including evaluating fasting and second-hour plasma glucose levels after overloading 75 g anhydrous glucose (14). Exclusion criteria were: systemic diseases, previous use of immunomodulatory, antilipidemic, antidiabetic, antihypertensive or antipsychotic medications, history of chronic inflammatory diseases, coronary heart disease, liver and kidney disease, and malignant diseases. The prediabetes patients were further classified into an IFG group and an IGT group according to Oral Glucose Tolerance Tests (OGTT). Blood samples were obtained from all of the participants and collected in 2 sterile tubes, one with the anticoagulant Ethylenediaminetetraacetic acid (EDTA) (for HbA1c analysis, genetic analysis, and detection of protein expression) and sodium fluoride (for glucose analysis), and the other had no anticoagulant (for routine biochemical and hormonal assays and ELISA tests). Routine tests, plasma glucose, and HbA1c levels were assayed in the test.

All subjects provided written informed consent to participate in the study which was approved by the Ethics Committee of the Affiliated Hospital of Istanbul University.

Laboratory examination of regulatory parameters

Plasma glucose, serum cholesterol, serum low-density lipoprotein (LDL)-cholesterol serum, high-density lipoprotein (HDL)-cholesterol, and serum triglyceride were determined using the enzymatic method (Roche Diagnostic, USA). The results were given as mg/dl. Serum insulin levels were assessed by sandwich-electrochemiluminescence immunoassay (ECLIA) using a Cobas e411 analyzer. The results were given as $\mu\text{U}/\text{ml}$. Furthermore, HbA1c levels were assessed by high-performance liquid chromatography (HPLC) (Bio-Rad Variant 2 Turbo). To assess beta-cell function and insulin resistance, homeostatic model assessment (HOMA) was used and HOMA-IR was calculated by formula as glucose in mg/dL, and fasting insulin in $\mu\text{U}/\text{mL}$ units.

WBC isolation and ELISA assay of preptin, mTOR, and FOXO-1

WBC was isolated from whole blood samples of 60

prediabetes cases and 25 control subjects. EDTA tube samples were used for leukocyte isolation. For total leukocyte isolation, phosphate Buffer Solution [(PBS) (pH: 7.4)] and ammonium chloride (0.17 M) solution were used.

Quantitative determinations of serum and leukocyte study parameters were performed using the corresponding commercially available ELISA kits from Sun Red, (China) according to the manufacturer's instructions.

A sandwich enzyme immunoassay for *in vitro* quantitative measurement of preptin in serum (Sun Red, China), mTOR in serum and leukocytes (Sun Red, China), and FOXO-1 in serum and leukocytes (Sun Red, China) were used. Intra and inter-assay CV for preptin, and mTOR preptin were found as 9% and 11%, respectively.

Statistical analysis

Statistical analyses were performed using SPSS software, version 15.0 (SPSS Inc., Chicago, IL, USA). Normal distribution of continuous variables was examined by the Shapiro-Wilk test and One-way ANOVA. In addition, the unpaired student's *t*-test was used for the comparison of meaningful in subgroup variables. Kruskal-Wallis test was used to compare the variables without normal distribution. Bonferroni-corrected Mann-Whitney U test was used to compare the meaningful variables in the subgroup. Pearson correlation coefficient was used to examine the relationship between normal variables with continuous variables, and Spearman correlation coefficient was used to determine the relationships between continuous variables without normal distribution. $P < 0.05$ was considered as a significant level. Regression analysis was performed in order to confirm our findings.

RESULTS

Patient characteristics

According to our results, there was no statistically significant difference between the three examined groups in demographic and clinical characteristics (age, BMI), and metabolic parameters (serum total cholesterol, HDL cholesterol, LDL cholesterol, and triglyceride).

However, HbA1c and 2nd-hour-after plasma glucose levels were significantly elevated in both the IFG and IGT groups compared to the healthy control

group ($P < 0.05$). Furthermore, increased Insulin and HOMA-IR were found in the IFG and IGT groups compared to the control group ($P < 0.05$). Serum insulin and HOMA-IR levels were significantly higher in the IGT group compared to the IFG group ($P < 0.05$).

mTOR, FOXO-1 and preptin levels in subjects with prediabetes and controls

Based on our findings, there was no significant difference in serum Preptin, mTOR, and FOXO-1 in the patient groups compared to the healthy control group ($P > 0.05$). In further analysis, there was a significant difference among the groups in leukocyte FOXO-1 and mTOR levels ($P < 0.05$). These results indicated that in IFG and IGT groups, leukocyte FOXO-1 levels were higher compared to the healthy control group (Table I). In addition, leukocyte mTOR levels was higher in the IGT group.

mTOR, preptin and FOXO-1 levels in gender-based classification

In subgroup analysis, our data showed that serum preptin, serum mTOR, serum FOXO-1 and leukocyte FOXO-1 levels were significantly more increased in females than males in the healthy control group ($P < 0.05$) (Table II). In addition, leukocyte mTOR levels were significantly elevated in females in comparison to males in the IFG group ($P < 0.05$). Likewise, leukocyte FOXO-1 levels statistically increased in the IFG and IGT groups compared to the healthy control group in males ($P < 0.05$). Moreover, leukocyte mTOR levels were elevated in females in the IFG and IGT groups compared to the healthy control group ($P < 0.05$).

Correlation analysis

According to our findings, there was a positive correlation between serum preptin level with serum mTOR ($P < 0.05$), serum FOXO-1 with serum preptin ($P < 0.05$) and serum mTOR with serum FOXO-1 ($P < 0.05$). Furthermore, a positive correlation between basal glucose levels with leukocyte mTOR and FOXO-1 levels, and between 2nd plasma glucose levels with leukocyte mTOR levels were present (Fig. 1). [$r = 0.216$, $P < 0.05$], ($r = 0.258$, $P < 0.05$)

and ($r = 0.320$, $P < 0.05$), respectively)]. Further statistical analysis showed no statistical correlation between other cardio vascular disease risk factor parameters ($P > 0.05$).

Based on our results, in the IFG, control and IGT groups, there was a positive correlation between serum mTOR with serum FOXO-1 ($P < 0.05$), serum FOXO-1 with serum preptin ($P < 0.05$) and serum preptin level with serum mTOR ($P < 0.05$). Regarding the parameters studied (leukocyte parameters in compared to serum parameters) in IFG and control groups, the correlation was not significant. However, in the IGT group, there was a negative correlation between leukocyte mTOR level with serum preptin and serum FOXO-1.

DISCUSSION

According to our results, there were no statistically significant differences in the preptin level between prediabetic patients and the healthy control group. Furthermore, there was no significant difference in preptin levels in gender-based classification analysis. This data suggested that serum preptin levels did not change relatively to the prediabetes state.

In the case of FOXO-1, despite the fact that there was no significant difference between serum FOXO-1

levels between IFG and IGT compared to the control group, leukocyte (cellular) FOXO-1 levels were higher in IFG and IGT groups than those of the control group. However, it is also important to notice that leukocyte FOXO-1 increasing pattern is only found in male patients, which suggests that cellular-related changes of FOXO-1 initiate in early-stage in impaired fasting glycemia males. This elevated FOXO-1 level could be an independent indicator of metabolic variables. Moreover, it could be deduced that cellular FOXO-1 level expression was regulated by different mechanisms in males compared to females.

We also noted that the change in leukocyte FOXO-1 level was independent from serum preptin. In further analysis, there was a weak correlation between basal glucose levels and leukocyte FOXO-1 levels. It could be suggested that cellular FOXO-1 pathways were stimulated by hyperglycemia. This observation supported our hypothesis that glycemic regulation mechanisms could take part in the control of FOXO-1 pathways at cellular level in prediabetic patients. However, it is unclear whether FOXO-1 affects prediabetes state or not.

In our study, mTOR levels were measured in serum and cellular levels. Although there was no statistically significant difference in serum level of mTOR between prediabetes individuals compared to the

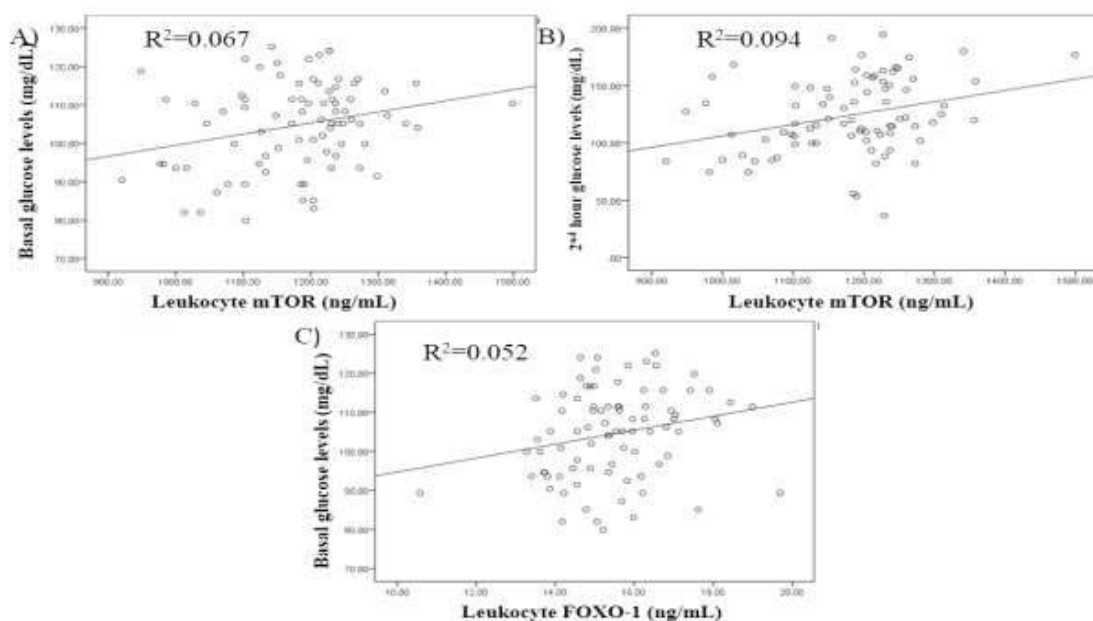


Fig. 1. Correlation graph between basal and 2^h glucose levels and leukocyte mTOR and FOXO-1 in all the groups.

Table I. *mTOR, FOXO-1 and preptin levels in prediabetes subjects and controls.*

	Control	IFG	IGT	p
Serum Preptin	537.0±258.6	481.8±251.5	524.0±244.5	P > 0.05
Serum mTOR	983.7±636.7	998.9±706.3	985.8±651.1	P > 0.05
Serum FOXO-1	10.0±7.6	10.1±7.8	9.9±7.2	P > 0.05
Leukocyte mTOR	1128.8±106.7	1182.6±90.01	1213. ±94.2	P < 0.05
Leukocyte FOXO-1	14.7±1.7	16.0±1.5	15.7±0.8	P < 0.05

IGT = impaired glucose tolerance, IFG = impaired fasting glucose, Data presented as mean ± SD, p<0.05 considered as significant level.

Table II. *mTOR, Preptin and FOXO-1 levels according to gender-based classification.*

	Male	Female	p
control	n = 6	n = 19	
Serum Preptin	355.6 ± 71.5	594.3 ± 270.8	P < 0.05
Serum mTOR	553.6 ± 149.3	1119.5 ± 673.1	P < 0.05
Serum FOXO-1	4.9 ± 2.0	11.6 ± 8.0	P < 0.05
Leukocyte mTOR	1127.5 ± 161.5	1129.3 ± 87.8	P > 0.05
eukocyte- FOXO-1	13.9 ± 0.4	14.9 ± 1.8	P < 0.05
IFG	n = 8	n = 22	
Serum Preptin	380.5 ± 173.0	518.7 ± 268.4	P > 0.05
Serum mTOR	727.4 ± 574.0	1097.6 ± 735.3	P > 0.05
Serum FOXO-1	7.4 ± 6.81	11.1 ± 8.0	P > 0.05
Leukocyte mTOR	1123.4 ± 75.7	1204.1 ± 86.5	P < 0.05
Leukocyte- FOXO-1	16.2 ± 1.3	15.9 ± 1.6	P > 0.05
IGT	n = 15	n = 15	
Serum Preptin	534.3 ± 253.9	513.6 ± 343.2	P > 0.05
Serum TOR	1020.9 ± 685.5	950.7 ± 636.7	P > 0.05
Serum FOXO-1	10.25 ± 7.6	9.5 ± 6.95	P > 0.05
Leukocyte mTOR	1124.6 ± 61.7	1221.4 ± 120.1	P < 0.05
Leukocyte- FOXO1	15.7 ± 0.8	15.6 ± 0.9	P > 0.05

IGT = impaired glucose tolerance, IFG = impaired fasting glucose, P < 0.05 considered as significant level.

healthy control group, in a further subgroup analysis in the prediabetes group, the leukocyte mTOR levels of IGT patients were significantly higher than the control group. Intergroup analysis revealed that in IGT and IFG groups, the leukocyte mTOR levels were higher in females compared to males. In an analysis using data between subgroups, in the IGT and IFG groups higher leukocyte mTOR levels were obtained in a female group compared to controls. The reason for this may be that during fasting hyperglycemia, changes in mTOR-related signaling (in cellular level) initiate earlier in females than males. To our knowledge, there was no study with which to compare our results. In correlation analysis of all studied subjects, there was a positive correlation between serum preptin level with serum mTOR, serum FOXO-1 with serum preptin, serum mTOR with serum FOXO-1. In subgroups correlation analysis, in IFG, control and IGT groups, similar correlation results were obtained. A negative correlation between leukocyte mTOR level with serum preptin and serum FOXO-1 was detected in the IGT group. These correlation analyses results are important as they confirm the evidence based on the hypothesis of interaction between the studied parameters in prediabetes and healthy controls.

However, in backward regression analysis, these correlation results were partially parallel with correlation analysis. Indeed, in total group analysis, when preptin was considered as the dependent variable among other parameters, the effect of serum mTOR on preptin level was confirmed. By considering serum mTOR as the dependent variable, the significant impact of serum preptin and serum FOXO-1 on serum mTOR was obtained. Also, by studying FOXO-1 as the dependent variable, serum mTOR was considered as an effective variable on serum FOXO-1 level. According to our presented data, there is a functional interaction between preptin and mTOR level with FOXO-1. Indeed, it could be suggested that elevated levels of cellular mTOR and preptin restrict the release of FOXO-1 into the circulation.

In conclusion, our findings demonstrate the altered levels of mTOR and FOXO-1 levels during prediabetes state. These changes could be triggered by adaptive response that leads to increased glucose level and diabetic-related complications. According

to our results, changes in the cellular levels of mTOR and FOXO-1 could occur based on gender. Indeed, mTOR-mediated mechanisms in female participants and FOXO-1-mediated mechanisms in male participants could play a primary role in the development of glucose intolerance. However, further studies are necessary in order to conclude a possible association between the gender-related changes in mTOR and FOXO-1 and other related mechanisms.

ACKNOWLEDGEMENTS

We would like to thank the Istanbul University Fikret Biyal laboratory staff for their participation in the survey and supporting our study.

This work was supported by Scientific Research Projects Coordination Unit of Istanbul University. Project Number: 54546.

REFERENCES

1. Bansal N. Prediabetes diagnosis and treatment: A review. *World J Diabetes* 2015; 6(2):296-303.
2. Tabák AG, Herder C, Rathmann W, Brunner EJ, Kivimäki M. Prediabetes: a high-risk state for diabetes development. *The Lancet* 2012; 379(9833):2279-90.
3. Hanna-Moussa A, Gardner MJ, Kurukulasuriya LR, Sowers JR. Dysglycemia/prediabetes and cardiovascular risk factors. *Rev Cardiovasc Med* 2009; 10(4):202-8.
4. Karve A, Hayward RA. Prevalence, diagnosis, and treatment of impaired fasting glucose and impaired glucose tolerance in nondiabetic U.S. adults. *Diabetes Care* 2010; 33(11):2355-59.
5. Buchanan C, Phillips A, Cooper G. Preptin derived from proinsulin-like growth factor II (proIGF-II) is secreted from pancreatic islet beta-cells and enhances insulin secretion. *Biochem J* 2001; 360(Pt 2):431-39.
6. Cornish J, Callon KE, Bava U, et al. Preptin, another peptide product of the pancreatic beta-cell, is osteogenic in vitro and in vivo. *Am J Physiol Endocrinol Metab* 2007; 292(1):E117-22.
7. Ozkan Y, Timurkan ES, Aydin S, et al. Acylated and desacylated ghrelin, preptin, leptin, and nesfatin-1 Peptide changes related to the body mass index. *Int J Endocrinol* 2013; 2013:236085.

8. Blagosklonny MV. TOR-centric view on insulin resistance and diabetic complications: perspective for endocrinologists and gerontologists. *Cell Death Dis* 2013; 4:e964.
9. Roberto Zoncu, David M. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol* 2012; 12(1):21-35.
10. Ueno M, Carnevali JB, Tambascia RC, et al. Regulation of insulin signalling by hyperinsulinaemia: role of IRS-1/2 serine phosphorylation and the mTOR/p70 S6K pathway. *Diabetologia* 2005; 48(3):506-18.
11. Wullschlegel S, Loewith R, Hall M. TOR signaling in growth and metabolism. *Cell* 2006; 124(3):471-84.
12. Tikhanovich I, Cox J, Weinman SA. Forkhead box class O transcription factors in liver function and disease. *J Gastroenterol Hepatol* 2013; 28 Suppl 1:125-31.
13. Ponugoti B, Dong G, Graves DT. Role of forkhead transcription factors in diabetes-induced oxidative stress. *Exp Diabetes Res* 2012; 2012:939751.
14. American Diabetes A. Diagnosis and classification of diabetes mellitus. *Diabetes Care* 2008; 31(Suppl 1):S55-60.

LETTER TO THE EDITOR

DECREASED PLASMA KALLIKREIN 5 CONCENTRATIONS IN PATIENTS WITH CHRONIC SPONTANEOUS URTICARIA

A. GRZANKA¹, A. DAMASIEWICZ-BODZEK², M. SZUMSKA²
and A. KASPERSKA-ZAJAC¹

¹Department of Internal Diseases, Dermatology and Allergology in Zabrze, SMDZ in Zabrze, Medical University of Silesia in Katowice, Poland; ²Department of Chemistry in Zabrze, SMDZ in Zabrze, Medical University of Silesia in Katowice, Poland

Received January 3, 2017 – Accepted April 18, 2017

Little is known about the role of the kallikrein-kinin system in chronic spontaneous urticaria (CSU). Kallikrein 5 (KLK5), a trypsin-like enzyme, is the most abundant in the skin and plays a role in itching and inflammatory reaction. In this study, we determined plasma KLK5 concentration, and its associations with acute phase response in CSU patients. Concentrations of KLK5 in plasma and CRP in serum were measured in patients with CSU of varying severity and in the healthy subjects. Plasma KLK5 concentrations were significantly lower in CSU (all) and moderate-severe CSU patients, as compared with the controls. There were no significant differences in KLK5 concentration in mild CSU patients as compared with the healthy subjects and moderate-severe CSU patients. No correlation was observed between KLK5 and CRP concentrations in the patients. It may be considered that circulating kallikrein 5 is down-regulated in CSU patients, however its potential role and the possible underlying mechanism are unknown.

To the Editor,

The pathogenesis of chronic spontaneous urticaria (CSU) is complex, involving interaction of various immune, infectious, neuroendocrine, metabolic, genetic and environmental factors. The disease is characterized by systemic inflammatory response and activation of coagulation/fibrinolysis system (1-4). Little is known about the role of the kallikrein-kinin system in CSU (5, 6). The system has been implicated in a wide range of processes, including inflammation, coagulation/fibrinolysis, and vessel permeability (7, 8).

This metabolic cascade, when activated by Hageman factor, releases kallikreins (KLKs), the serine proteases, into tissues and plasma, which

triggers the release of kinins from kininogen. KLKs may be directly or indirectly involved in the pathogenesis of different immune-inflammatory diseases (7-9).

KLK5, a trypsin-like enzyme, is the most abundant in the skin (10) and plays a role in itching (11), skin desquamation and inflammatory reaction (11, 12). In this study, we determined plasma KLK5 concentration, and its associations with acute phase response in CSU patients.

MATERIALS AND METHODS

Fifty-eight CSU patients (18 men and 40 women; median age: 38 years, range: 24-52) with a median disease

Key words: kallikrein 5, chronic spontaneous urticaria, acute phase response

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.

duration of 3 years were studied (13). The study comprised 32 mild and 26 moderate-severe CSU patients as well as 22 (7 men and 15 women; median age: 38 years, range: 24-50) healthy subjects (13). The Ethics Committee of the Medical University of Silesia (KNW-640-2-1-004/15) approved the study, and written, informed consent was obtained from all the subjects.

Blood collection

Fasting blood samples were taken from elbow veins, and the plasma stored at -85°C until the tests were performed.

Assay of KLK5

Concentrations of KLK5 in plasma samples were measured by ELISA method using commercially available kits (Quantikine ELISA from R&D Systems, Inc., MN, USA) according to the manufacturers' detailed instructions. The coefficients of variance for intra-assay and inter-assay were below 8%.

CRP assay

Serum C-reactive protein (CRP) concentrations were measured using Roche/Hitachi cobas c system. Normal ranges: $< 5.0 \text{ mg/L}$.

Statistical analysis

Normal distribution was measured using Shapiro-Wilk's test. Independent data between the patient groups and the healthy subjects, and between the patients with mild and moderate-severe CSU symptoms, were compared using non-parametric U Mann-Whitney test. The Spearman's rank test was used for correlations. $p < 0.05$ was considered statistically significant.

RESULTS

Plasma KLK5 concentration

Plasma concentration of KLK5 was significantly lower in the CSU patients as compared with the controls (median: $97.6 \text{ vs } 130.6 \text{ pg/ml}$; $p=0.028$) (Fig. 1).

Plasma KLK-5 concentrations were not significantly lower in female patients as compared with males (median: $81.4 \text{ vs } 102.6 \text{ pg/ml}$; $p=0.061$) in the CSU group. However, plasma KLK-5

concentration was significantly lower in females compared to the males in the control group (median: $115.4 \text{ vs } 141.6 \text{ pg/ml}$; $p=0.044$).

KLK5 plasma concentration was significantly lower in moderate-severe CSU as compared with the controls, but not with mild CSU patients (median: $92.9 \text{ vs } 130.6 \text{ vs } 111.7 \text{ pg/ml}$, $p=0.012$ and $p=0.35$, respectively).

No significant differences were observed in plasma KLK5 concentration between mild CSU patients and the healthy subjects (median: $111.6 \text{ vs } 130.6 \text{ pg/ml}$, $p=0.15$); KLK5 concentrations were similar in ASST(+) and ASST(-) CSU patients (selected according to the similar UAS) (Table I).

Serum CRP concentration

CRP concentration was significantly higher in CSU patients compared to the healthy subjects (median: $3.7 \text{ vs } 0.35 \text{ mg/l}$; $p < 0.0001$). We observed significant differences in serum CRP concentration between patients with mild, moderate-severe CSU symptoms and the controls (median: $1.35 \text{ vs } 9.8 \text{ vs } 0.35 \text{ mg/l}$, respectively; $p < 0.001$) (13).

DISCUSSION

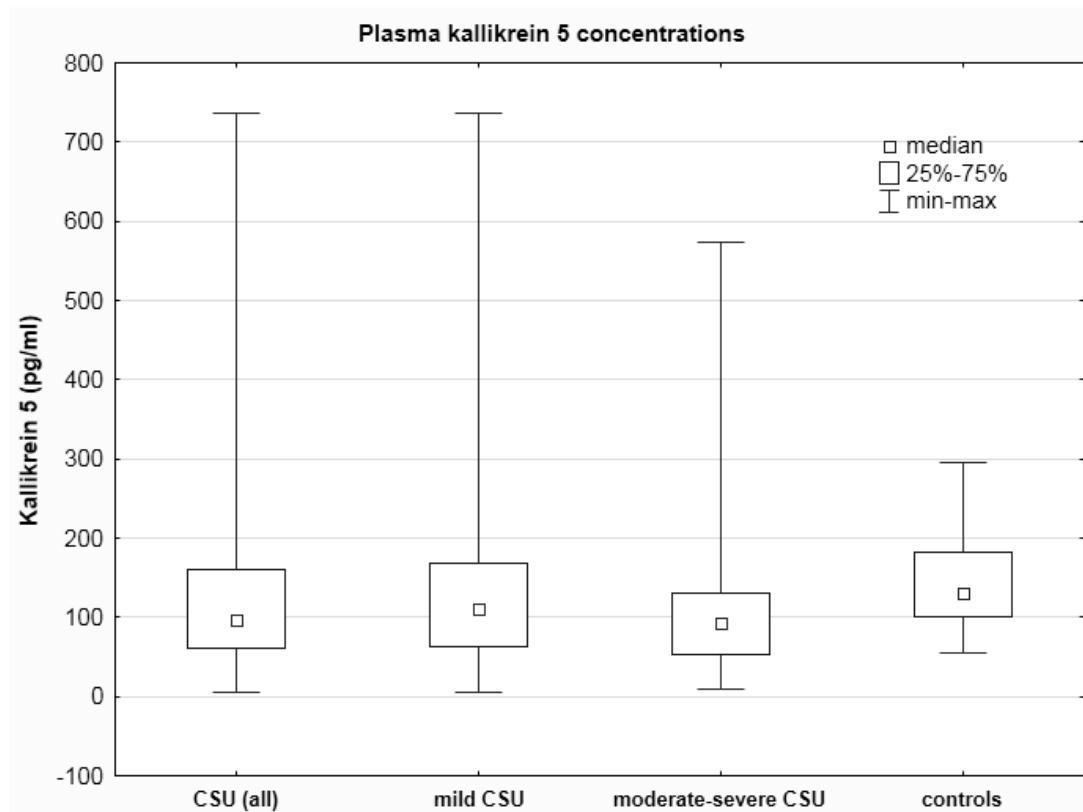
This study is the first one to show abnormal plasma KLK5 concentrations in patients with CSU. Plasma KLK5 concentrations were significantly lower in CSU (all) and moderate-severe CSU patients as compared with the controls. There were no significant differences in KLK5 concentration in mild CSU patients as compared with the healthy subjects and moderate-severe CSU patients. Plasma KLK5 did not correlate with serum CRP concentration, the established biomarker of CSU activity/severity, thus demonstrating that KLK5 provides independent information from acute phase response in CSU.

It may be suggested that circulating KLK5 is down-regulated in CSU patients, however its potential role in urticarial processes is unknown.

Lower circulating KLK5 concentrations in CSU may represent a non-specific inflammatory response, as similar changes were also observed in other inflammatory skin diseases, including psoriasis (14)

Table I. *KLK5 and CRP concentrations.*

Studied parameter mean±SD (95% CIs) (SE) the Shapiro-Wilk's normality test	CSU (all) n=58	mild CSU n=32	moderate-severe CSU n=26	controls n=22
KLK5 (pg/ml)	127.0±119.2 (95.7-158.4) (15.6) p=0.00000	136.3±128.8 (89.8-182.7) (22.8) p=0.00000	115.7±107.5 (72.2-159.1) (21.1) p=0.00000	148.3±64.2 (119.8-176.8) (13.7) p=0.078
CRP (mg/l)	7.9±10,6 (5.1-10.7) (1.4) p=0.00000	1.8±1,1 (1.5-2.2) (0.2) p=0.00019	15.4±2.4 (10.5-20.3) (2.4) p=0.00036	0.3±0.1 (0.3-0.4) (0.03) p=0.387

**Fig. 1.** Plasma KLK5 concentrations were significantly lower in CSU (all) and moderate-severe CSU patients as compared with the controls (* $\square$ < 0.05).

and atopic dermatitis (AD) (15). The concentrations of KLK5 were decreased in these diseases, but some other KLKs were increased as compared with the healthy subjects (14, 15). In addition, the trypsin- and chymotrypsin-like enzymes (KLK1-KLK15) are also differentially expressed in the skin of psoriasis and AD patients, suggesting different roles for members of the KLK family in the diseases (14, 15). Unfortunately, tissue and plasma concentrations of multiple KLKs in CSU patients have not been investigated.

Additional studies, should be performed to determine why CSU patients have lower plasma KLK5 concentration.

The lower circulating KLK5 concentration might result from the dysregulation of the kallikrein-kinin system (rate of synthesis/transformation/consumption of proenzymes, enzymes and/or end products of the cascade). Human plasma KLK5, is synthesized in the liver and circulates bound to high molecular weight kininogen or as a free zymogen. Thus, KLK5 may be expressed/secreted at low levels or may be its enhanced consumption. The amount of KLK5 in plasma might depend on the balance between the inflammatory and antiinflammatory stimuli; we observed lower KLK5 concentration in CSU patients with higher disease activity/severity. Alternatively, binding of KLK5 with serum proteinase inhibitors may lead to complexes that are not recognized by ELISA, similarly to other diseases (8, 16).

It has been demonstrated that KLK5 is partially complexed with alpha(1)-antitrypsin and alpha(2)-macroglobulin (17), which belong to acute phase proteins. On the other hand, it is known that CSU is associated with activation of acute phase response. CRP correlates with the disease severity/activity.

Among the human kallikrein-like peptidase, KLK5 was detected predominantly in the skin (10). KLKs may take part in proteinase-activated receptor-2 (PAR2) activation in the epidermis and thereby in PAR2-mediated inflammatory responses, including epidermal barrier repair and pruritus (18). It should be noted that plasma concentrations of KLK5 might not reflect the skin expression level of the enzyme. The discrepancy between expression in the skin and circulating concentrations have been

described in other diseases, including psoriasis and AD (14, 15).

It is known that expression of KLK5 is hormonally regulated and dependent of gender. It has been demonstrated that KLK5 is up-regulated by estrogens and progesterones in the breast carcinoma cell line (19, 20). Plasma KLK5 concentration differed by gender among the controls, but not in CSU patients, which is in accordance with the previous observation (16).

This discrepancy between gender in the CSU and healthy groups may be attributed to relatively small groups of patients with CSU with different disease activity and individual activity of the acute phase response that results in binding of KLK5, as mentioned above.

Limitations

This study had several limitations. Firstly, the number of patients was relatively small. Secondly, circulating KLK5 concentrations were measured in symptomatic CSU patients. Therefore, we could not determine whether reduced plasma KLK5 concentration was a predictor or consequence of the disease process.

In conclusion, it may be considered that circulating kallikrein 5 is down-regulated in CSU patients, however its potential role and the possible underlying mechanisms are unknown. It seems that proteolytic enzymes, might be involved in the pathogenesis of CSU. Further studies evaluating the expression of members of the KLK family and its association with activation of coagulation/fibrinolysis in CSU may help understanding the role of the kallikrein-kinin cascade in this disease.

ACKNOWLEDGEMENTS

This study was supported by a research grant from the Committee for Scientific Research (KNW-640-2-1-004/15).

REFERENCES

1. Marzano AV, Pigatto P, Cristaudo A, et al. Management of chronic spontaneous urticaria: practical parameters.

- G Ital Dermatol Venereol 2015; 150:237-46.
2. Kasperska-Zajac A, Damasiewicz-Bodzek A, Tyrpien-Golder K, Zamlynski J, Grzanka A. Circulating soluble receptor for advanced glycation end products is decreased and inversely associated with acute phase response in chronic spontaneous urticaria. *Inflamm Res* 2016; 65:343-46.
 3. Kasperska-Zajac A, Grzanka A, Misiolek M, Mazur B, Machura E. Pentraxin-3 as a local inflammatory marker in chronic spontaneous urticaria. *Cytokine* 2015; 76:566-8.
 4. Kasperska-Zajac A, Grzanka A, Machura E, et al. Analysis of procalcitonin and CRP concentrations in serum of patients with chronic spontaneous urticaria. *Inflamm Res* 2013; 62:309-12.
 5. Berrens L, Jankowski E, Jankowski-Berntsen I. Complement component profiles in urticaria, dermatitis herpetiformis, and alopecia areata. *Br J Dermatol* 1976; 95:145-52.
 6. Monteseirín J, Bobadilla P, Galindo PA, Delgado J, Palma JL, Llamas E, Conde J. Plasma kallikrein amidolytic activity in patients with urticaria. *J Investig Allergol Clin Immunol* 1993; 3:142-5.
 7. Moreau ME, Garbacki N, Molinaro G, Brown NJ, Marceau F, Adam A. The kallikrein-kinin system: current and future pharmacological targets. *J Pharmacol Sci* 2005; 99:6-38.
 8. Ponticelli C, Meroni PL. Kallikreins and lupus nephritis. *J Clin Invest* 2009; 119:768-71.
 9. Chao J, Yin H, Gao L, Hagiwara M, Shen B, Yang ZR, Chao L. Tissue kallikrein elicits cardioprotection by direct kinin b2 receptor activation independent of kinin formation. *Hypertension*. 2008; 52:715-20.
 10. Shaw JL, Diamandis EP. Distribution of 15 human kallikreins in tissues and biological fluids. *Clin Chem* 2007; 53:1423-32.
 11. Andoh T, Tsujii K, Kuraishi Y. Increase in pruritogenic kallikrein 5 in the skin of NC mice with chronic dermatitis. *Exp Dermatol*. 2015 doi: 10.1111/exd.12828.
 12. Caubet C, Jonca N, Brattsand M, Guerrin M, Bernard D, Schmidt R, Egelrud T, Simon M, Serre G. Degradation of corneodesmosome proteins by two serine proteases of the kallikrein family, SCTE/KLK5/hK5 and SCCE/KLK7/hK7. *J Invest Dermatol* 2004; 122:1235-44.
 13. Kasperska-Zajac A, Grzanka A, Damasiewicz-Bodzek A. IL-6 transsignaling in patients with chronic spontaneous urticaria. *Plos One* 2015; 10:e0145751.
 14. Komatsu N, Saijoh K, Kuk C, Shirasaki F, Takehara K, Diamandis EP. Aberrant human tissue kallikrein levels in the stratum corneum and serum of patients with psoriasis: dependence on phenotype, severity and therapy. *Br J Dermatol* 2007; 156:875-83.
 15. Komatsu N, Saijoh K, Kuk C, Liu AC, Khan S, Shirasaki F, Takehara K, Diamandis EP. Human tissue kallikrein expression in the stratum corneum and serum of atopic dermatitis patients. *Exp Dermatol* 2007; 16:513-9.
 16. Planque C, Li L, Zheng Y, et al. A multiparametric serum kallikrein panel for diagnosis of non-small cell lung carcinoma. *Clin Cancer Res* 2008; 14:1355-62.
 17. Yousef GM, Kapadia C, Polymeris ME, Borgono C, Hutchinson S, Wasney GA, Soosaipillai A, Diamandis EP. The human kallikrein protein 5 (hK5) is enzymatically active, glycosylated and forms complexes with two protease inhibitors in ovarian cancer fluids. *Biochim Biophys Acta*. 2003; 1628:88-96.
 18. Stefansson K, Brattsand M, Roosterman D, Kempkes C, Bocheva G, Steinhoff M, Egelrud T. Activation of proteinase-activated receptor-2 by human kallikrein-related peptidases. *J Invest Dermatol* 2008; 128:18-25.
 19. Diamandis EP, Yousef GM, Luo LY, Magklara A, Obiezu CV. The new human kallikrein gene family: implications in carcinogenesis. *Trends Endocrinol Metab* 2000 11:54-60.
 20. Yousef GM, Diamandis EP. The new kallikrein-like gene, KLK-L2. Molecular characterization, mapping, tissue expression, and hormonal regulation. *J Biol Chem* 1999; 274:37511-6.

LETTER TO THE EDITOR

EXPRESSION OF INFLAMMATORY CYTOKINES IN OVARIAN EPITHELIAL CANCER AND THEIR CORRELATION

YJ. TANG^{1,2}, HT. DOU³, QH. ZHANG³, JJ. LIU⁴ and XS. YANG⁵¹Department of Obstetrics and Gynecology, Qilu Hospital, Shandong University, Shandong, China;²Department of Obstetrics and Gynecology, Zibo Central Hospital, Shandong, China; ³Department of Obstetrics and Gynecology, Zibo Central Hospital, Shandong, China; ⁴Department of Obstetrics and Gynecology, Binzhou Medical School Affiliated Hospital of Yantai, Shandong, China;⁵Department of Obstetrics and Gynecology, Qilu Hospital, Shandong University, Shandong, China*Received March 22, 2017 – Accepted April 12, 2017*

This study investigates the expression of interleukin (IL)-17 (IL-17A and IL-17D) and its role in ovarian epithelial tumor and its correlation with the clinical characteristics. Fifty malignant ovarian epithelial tumor specimens were collected after surgery and set as group A. Ten benign epithelioma specimens and ten normal epithelial specimens were set as group B and C, respectively. The expression of IL-17A and IL-17D in the three groups was detected using the EnVision method, and the correlation of positive expression rate with pathological type, clinical stage, presence of ascites and differentiation degree was analyzed. Group A had higher expression of IL-17A and IL-17D compared to group B and C ($p < 0.05$). The expression rate was in correlation to clinical stage and differentiation degree, but in no correlation to pathological type and presence of ascites. The expression rate of malignant epithelioma in the early stage was lower than that in the late stage ($p < 0.05$). Lower differentiation degree indicated higher expression rate ($p < 0.05$). Pathological type and presence of ascites had no remarkable influence on the expression rate ($p > 0.05$). Study of the expression of IL-17 provides theoretical support for the clinical treatment and prognosis prediction of ovarian epithelial tumor.

To the Editor,

Ovarian tumor is a disease that can severely threaten the health of females, and epithelial ovarian tumor (1, 2) is the most common type. In recent years, many studies have confirmed the close correlation between interleukin (IL)-17 and tumor, but its specific role in the development process of tumor is controversial. IL-17 (3) is an inflammatory cytokine produced by activated T cells, and its family members include IL-17A~IL-17F. Currently, many experts and scholars have

studied the influence of IL-17 on tumor. Kato et al. (4) detected the expression of IL-17 mRNA through real-time Polymerase Chain Reaction (RT-PCR) and investigated its correlation with microvessel density of patients with ovarian carcinoma. Miyahara et al. (5) reported the increased expression of CD4+ T cells producing IL-17 within tumor environment. Wu et al. (6) investigated the increased expression of IL-17A in human gastric cancer and its potential roles in gastric carcinogenesis. Cappello et al. (7) studied the roles of IL-17 and Th17 in the carcinogenesis of

Key words: inflammatory cytokines, ovarian epithelial tumor, IL-17A, IL-17D, expression, correlation

Mailing address:

MD Xingsheng Yang,
Department of Obstetrics and Gynecology,
Qilu Hospital,
Shandong University,
No. 107, Wenhua West Road, Jinan, Shandong, 250012, China.
e-mail: xingsheng6615@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.

pancreatic ductal adenocarcinoma and anti-tumor responses.

Taking IL-17A and IL-17D as examples, this study investigated the expression of IL-17 in ovarian epithelial tumor and its correlation with various clinical characteristics and found they were highly expressed, and tumors with higher clinical stage suggested lower differentiation and higher positive expression rate, which is valuable to the malignancy determination and prognosis prediction. Study of the expression of IL-17 in ovarian epithelial tumor can provide a theoretical support for the clinical treatment and prognosis prediction.

MATERIALS AND METHODS

Research subjects

Fifty postoperative tissue specimens were collected from the malignant ovarian epithelial tumor patients admitted to the Department of Gynecology of Qilu Hospital of Shandong University, China between January 2013 and December 2015. They were all diagnosed as malignant ovarian epithelial tumor by physical examination and had not previously undergone chemoradiotherapy or biotherapy.

Moreover, ten normal ovarian tissue specimens and ten benign ovarian epithelial tumor specimens were collected and taken as controls. All the specimens were washed, fixed, embedded by paraffin, and sliced into pathological sections. All the sections were confirmed as malignant ovarian epithelial tumor tissues by a professional pathologist after being processed by hematoxylin-eosin (HE) staining. The general data, clinical information, pathological data and invasion depth of each patient were recorded in detail. All the patients and their family members gave informed consent to participate in the study.

Main reagents

The main reagents included sterile Dulbecco's modified eagle medium (HyClone, US), rabbit anti-human polyclonal antibody (Santa Cruz, USA), IL-17D antibody (R & D Systems, USA), mouse anti-human monoclonal antibody (Shanghai Institute of Enzyme-linked Biology, China), anti-mouse second antibody immunohistochemical kit (Vector, USA), DAB color development kit (Wuhan Boster Bioengineering Co., Ltd., China), APES (Union Carbide Corporation, USA) and

citrate buffer (Shanghai Rongbo Biotechnology Co., Ltd., China).

Main instruments

The main instruments included optical microscope (Olympus, Japan), cell culture plate (Corning, USA), freezing microtome (Leica, Germany), slide drier (Leica, Germany), pathological image analyzer (Leica, Germany), electronic analytical balance (Mettler, Switzerland), table model high-speed centrifuge (Hettich, Germany), tissue embedder (Sakura, Japan), flow cytometry (Beckman Coulter, USA), low-temperature refrigerator (Thermo, USA), bechtop (Esco Micro Pte Ltd, Singapore) and vacuum negative pressure drying oven (Shanghai Lanhao Instrument Co., Ltd., China).

Experimental methods

Immunohistochemical EnVision method (8) was adopted. IL-17A was taken as the primary antibody, and IL-17D was taken as the second antibody. Phosphate buffer saline (PBS) was taken as the negative control. The sections with known positive antigen were taken as positive controls.

Section preparation

The glass slides used for immunohistochemistry (9) were immersed in strong acid. Forty-eight hours later, they were taken out, rinsed repeatedly, immersed in 95% ethyl alcohol for 2 h, dried, and immersed in APES for 5 min, to effectively prevent slide flaking. Then they were immersed in absolute ethyl alcohol and dried at room temperature. The tissue specimens were collected from the most diseased site of the tumor tissues. After fixation and dehydration, they were immersed in a mixture of absolute ethyl alcohol and xylene, followed by xylene. The operation was repeated after xylene was changed. Then the specimens were embedded using a tissue embedding machine. The embedded paraffin specimens were cut into 4- μ m thick slices using a freezing microtome, laid horizontally on the glass slides and dried.

Dewaxing

The sections were immersed in xylene for 5-15 min. The procedure was repeated after xylene was changed. Then they were immersed with 100% ethyl alcohol twice, 5 min each time, with 90% ethyl alcohol twice, 2 min each time,

with 80% and 70% ethyl alcohol, once each, 2 min each time, washed by distilled water, and dewaxed. To eliminate the activity of endogenous peroxidase, they were incubated with 3% H₂O₂. After 10-min incubation, they were washed by distilled water and PBS.

Antigen retrieval

Vacuum negative pressure antigen repair method (10) was used. The dewaxed sections were processed by 0.3% H₂O₂ methyl alcohol using vacuum negative pressure. After being washed by distilled water, the sections were added with trace amount of citrate buffer and dried in a vacuum negative pressure drying oven whose temperature was preset at 10 min. After the temperature of the repair liquid recovered to 25°C, it was immersed in PBS.

Antibody and color development with DAB

Firstly, 50 µl of diluted primary antibody was added. Glass slides were preserved at 0~5°C overnight, then placed at 25°C for 1 h, and washed by PBS. Second antibody was added twice, 50 µl each time. Then it was placed at 25°C for 1 h and washed by PBS. After that, color was developed using a DAB color development kit. The color development time was strictly controlled. Then it was observed under an optical microscope, and the positive cells were brown.

Staining

After color development with DAB reagent, the sections were redyed with hematoxylin for 5 to 10 min (11). Then they were washed by distilled water; the color was separated by hydrochloric acid alcohol. After being washed by water, the sections were observed under an optical microscope, and then stained with 0.3% eosin for 5 min. Several drops of glacial acetic acid were added to accelerate staining. After being washed by distilled water, the sections were dehydrated gradiently in a conventional way. After dehydration, the sections were washed by ethyl alcohol in different concentrations and processed by transparency disposal using xylene. The residual solution was removed, and the sections were mounted.

Microscopic examination and statistical processing

The slides were examined, analyzed and determined under a microscope. The orientation of positively expressed signals of target proteins was observed. The results were determined using a double-blinded method (12) to avoid

extra variables induced by subjective expectation and enhance accuracy. The experimental data were processed by SPSS. For the immunohistochemical results, non-parametric Kruskal-Wallis H test was performed on multiple independent samples (13). Difference was considered as statistically significant if $p < 0.05$. Spearman correlation coefficient analysis (14) was also used.

RESULTS

The expression of IL-17 in epithelial ovarian tumor

The specimens collected from malignant epithelial ovarian tumors were set as group A; the specimens from benign epithelial ovarian tumors were set as group B; the specimens from normal ovarian tissues were set as group C.

Expression of IL-17A

Upon testing, it was found that IL-17A was located in cytoplasm and turned brownish yellow after staining. IL-17A indicated a high positive expression in group A and nearly no expression in groups B and C. The immunohistochemical results demonstrated that the positive expression of IL-17A was different in groups A, B and C, 62%, 19% and 8%, respectively. Obviously, group A had higher positive expression rate than groups B and C, and the difference had statistical significance ($p < 0.05$); but no remarkable difference was observed between groups B and C ($p > 0.05$).

Expression of IL-17D

Through detection, it was found that IL-17D was located in the cytomembrane and cytoplasm and turned light yellow after staining. As shown in Fig.1, IL-17D was highly expressed in group A and nearly had no expression in groups B and C. The immunohistochemical results demonstrated that the positive expression rate of IL-17D was 75%, 42% and 0% respectively in groups A, B and C; the difference of group A with group B and C was statistically significant ($p < 0.05$); but there was no difference between group B and C ($p > 0.05$).

Correlation study

According to the immunohistochemical results,

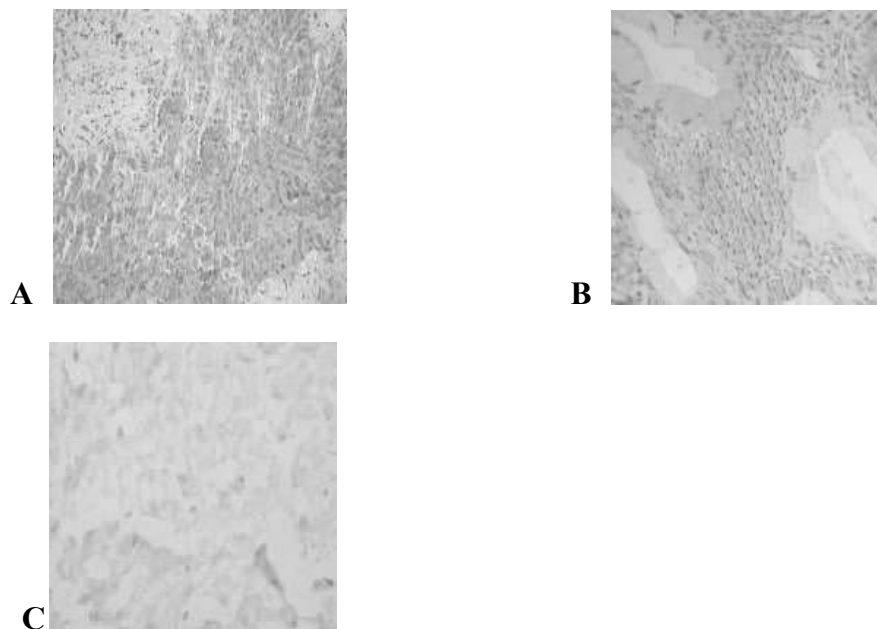


Fig. 1. The expression of IL-17A in group A (A), group B (B) and group C (C) (X200).

the sections from 50 cases of malignant epithelial ovarian tumor were divided into IL-17A positive expression group, IL-17D positive expression group and a negative group. The correlation between the expression of IL-17A and IL-17D tissues and various clinical characteristics was statistically analyzed.

Ovarian epithelial tumor can be classified as serous ovarian tumor, mucinous ovarian tumor and endometrioid tumor according to pathological types. The number of cases of serous ovarian tumor was 25% that of benign ovarian tumors, and most of the tumors are unilateral. Serous ovarian tumors with smooth surface are different in size and irregular in shape and can be observed with light yellow clear serous fluid. Serous ovarian tumor is divided into simple serous ovarian tumor and papillary serous ovarian (15). Mucinous ovarian tumor, also called mucinous cystadenoma, has an incidence of 20% among benign ovarian tumors, and most of the tumors are unilateral; they are featured by grey-white surface, large volume, multilocular section and different-size cyst cavity. The jellylike cyst fluid of mucinous ovarian tumor contains proteins and glycoprotein. The malignancy probability of mucinous ovarian tumor is around 10% (16). Endometrioid tumor is induced

by hydronephrosis and hydroureter or renal atrophy caused by metrorrhagia and ureter compression and manifests as anemia, emaciation, fever and dyscrasia (17). In this study, there were 27 cases of serous ovarian tumor, 10 cases of mucinous ovarian tumor and 13 cases of endometrioid tumor. The positive expression rate of IL-17A in the serous ovarian tumors, mucinous ovarian tumors and endometrioid tumors was 66.7%, 60% and 53.8% respectively, while the positive expression rate of IL-17D was 66.7%, 70% and 92.3% respectively. The statistical analysis suggested no significant difference in the positive expression rate and pathological type between IL-17A and IL-17D ($p > 0.05$).

There were 16 cases of absence of ascites or ascetic volume no more than 500 ml and 34 cases of ascetic volume more than 500 ml. Through detection before surgery, it was found that the positive expression rate of IL-17A was 56.3% in the group with no ascites or ascetic volume no more than 500 ml and 61.8% in the group with ascetic volume more than 500 ml. The positive expression rate of IL-17D was 68.8% in the group with no ascites or ascetic volume no more than 500 ml and 79.4% in the group with ascetic volume more than 500 ml. The difference between them had

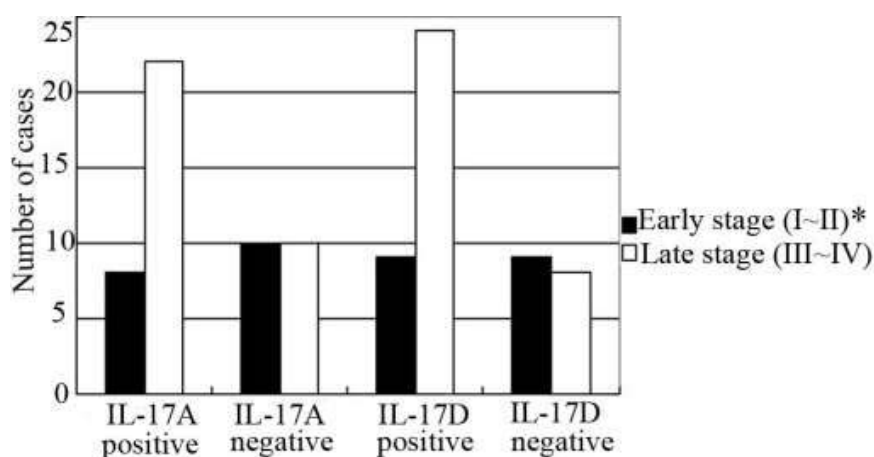


Fig. 2. The correlation between the expression of IL-17A and IL-17D in malignant ovarian epithelial tumors and clinical stages ($p < 0.05$ compared to tumor at the late stage).

no statistical significance ($p > 0.05$).

As shown in Fig. 2, the clinical stages of ovarian epithelial tumor can be divided into early stage (I ~ II), ($n = 18$) and late stage (III ~ IV) ($n = 32$). The positive expression of IL-17A and IL-17D in tumors with different clinical stages were 44.4% and 50%, respectively, in tumor at early stage and 68.8% and 75%, respectively, in tumor at late stage. It was found that the positive expression rate in tumor at early stage was lower than that at late stage, and the difference had statistical significance ($p < 0.05$). Malignant ovarian epithelial tumor was further classified according to differentiation degree, the positive expression rates of IL-17A in lowly differentiated tissues and moderately and highly differentiated tissues were 90.3% and 57.3%, respectively, while those of IL-17D were 91.1% and 61.6%, respectively. Thus it could be concluded that the positive expression rates of IL-17A and IL-17D in lowly differentiated tissues were higher than those in moderately and highly differentiated tissues, and the differences had statistical significance ($p < 0.05$). The positive expression of IL-17A and IL-17D was in a correlation with clinical stage and differentiation degree.

DISCUSSION

IL-17, the major effector molecule of TH17, is generated by proinflammatory cytokines secreted by activated T cells (18). The tumor immunity of IL-

17A in ovarian epithelial tumor tissues is weak, and its effect in counteracting tumors is also weak (19). Gulubova et al. (20) considered that IL-17 could resist tumor through inducing multiple cytokines. The results of this experiment demonstrated that IL-17A and IL-17D had high positive expression rates in malignant ovarian epithelial tumor, but almost had no expression in benign ovarian epithelial tumor and normal ovarian tissues, and its promotion effect on tumors was stronger than the immunoreaction. The further study suggest tumors with higher clinical stage had lower differentiation and higher expression rates of IL-17A and IL-17D, indicating IL-17A and IL-17D could be regarded as the evaluation criteria for the determination of biological behaviours of ovarian epithelial tumor and prognosis prediction. Compared to other methods, the experimental method was easy to carry out and obtained more accurate results, which provides certain assistance to the clinical treatment of ovarian epithelial tumor; however, further exploration is needed as this study also had deficiencies.

In conclusion, the investigation on the expression of IL-17 in ovarian epithelial tumor and its correlation with various clinical characteristics can provide an effective basis for the diagnosis and the malignancy determination of ovarian epithelial tumor.

REFERENCES

1. Chen XD. Common pathological diagnosis problems

- of ovarian epithelial tumor and clinical significance. *J Pract Obstetr Gynecol* 2015; 31(11):808-10.
2. Tural D, Serdengeçti S, Demirelli F, et al. Clinical significance of p95HER2 overexpression, PTEN loss and PI3K expression in p185HER2-positive metastatic breast cancer patients treated with trastuzumab-based therapies. *Brit J Cancer* 2014; 110(12):1968.
 3. Maione F. Commentary: IL-17 in chronic inflammation: from discovery to targeting. *Front Pharmacol* 2016; 7:250.
 4. Kato T, Furumoto H, Ogura T, et al. Expression of IL-17 mRNA in Ovarian Cancer. *Biochem Biophys Res Comm* 2001; 282(3):735-38.
 5. Miyahara Y, Odunsi K, Chen W, et al. Generation and regulation of human CD4+ IL-17-producing T cells in ovarian cancer. *Proc Nat Acad Sci* 2008; 105(40):15505-10.
 6. Wu X, Zeng Z, Xu LX, et al. Increased expression of IL17A in human gastric cancer and its potential roles in gastric carcinogenesis. *Tumor Biol* 2014; 35(6):5347-56.
 7. Cappello P, Curto R, Mucciolo G, Rolla S, Tonoli E, Novelli F. Abstract 3170: IL17 favours carcinogenesis and limits the anti-tumor responses in pancreatic cancer. *Cancer Res* 2015; 75(15 S):3170-70.
 8. Sugita S, Aoyama T, Kondo K, et al. Diagnostic utility of NCOA2 fluorescence in situ hybridization and Stat6 immunohistochemistry staining for soft tissue angiofibroma and morphologically similar fibrovascular tumors. *Human Pathol* 2014; 45(8):1588-96.
 9. Massi D, Simi L, Sensi E, et al. Immunohistochemistry is highly sensitive and specific for the detection of NRASQ61R mutation in melanoma. *Mod Pathol* 2014; 28(4):487-97.
 10. Ju XP. Application of different antigen retrieval methods in immunohistochemical staining. *Chin Health Stand Manag* 2015; (14):174-75.
 11. Chan JK. The wonderful colors of the hematoxylin-eosin stain in diagnostic surgical pathology. *Int J Surg Pathol* 2014; 22(1):12-32.
 12. Törökné AK, László E, Chorus I, Sivonen K, Barbosa FAR. Cyanobacterial toxins detected by Thamnotoxkit (a double blind experiment). *Environm Toxicol* 2015; 15(5):549-53.
 13. Qi MP, Shen XP. The realization of mean rank's post hoc multiple comparisons of Kruskal-Wallis H test by using SPSS software. *J Lanzhou Higher Polytech Coll* 2015; (2):76-78.
 14. Sedgwick P. Spearman's rank correlation coefficient. *BMJ* 2014; 349(1):7327-27.
 15. Sayhan S, Diniz G, Karadeniz T, Ayaz D. Expression of caveolin-1 in peritumoral stroma is associated with histological grade in ovarian serous tumors. *Ginek Pol* 2015; 86(6):424-28.
 16. Zeppernick F, Meinhold-Heerlein I, Iem S. Precursors of ovarian cancer in the fallopian tube: serous tubal intraepithelial carcinoma--an update. *J Obstetr Gynaecol Res* 2015; 41(1):6-11.
 17. Wu CH, Mao TL, Vang R, Ayhan A, Wang TL, Kurman RJ, Shih IeM. Endocervical-type mucinous borderline tumors are related to endometrioid tumors based on mutation and loss of expression of ARID1A. *Int J Gynecol Pathol Offic J Int Soc Gynecol Pathol* 2012; 31(4):297.
 18. Shimizu J, Takai K, Takada E, et al. Possible association of proinflammatory cytokines including IL1 β and TNF α with enhanced Th17 cell differentiation in patients with Behcet's disease. *Clin Rheumatol* 2016; 35(7):1857-63.
 19. Wang G, Gu XL, Chen L, Wang YM, Cao B, E Q. Comparison of the expression of 5 heat shock proteins in benign and malignant salivary gland tumor tissues. *Oncol Lett* 2013; 5(4):1363-69.
 20. Gulubova M, Ananiev J, Ignatova M, Halacheva K. Pro-tumor and anti-tumor functions of IL-17 and of TH17 cells in tumor microenvironment. *Acta Medica Bulg* 2016; 43(2):68-79.

LETTER TO THE EDITOR

**IN VITRO ANTIOXIDANT POTENTIAL
OF SELECTED APHRODISIAC MEDICINAL PLANTS**M. RIAZ¹, M. SHAHID¹, A. JAMIL¹ and M. SAQIB²¹Department of Biochemistry, University of Agriculture, Faisalabad-Pakistan; ²Department of Clinical Medicine and Surgery, University of Agriculture, Faisalabad-Pakistan

Received March 31, 2017 – Accepted April 26, 2017

The present study aimed to evaluate the antioxidant activity of six selected aphrodisiac medicinal plants. Useful parts of the selected medicinal plants were collected and extracted in methanolic solvent. The antioxidant activity of selected plant extract was determined through different antioxidant assays, namely DPPH radical scavenging assay and ferric reducing antioxidant assay. Moreover, antioxidant compounds, like total phenolics and total flavonoids contents, were also determined. Results showed that *Mucuna pruriens* seed extract displayed high contents of phenolic compounds with total phenolic content of 683.15 ± 4.28 mg GAE/g dry plant material while the least phenolic content was observed in *Asparagus racemosus* (195.5 ± 3.02 mg GAE/g dry plant material). Highest total flavonoids content was found in *Anacyclus pyrethrum* roots (156.58 ± 4.01 µg CE/g) and the least content was found in *Asparagus racemosus* roots. Among the studied plant extracts, the highest radical scavenging activity was shown by *Mucuna pruriens* seed extract (82.05 ± 0.55 %) and the least percent scavenging activity was observed in *Tribulus terrestris* extract (36.40 ± 2.01 %). Vitamin C was used as positive control for antioxidant assays showing 93.54 ± 0.95 % percent radical scavenging activity. The plant extract also exhibited a strong reducing potential against free radicals. Therefore, the present study concluded that all the studied medicinal plants possess varying concentrations of secondary active metabolites responsible for the antioxidant properties of the tested plant extracts.

To the Editor,

Aphrodisiacs are substances with potential to boost the couple's aspiration toward reproduction. The bioactive aphrodisiacs derived from plants and animals have fascinated mankind throughout recorded history. Sexual interactions are the pivotal biological and social link in human life. The central nervous system is involved in controlling and regulating sexual desire through integration of olfactory, tactile and mental stimuli (1). Some medicinal plants have been reported to have aphrodisiac potential that can enhance sexual activities and libido (2). Free radicals are produced as a result of oxidation reactions which start chain

reactions and subsequently can cause cellular damage leading to a large number of diseases in humans (3). Antioxidants are involved in preventing cellular damage which is the common pathway for a variety of diseases, such as cancer, aging and infertility. Vital sources of natural antioxidants are medicinal plants containing many bioactive antioxidant compounds such as flavonoids and polyphenols (4).

Asparagus racemosus, an aphrodisiac medicinal plant from the *Liliaceae* family possesses antioxidant properties due to the presence of active phytoconstituents like Isoflavons, particularly racemofuran, asparagamine A and racemosol (5).

Key words: antioxidant, phenolics, flavonoids, aphrodisiac

Mailing address:

Dr Riaz Muhammad
Department of Biochemistry,
University of Agriculture,
Faisalabad, 38040-Pakistan
e-mail: riazmlt786@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.**

Mucuna pruriens (Fabaceae family) is a well-known herbal drug used for the management of a variety of disorders including male infertility, nervous disorders and also as an aphrodisiac. The seeds of this plant are of medicinal importance due to presence of active secondary metabolites. The seed powder contains high concentration of L-DOPA (6). *Chlorophytum borivillianum* from the Liliaceae plant family is a highly valued medicinal plant that possesses a number of biological activities including those antioxidant, antimicrobial, hepatoprotective and hypolipidemic. Conventionally, *C. borivillianum* roots are being used in treating male impotence, oligozoospermia and erectile dysfunction (7). *Anacyclus pyrethrum* from the Asteraceae family is known to have medicinal properties. The plant root has been reported to have anti-inflammatory, antimicrobial, immunostimulating and aphrodisiac potential (8). *Tribulus terrestris* from the Zygophyllaceae family possesses antioxidant, antihypertensive and aphrodisiac potential (9). The main aim of the present study was to explore the potential of selected medicinal plants as a source of natural antioxidants.

MATERIALS AND METHODS

The current research work was carried out in the Bioassay Section, Medicinal Biochemistry Laboratory, Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan.

Collection and preparation of plant extracts

Medicinal plants for the present study were collected from the Botanical Garden, University of Agriculture, Faisalabad, Pakistan and from a local area of Faisalabad. Plants and their different parts were taxonomically identified and authenticated by the Department of Botany of the same University. The useful plant parts used in the present study were *Asparagus racemosus* (roots), *Chlorophytum borivillianum* (roots), *Mucuna pruriens* (seeds), *Anacyclus pyrethrum* (roots), *Tribulus terrestris* (fruits) and *Cichorium intybus* (aerial parts). The washed air-dried plant materials were ground into fine powder using the Grinder, Model CB 222, Cambridge, UK. The powdered plant materials (1 Kg) were extracted with methanol in water (70:30 v/v) using an orbital shaker

(Gallenkamp, UK) for 72 h at room temperature. Whatman No. 1 filter paper was used for filtration, and the filtrate was concentrated using a rotary evaporator (Heidolph, model Laborata 4000, Germany) under reduced pressure at 45-50°C. The concentrated extracts were weighed and percentage yield (g/100 g of dry plant) was calculated.

Yield (%) = (Weight of dried extract / Weight of dried plant material) * 100

Antioxidant potential of plant extracts through different assays

Determination of total phenolic contents (TPC)

The total phenolic contents in methanolic plant extracts were determined by the Folin-Ciocalteu method as described by Jain et al. (10). Different concentrations of gallic acid (50, 100, 150, 200, 250 and 300 mg/mL) were used to construct the standard curve. One milliliter of standard solutions and plant extract (10 mg/mL) was added to Folin-Ciocalteu reagent (5mL) and 20% sodium carbonate (4mL). The mixture was incubated for 1 h to measure the absorbance of colored complex at 765 nm. Total phenolics in methanolic extracts were calculated as gallic acid equivalents (GAE) using the formula:

$$T = C \times V / M$$

Where, T = total phenolic contents in mg GAE/g extract; C = Gallic acid concentration in mg/mL; V = extract volume in mL; M = plant extract weight in grams.

Determination total flavonoids contents (TFC)

The plant extract was tested for total flavonoid contents following the protocol described by Pranuthi et al. (11). Briefly, 0.5 mL of plant extract was added to distilled water (2 mL) and 0.15 mL of 5% NaNO₂ solution and incubated for 6 min. After that 0.15 mL of 10% AlCl₃ solution was added and again incubated for 6 min followed by the addition of 4% NaOH solution to the mixture. Volume of the reaction mixture was made up to 5 mL by the addition of methanol and mixed well. The reaction absorbance was taken at 510 nm after incubation at room temperature for 15 min. Total flavonoid contents (TFC) of extracts were expressed as µg catechin equivalents per g of plant extract from the linear regression curve of catechin.

DPPH scavenging activity

Antioxidant potential of plant extract was determined by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical

scavenging assay as described by Ayaz et al. (12). According to this method, 1 mL of 0.004% DPPH in methanol solution (freshly prepared) was added to 3 mL of plant extract and the mixture was kept in the dark for 30 min. The absorbance at 517 nm was noted. Absorbance of reaction mixture was inversely related to scavenging activity of free radicals. The antioxidant activity of ascorbic acid and BHT as standard was also analyzed. The reagent blank was used as control. The percentage DPPH radical inhibition by samples was calculated.

$$\text{DPPH Inhibition (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

Where

A_1 = Absorbance of sample,

A_0 = Absorbance of blank.

Measurement of reducing power

The reductive potential of the extract was measured by the principle of direct electron donation in the reduction of $\text{Fe}^{3+}(\text{CN})_6$ to $\text{Fe}^{2+}(\text{CN})_6$ as described by Yadav et al. (13). The reaction mixture contained 1 mL of plant extract, 0.2 M phosphate buffer with pH 6.6 (2.5 mL) and 1% potassium ferricyanide (2.5 mL). The control contained all the reaction reagents except plant extract. The mixture was kept at 50°C for 20 min. The reaction was stopped by adding 2.5 mL of TCA (1% w/v), in each reaction mixture, followed by the centrifugation at 3000 rpm for 10 min. Then 2.5 mL each of supernatant and distilled water was mixed, then 0.5 mL of 0.1% w/v ferric chloride was added. The reaction absorbance was measured at 700 nm.

Statistical analysis

The data obtained were analyzed by applying one way analysis of variance (ANOVA) followed by multiple comparison tests using the statistical software Statistix 8.1.

RESULTS

The highest percent yield was obtained from methanolic extract of *A. racemosus* roots while the least percent yield was given by the methanolic extract of *M. pruriens* seeds. The obtained methanolic extract yield of studied medicinal plants in decreasing order was *A. racemosus* roots > *A. pyrethrum* roots > *C. intybus* aerial parts > *T. terrestris* fruits > *C. borivilianum* roots > *M. pruriens*

seeds (28.16, 18.44, 10.01, 9.76, 7.0 and 4.14) g/100g dry weight of plants material, respectively.

The total flavonoid and phenolic contents of the studied medicinal plant methanolic extracts were determined in the current study and the mean $\pm$ SE (Standard error of mean) values of their results are given in Table I. These phytochemical compounds revealed significant ($p < 0.05$) differences in their total contents on the medicinal plants methanolic extract studied. The seed extract of *M. pruriens* showed the highest total phenolic contents followed by *A. pyrethrum* roots extract, fruit extract of *T. terrestris*, aerial parts of *C. intybus*, *C. borivilianum* roots and root extract of *A. racemosus*. The highest total flavonoid content was found in the methanolic extract of *A. pyrethrum* roots followed by fruit extract of *T. terrestris*, *M. pruriens* seeds, *C. intybus* aerial parts, roots extract of *C. borivilianum* and methanolic roots extract of *A. racemosus*.

DPPH scavenging activity, expressed in percentage, caused by methanolic extracts of the studied plant materials is also given in Table I. Vitamin C was used as positive control that showed $93.54 \pm 0.95\%$ percent radical scavenging activity. Among the tested medicinal plant extracts, the highest scavenging activity was shown by *M. pruriens* seed extract followed by *A. pyrethrum* root extract, aerial parts of *C. intybus*, root extract of *C. borivilianum*, *A. racemosus* root extract and fruit extract of *T. terrestris* in order of scavenging percentage (82.05 ± 0.55 , 75.66 ± 1.09 , 65.28 ± 2.05 , 44.06 ± 2.68 , 39.54 ± 4.77 and 36.40 ± 2.01), respectively. These results are in favor of the implication of phenolic and flavonoid compounds in the antioxidant activity of the medicinal plants studied.

In the present study, methanolic extracts of selected medicinal plants were tested for their reducing potential ability to reduce the ferric ions into ferrous form. Methanolic extract of *C. intybus* aerial parts showed the highest reducing power compared to the other studied medicinal plants, while the least reducing potential was observed in the methanolic root extract of *A. racemosus*. The reductive capacity of methanolic extract of selected parts of tested plant extract in decreasing

Table I. Antioxidant potential of methanolic extract of studied plant material.

Plants\Parameters	TPC (mg GAE/g dry plants material)	TFC (μ g CE/g dry plants material)	DPPH Scavenging activity (%)
<i>A. racemosus</i> roots	195.58 $\pm$ 3.02 ^E	6.36 $\pm$ 0.32 ^D	44.06 $\pm$ 2.68 ^D
<i>C. borivilianum</i> roots	238.88 $\pm$ 1.75 ^D	11.33 $\pm$ 0.28 ^D	39.54 $\pm$ 4.77 ^{DE}
<i>M. pruriens</i> seeds	683.15 $\pm$ 4.28 ^A	35.47 $\pm$ 1.53 ^C	82.05 $\pm$ 0.55 ^B
<i>A. pyrethrum</i> roots	641.76 $\pm$ 4.16 ^B	156.58 $\pm$ 4.01 ^A	75.66 $\pm$ 1.09 ^B
<i>T. terrestris</i> fruits	325.33 $\pm$ 4.64 ^C	57.91 $\pm$ 0.79 ^B	36.40 $\pm$ 2.01 ^E
<i>C. intybus</i> aerial parts	315.70 $\pm$ 1.97 ^C	34.61 $\pm$ 0.51 ^C	65.28 $\pm$ 2.05 ^C
Vitamin C	--	--	93.54 $\pm$ 0.95 ^A

TPC: total phenolic contents; GAE: gallic acid equivalent; TFC: total flavonoids contents; CE: catechin equivalent; (–): not tested. All data are presented as mean $\pm$ SEM of multiple determinations of each experiment. Mean with different superscript letters in the same column indicate significant ($p < 0.05$) differences among tested plants extract and controls.

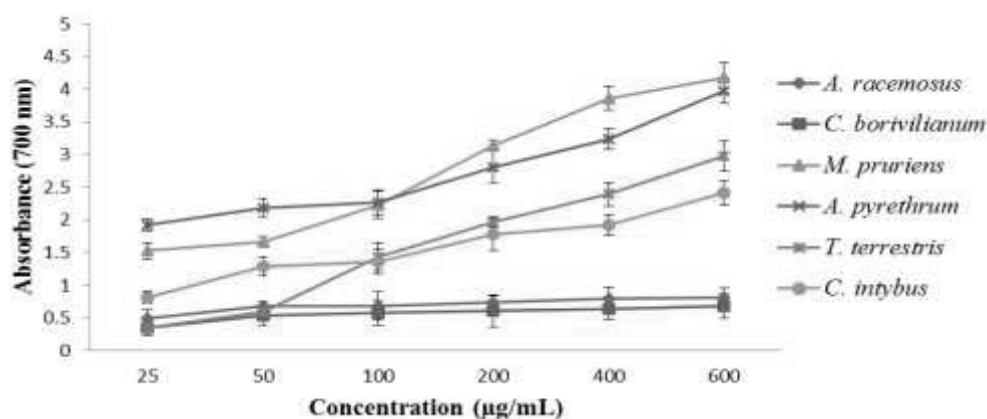


Fig. 1. Reducing potential of the studied medicinal plants using different concentrations of extracts by FRAP method. Data are presented as mean $\pm$ SEM of replicate measurements.

order of reducing ability is *C. intybus*, *T. terrestris*, *A. pyrethrum*, *M. pruriens*, *C. borivilianum* and *A. racemosus*. The increase in the reductive capacity of medicinal plants extract depends on the concentration of plant extract. The reducing potential increases with increasing concentration of extract as shown in Fig. 1.

DISCUSSION

Antioxidant molecules are capable of decreasing the oxidation process of substrates. Free radicals, like reactive oxygen species (ROS), are produced during oxidation reactions. Oxidative stress results due to the defect in the body's natural antioxidant defense

system which might be associated with various human ailments and other health-related problems (14). Nowadays, there is great interest in the use of natural antioxidants like flavonoids, carotenoids and vitamins A, C and E in food supplements which help to prevent oxidative stress in the human body (15).

The most commonly found phytoconstituents in medicinal plants are flavonoids and phenolic compounds reported to have numerous biological activities including antioxidant potential. The total flavonoid and phenolic contents of the medicinal plant methanolic extracts determined in the present study revealed significant differences in their total contents. These phytochemical compounds have antioxidant potential not only because of their ability to donate hydrogen or electrons but also because of being stable radical intermediates. Previous studies revealed that the antioxidant potential of medicinal plants is associated with the contents of phenolic compounds (16). The present study also revealed the presence of flavonoid compounds as high total flavonoids contents were found in the methanolic extract of the studied plants. High flavonoid content in the tested plant extract revealed the antioxidant activity. Mujeeb et al. (17) studied the root extract of *M. pruriens* and reported that the plant has good antioxidant potential due to the presence of phenolic and flavonoid compounds as the active phytoconstituents. In the current study, high phenolic and flavonoid contents in the seed extract of *M. pruriens* proved the antioxidant activity of this plant.

Radical scavenging activity of medicinal plants is very important for the determination of their antioxidant capacity. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) test system was used for analysis of methanolic plant extract for their radical scavenging activity determination. The DPPH assay primarily functions through measuring the direct transfer of electrons or hydrogen atoms to the free radical. The radical scavenging assay reaction extent depends mainly on the ability of antioxidants to donate hydrogen governed primarily by their degree of hydroxylation and structure (18). The results of scavenging activity of the tested medicinal plant extracts are in favor of the implication of phenolic and flavonoid compounds in the antioxidant activity.

Selles et al. (19) evaluated the antioxidant activities of *A. pyrethrum* extracts and found an increased radical scavenging activity with increasing concentration of plants extract. They reported that *A. pyrethrum* has good antioxidant potential and should be used as an alternative source of natural antioxidant in food, cosmetics and pharmaceutical industries. Therefore, the present results are in agreement with their findings as *A. pyrethrum* extract was also observed with good radical scavenging activity. Sujith et al. (8) reported the DPPH absorption as the ability of test compounds for free radical scavenging.

The reducing power estimation of natural antioxidants is very informative in context for their ranking as reducing agents and to predict the possibility of electron transfer to reactive oxygen. The reducing power assay principle based on the ability of phenolic compounds to reduce ferric (Fe^{+3}) ion to ferrous (Fe^{+2}) form by electron donation. Different methods were used to assay the antioxidant capacity of selected medicinal plants. The ferric reducing antioxidant power (FRAP) method used in the current study is quite rapid and easy to perform. In this assay, Fe^{3+} /ferricyanide complex is reduced to the ferrous form by antioxidants and can be checked by measuring the intensity of navy blue color formation at 700 nm (20). In the present study, methanolic extract of selected medicinal plants were tested for their reducing potential ability to reduce the ferric ions into ferrous form. Methanolic extract of *C. intybus* aerial parts showed the highest reducing power compared to the other plants studied, while the least reducing potential was observed in the methanolic root extract of *A. racemosus*. Sujith et al. (8) determined the antioxidant potential of *A. pyrethrum* roots and reported good reducing potential.

The study concludes that the selected aphrodisiac medicinal plants possess antioxidant activities due to the presence of active secondary metabolites. Further comprehensive studies are needed to detect and identify the active phytoconstituents present in the studied medicinal plant methanolic extracts using modern detection and identification techniques.

ACKNOWLEDGEMENTS

The authors are grateful to the Bioassay section, Medicinal Biochemistry Lab, Department of Biochemistry, UAF for providing the facilities of experimental work and technical support. We also thank the Department of Biochemistry administration for support and assistance during the research work.

REFERENCES

1. Patel D, Kumar R, Prasad S, Hemalatha S. Pharmacologically screened aphrodisiac plant-A review of current scientific literature. *Asian Pac J Trop Biomed* 2011; 1(1):S131-S38.
2. Singh R, Ali A, Gupta G, Semwal A, Jeyabalan G. Some medicinal plants with aphrodisiac potential: A current status. *J Acute Dis* 2013; 2(3):179-88.
3. Riaz M, Mahmood Z, Saeed M, Abbas M, Shahid M. Lipid peroxidation and antioxidant enzymes in relation to semen quality. *Oxid Commun* 2016; 39(4):2990-98.
4. Sebai H, Selmi S, Rtibi K, Souli A, Gharbi N, Sakly M. Lavender (*Lavandula stoechas* L.) essential oils attenuate hyperglycemia and protect against oxidative stress in alloxan-induced diabetic rats. *Lipids Health Dis* 2013; 12(1):189.
5. Sahrawat AK, Sharma PK, Sahrawat A. Asparagus racemosus-wonder plant. *Int J Adv Res* 2014; 2(4):1039-45.
6. Sharma T, Ramamurthy A, Nathani S, Sharma G. A comparative pharmacognosy study of black and white seeds of kapikacchu (*Mucuna pruriens* (L.) DC.). *Int J Pharm Sci Res* 2017; 8(2):838.
7. Giribabu N, Kumar KE, Rekha SS, Muniandy S, Salleh N. Chlorophytum borivilianum (Safed Musli) root extract prevents impairment in characteristics and elevation of oxidative stress in sperm of streptozotocin-induced adult male diabetic Wistar rats. *BMC Complement Altern Med* 2014; 14(1):291.
8. Sujith K, Darwin CR, Suba V. Antioxidant activity of ethanolic root extract of *Anacyclus pyrethrum*. *Int Res J Pharm* 2011; 2(12):2222-26.
9. Shahid M, Riaz M, Talpur M, Pirzada T. Phytopharmacology of *Tribulus terrestris*. *J Biol Regul Homeost Agents* 2016; 30(3):785-88.
10. Jain S, Jain A, Vaidya A, Kumar D, Jain V. Preliminary phytochemical, pharmacognostical and physico-chemical evaluation of *Cedrus deodara* heartwood. *J Pharmacogn Phytochem* 2014; 3(1):91-95.
11. Pranuthi EK, Narendra K, Swathi J, et al. Qualitative Assessment of Bioactive Compounds from a Very Rare Medicinal Plant *Ficus dalhousiae* Miq. *J Pharmacogn Phytochem* 2014; 3(1):57-61.
12. Ayaz M, Junaid M, Ahmed J, et al. Phenolic contents, antioxidant and anticholinesterase potentials of crude extract, subsequent fractions and crude saponins from *Polygonum hydropiper* L. *BMC Complement Altern Med* 2014; 14(1):145.
13. Yadav M, Yadav A, Yadav JP. In vitro antioxidant activity and total phenolic content of endophytic fungi isolated from *Eugenia jambolana* Lam. *Asian Pac J Trop Med* 2014; 7:S256-S61.
14. Smolskaitė L, Venskutonis PR, Talou T. Comprehensive evaluation of antioxidant and antimicrobial properties of different mushroom species. *LWT- Food Sci Technol* 2015; 60(1):462-71.
15. Jayakumar R, Chennazhi KP, Srinivasan S, Nair SV, Furuike T, Tamura H. Chitin scaffolds in tissue engineering. *Int J Mol Sci* 2011; 12(3):1876-87.
16. Jain A, Soni M, Deb L, et al. Antioxidant and hepatoprotective activity of ethanolic and aqueous extracts of *Momordica dioica* Roxb. leaves. *J Ethnopharmacol* 2008; 115(1):61-66.
17. Mujeeb M, Bagri P, Aeri V, Khan S. Hepatoprotective and antioxidant activity of the methanolic extract of *Mucuna pruriens* Bak. roots. *Orient Pharm Exp Med* 2010; 10(2):79-85.
18. Woldegiorgis AZ, Abate D, Haki GD, Ziegler GR. Antioxidant property of edible mushrooms collected from Ethiopia. *Food Chem* 2014; 157:30-36.
19. Selles C, Dib MEA, Allali H, Tabti B. Evaluation of antimicrobial and antioxidant activities of solvent extracts of *Anacyclus pyrethrum* L. from Algeria. *Mediterr J Chem* 2012; 2(2):408-15.
20. Gupta S, Prakash J. Studies on Indian green leafy vegetables for their antioxidant activity. *Plant Foods Hum Nutr* 2009; 64(1):39-45.

LETTER TO THE EDITOR

IMMUNOMODULATORY POTENTIAL OF *ANACYCLUS PYRETHRUM* (L.) AND *MUCUNA PRURIENS* (L.) IN MALE ALBINO RATS

F. YOUSAF¹, M. SHAHID¹, M. RIAZ^{1,2}, A. ATTA³ and H. FATIMA¹

¹Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan; ²Department of Allied Health Sciences, Sargodha Medical College, University of Sargodha, Sargodha, Pakistan;

³Department of Biochemistry, Bahauddin Zakaria University, Multan, Pakistan

Received March 6, 2017 – Accepted April 27, 2017

The present study was designed to investigate the immunomodulatory potential of *Anacyclus pyrethrum* roots and *Mucuna pruriens* seeds in male albino rats. The roots of *A. pyrethrum* and seeds of *M. pruriens* were extracted with methanolic solvent (70:30) and administered at dose concentrations of 50, 100 and 200 mg/Kg body weight to healthy male rats. The immune system of rats was suppressed by injecting carbon tetrachloride to animals in the toxic control group and test group animals. Cell-mediated immune response of animals was examined by performing neutrophil adhesion test and the humoral immune response was evaluated by determining serum immunoglobulin levels of the animals under study. The administration of methanolic extracts of *A. pyrethrum* roots and *M. pruriens* seeds significantly ($p < 0.05$) increased the neutrophil adhesion to the nylon fiber. Increase in % neutrophil adhesion was observed in animals treated with 200 mg of each plant extract. Significant ($p < 0.05$) improvement in immunoglobulin levels was recorded in the extract treated group animals, showing that the root extract of *A. pyrethrum* and seed extract of *M. pruriens* have immunomodulatory potential. We therefore conclude that the tested extracts can be used as immunomodulatory agents to stimulate the immune system.

To the Editor,

The immune system is a remarkably sophisticated defense system designed to protect the vertebrate body against invading microbes. It is made up of many interdependent cell types protecting the body from microbial infections and tumor cell growth (1). The immune system is the part of the body which detects pathogens with the help of specific receptors to produce immediate response by activation of cytokines and releasing inflammatory mediators (2). In developing nations, malnutrition and infectious diseases are challenging as the body's defense system is compromised in infected individuals. The

physiology of the immune system is greatly affected by malnutrition and in many cases stimulation is necessary in immunosuppressed individuals or suppression in over exaggerated individuals in conditions of autoimmune diseases (3).

Agents that enhance the resistance against foreign invading organisms are called immunomodulators. Natural immunomodulators by plant sources are economic, easily available, biocompatible and can restore the weakened immune system or by subdue the excessive immune system (4, 5). Plants with immunomodulatory potential enhance the immune system by increasing the activity of

Key words: immunomodulatory, neutrophil adhesion, immunoglobulins, *A. pyrethrum*

Mailing address:

Dr Muhammad Riaz,
Department of Allied Health Sciences,
Sargodha Medical College,
University of Sargodha,
Sargodha, 40100-Pakistan
e-mail: riazmlt786@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.**

macrophages and by killing antigens with the formation of free radicals, cytokines and nitric oxides (6, 7). Roots of *A. pyrethrum* are widely used as aphrodisiac, hepatoprotective, anti-inflammatory and immunomodulatory agents due to the presence of secondary active metabolites and antioxidant compounds (8, 9). *M. pruriens* (L.) DC from the *Fabaceae* family possesses several biological activities such as those antioxidant, antimicrobial, anti-inflammatory and neurorestorative (10). The present study was planned to document the immunomodulatory potential of *A. pyrethrum* and *M. pruriens* in male rats.

MATERIALS AND METHODS

Study design and setting

This preclinical laboratory-based experimental study was conducted in the Bioassay Section, Medicinal Biochemistry Laboratory, Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan.

Collection and extraction of plants

A. pyrethrum and *M. pruriens* were collected from the Faisalabad region, taxonomically identified and authenticated by the Department of Botany, University of Agriculture, Faisalabad, Pakistan. Seeds of *M. pruriens* and roots of *A. pyrethrum* were used for extract preparation. The dried plants parts were ground into fine powder using a grinder (Model CB 222, Cambridge UK). The powdered plant materials (1 Kg) were extracted with methanol in water (70:30 v/v) using an orbital shaker (Gallenkamp, UK) for 72 h at room temperature, filtered through Whatman No. 1 filter paper, concentrated in a rotary evaporator (Heidolph, model Laborata 4000, Schwabach, Germany), freeze dried under vacuum and stored at 4°C until experimental bioassays were carried out. The percentage yield of extract (g/100 g of dry plant) was determined. Different concentrations of plant extracts (50, 100 and 200 mg/Kg body weight) were prepared. Control solutions were normal saline (as normal control), 20% CCl₄ (toxic control) and vitamin C (100 mg/Kg body weight) as positive control.

Laboratory animals

Thirty-six male rats aged between 8-12 weeks

were randomly divided into nine experimental groups with four animals in each group. All the study animals were maintained under standard laboratory conditions and temperature (25±1°C) and 12 h light/dark cycle. They received rat pellets and clean water ad libitum. The animals were acclimatized for 2 weeks before the experimental study was carried out. All procedures were conducted in accordance with the guidelines framed by institutional animal care and use committee of the University of Agriculture, Faisalabad, Pakistan. The study was approved by the Graduate Studies and Research Board, University of Agriculture, Faisalabad, Pakistan.

Treatment groups and dosing of animals

Rats in the normal control group (Group-1) received normal saline and diet throughout the six-week study period. Rats in the toxic control group (Group-2) and positive control group (Group-3) received 0.5 mL of 20% CCl₄ and 100 mg/Kg bw of Vitamin C, respectively. The test group rats (Groups 4-6 and 7-9) received 50, 100 and 200 mg/Kg bw methanolic root extract of *A. pyrethrum* and seed extract of *M. pruriens*, respectively, after CCl₄ intoxication.

Neutrophil adhesion test

After 42 days of treatment to all the rats under study, blood samples were collected by heart puncture in Tri K EDTA vials and subjected to total as well as differential leukocyte count. After initial counts, the blood samples were incubated with 80 mg mL⁻¹ of nylon fibers at 37°C for 15 min. The incubated samples were again analyzed for total and differential leukocyte count. The product of total leukocyte count and % neutrophil known as neutrophil index was determined for each of the respective groups. The % neutrophil adhesion for each of the test groups was determined as follows (11):

Neutrophil Index = TLC X % Neutrophil

Neutrophil adhesion (%) = (NIu-NIt) / NIu x 100

Where,

NIu: Neutrophil Index before incubation with nylon fibers

NIt: Neutrophil Index after incubation with nylon fibers

Determination of Serum Immunoglobulins (IgG and IgM)
Serum IgG and IgM were determined by enzyme linked immunosorbent assay (ELISA). The total IgG and IgM levels in the serum sample were determined by ELISA

method using rat IgG and IgM ELISA kits designed for measurement of IgG and IgM, respectively, in rat serum following the procedure adopted by Pastorelli et al. (12).

Statistical analysis

Graphs and Average $\pm$ SEM were prepared in Microsoft® Excel Version 2010 (10.2614.2625). The data obtained were analyzed by applying two sample *t*-tests using Minitab version 17.1.30.0 to compare significant differences between studied parameters among the different study groups (13).

RESULTS

The methanolic root extract of *A. pyrethrum* gives more percent yield (18.44g/100g dry weight) than the methanolic seed extract of *M. pruriens* (4.14g/100g dry weight). Significant ($p < 0.05$) increase in percent neutrophil adhesion in Vitamin C-treated male albino rats, rats treated with 200 mg/Kg of *A. pyrethrum* root, 100 and 200 mg/Kg of *M. pruriens* seed per Kg body weight of rats was observed when compared with the normal control group rats. On comparing with the CCl₄-intoxicated group of animals, significant ($p < 0.05$) increase in %NA was also observed in animals treated with 200 mg/Kg of each plant extract. The results of

neutrophil adhesion test are shown in Fig. 1.

The immunoglobulins, namely IgG and IgM, in serum were determined as marker for the evaluation of immunomodulatory activity of methanolic root extract of *A. pyrethrum* and seed extract of *M. pruriens*. Significant ($p < 0.05$) improvement in levels of IgG and IgM in the extract-treated rats compared to CCl₄-intoxicated rats was observed. Significant difference was also observed on comparing immunoglobulin levels in the extract-treated rats with the normal control rats, while a significant ($p < 0.05$) decrease in immunoglobulin levels was observed in the extract-treated group rats compared to positive control group rats treated with Vitamin C. Significant ($p < 0.05$) variation in IgG levels was observed in rats treated with 50 and 200 mg of *A. pyrethrum* root extract. No significant improvement in IgG level was observed in the extract-treated rats in comparison with the normal control and CCl₄-intoxicated group rats. Overall improvement in immunoglobulin levels was observed in the positive control group and extract-treated group animals in our present study suggests that the root extract of *A. pyrethrum* and seed extract of *M. pruriens* has immunomodulatory potential. Variations in mean $\pm$ SEM of the obtained results are shown in Table I.

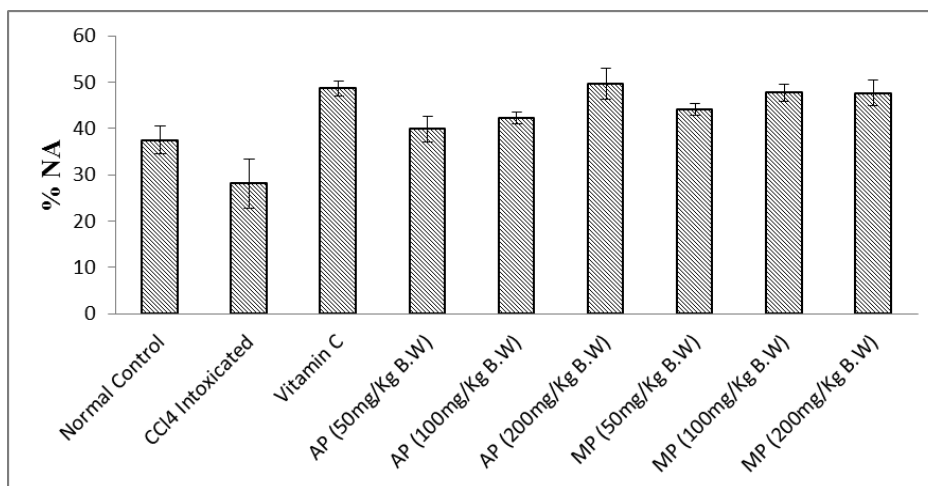


Fig. 1. Effect of methanolic root extract of *A. pyrethrum* and seed extract of *M. pruriens* on percent neutrophil adhesion (%NA).

Table I. Mean $\pm$ SEM values of serum immunoglobulins (IgM and IgG) in controls and experimental group of animals.

Groups\Parameters	IgM (mg/dL) Mean $\pm$ SEM	IgG (mg/dL) Mean $\pm$ SEM
Normal Control	83.03 $\pm$ 4.0	181.47 $\pm$ 3.2
CCl ₄ Intoxicated	56.1 $\pm$ 6.2 ^A	173.83 $\pm$ 2.7
Vitamin C	98.07 $\pm$ 2.2 ^{AB}	191.45 $\pm$ 3.0 ^B
AP (50mg/Kg B.W)	67.83 $\pm$ 3.1 ^{AC}	112.87 $\pm$ 4.3 ^{ABC}
AP (100mg/Kg B.W)	79.3 $\pm$ 5.7 ^{BC}	205.0 $\pm$ 10 ^B
AP (200mg/Kg B.W)	65.7 $\pm$ 4.3 ^{AC}	125.33 $\pm$ 4.2 ^{ABC}
MP (50mg/Kg B.W)	44.15 $\pm$ 3.5 ^{AC}	293.9 $\pm$ 8.8 ^{BC}
MP (100mg/Kg B.W)	83.5 $\pm$ 2.9 ^{BC}	100.83 $\pm$ 5.2 ^{ABC}
MP (200mg/Kg B.W)	107.6 $\pm$ 4.2 ^{AB}	109.95 $\pm$ 4.8 ^{ABC}

Values are expressed as Mean $\pm$ SEM (n = 4).

^Ap < 0.05 as compared with respective normal control; ^Bp < 0.05; ^Cp < 0.05 as compared with CCl₄ intoxicated and Vitamin C treated group animals respectively

IgM: immunoglobulin M; IgG: immunoglobulin G; AP: *Anacyclus pyrethrum*; MP: *Mucuna pruriens*.

DISCUSSION

Immunopharmacology is a relatively new branch of pharmacological science and is developing with the aims for searching immunomodulators. Neutrophils in the intravascular blood sense the infection by invading microorganisms and adhere to the endothelial capillaries, migrating through the vessel walls thus engulfing and digesting the invading organisms (14). In the neutrophil adhesion test, nylon fiber was used for neutrophil adhesion which determines the migration of lymphocytes through blood vessel walls and the macrophage numbers reaching the inflammation site under neutrophil influence (15). Significant variation in percent neutrophil adhesion was observed in the different study group rats treated with plant extracts and Vitamin C compared to normal control rats. CCl₄ intoxication to rats decreased %NA in our current study. Fulzele et al. (11) reported immunomodulatory activity of *Haridradi ghrita* in respect to neutrophil adhesion test. To the best of our knowledge, no

literature report describing the immunomodulatory potential of *A. pyrethrum* roots and *M. pruriens* seeds extract in respect to neutrophil adhesion test has been published.

The immune system plays a key role in maintaining the body's homeostasis in such a way as to eliminate the foreign substances not recognized as self by the body (16). Extensive emphasis in research programs has been laid globally for the past two decades in order to explore the plant kingdom for the extraction of natural agents from plants to cope with diseases for which modern medicine has no effective treatment regimen (12). In the present study, significant improvement in serum IgG and IgM levels in the extract-treated rats was observed when compared with the CCl₄-intoxicated rats. We also observed a significant decrease in immunoglobulin levels in the extract-treated rats compared to the positive control rats treated with Vitamin C. No dose-dependent improvement was observed in immunoglobulins level of rats after extract treatment. Overall improvement in immunoglobulin levels observed in the positive

control and extract-treated rats in our study suggests that the root extract of *A. pyrethrum* and seed extract of *M. pruriens* have immunomodulatory potential. Pastorelli (12) determined the immunoglobulin levels in plant extract-treated rats and found that the plants with antioxidant potential have immunomodulatory activity. We also observed an improvement in immunoglobulin levels in the methanolic root extract of *A. pyrethrum* and seed extract of *M. pruriens*. The current finding suggests the immunomodulatory activity of the tested plant extracts may be due to the presence of antioxidant compounds reported in the literature. Bharani et al. (17) reported significantly increased serum immunoglobulins in mice administered with methanolic extract of *Morus alba* leaves. Fewer reports are available on the immunomodulatory activity of *A. pyrethrum* and *M. pruriens* tested in the present study.

The present study concluded that methanolic extracts of *A. pyrethrum* roots and *M. pruriens* seeds possess immunomodulatory activities. Further studies are required to explore the immunomodulatory potential of the plants studied in the future.

ACKNOWLEDGEMENTS

The Authors are extremely grateful to the staff of the Medicinal Biochemistry Laboratory, Department of Biochemistry, UAF for providing the facilities in conducting this research study.

REFERENCES

1. Nagarathna P, Reena K, Sriram Reddy JW. Review on Immunomodulation and Immunomodulatory activity of some herbal plants. *Int J Pharm Sci Rev Res* 2013; 22(1):223-30.
2. Shivaprasad H, Kharya M, Rana A, Mohan S. Preliminary immunomodulatory activities of the aqueous extract of *Terminalia chebula*. *Pharm Biol* 2006; 44(1):32-4.
3. Nfambi J, Bbosa GS, Sembajwe LF, Gakunga J, Kasolo JN. Immunomodulatory activity of methanolic leaf extract of *Moringa oleifera* in Wistar albino rats. *J Basic Clin Physiol Pharmacol* 2015; 26(6):603-11.
4. Ortuno J, Cuesta A, Rodríguez A, Esteban MA, Meseguer J. Oral administration of yeast, *Saccharomyces cerevisiae*, enhances cellular innate immune response of gilthead seabream (*Sparus aurata* L.). *Vet Immunol Immunopathol* 2002; 85(1):41-50.
5. Małaczewska J, Wojcik R, Jung L, Siwicki AK. Effect of Biolex β -HP on selected parameters of specific and non-specific humoral and cellular immunity in rats. *Bull Vet Inst Pulawy* 2010; 54:75-80.
6. Ismail S, Asad M. Immunomodulatory activity of *Acacia catechu*. *Indian J Physiol Pharmacol* 2009; 53(1):25-33.
7. Shahid M, Riaz M, Talpur M, Pirzada T. Phytopharmacology of *Tribulus terrestris*. *J Biol Regul Homeost Agents* 2016; 30(3):785.
8. Selles C, Medjdoub H, Dib MEA, Zerriouh M, Tabti B. Anti-diabetic activity of aqueous root extract of *Anacyclus pyrethrum* L. in streptozotocin-induced-diabetic rats. *J Med Plants Res* 2012; 6(16):3193-8.
9. Kumar VK, Lalitha K. Pharmacognostical studies on the root of *Anacyclus pyrethrum* DC. *Indian J Nat Prod Resour* 2012; 3(4):518-26.
10. Manyam BV, Dhanasekaran M, Hare TA. Neuroprotective effects of the antiparkinson drug *Mucuna pruriens*. *Phytother Res* 2004; 18(9):706-12.
11. Fulzele S, Bhurchandi P, Kanoje V, Joshi S, Dorle A. Immunostimulant activity of *Ashtamangal ghrita* in rats. *Indian J Pharmacol* 2002; 34(3):194-7.
12. Pastorelli G, Rossi R, Corino C. Influence of *Lippia citriodora* verbascoside on growth performance, antioxidant status, and serum immunoglobulins content in piglets. *Czech J Anim Sci* 2012; 57:312-22.
13. Montgomery DC. Design and analysis of experiments: John Wiley & Sons; 2008.
14. Malech HL, Gallin JI. Neutrophils in human diseases. *N Engl J Med* 1987; 317(11):687-94.
15. Sibi P, Varghese P. Evaluation of in-vivo immunomodulatory activity of *Punica granatum*. *Linn. Int J Res Ayur Pharm* 2014; 5(2):175-7.
16. Kumar SV, Kumar SP, Rupesh D, Nitin K. Immunomodulatory effects of some traditional medicinal plants. *J Chem Pharm Res* 2011; 3(1):675-84.
17. Bharani S, Asad M, Dhamanigi SS, Chandrakala GK. Immunomodulatory activity of methanolic extract of *Morus alba* Linn.(mulberry) leaves. *Pak J Pharm Sci* 2010; 23(1):63-8.

LETTER TO THE EDITOR

ANALYSIS OF HEMORRHAGIC FEVER WITH RENAL SYNDROME AND ITS PATHOGENIC GENE SEQUENCE BASED ON GEOGRAPHIC INFORMATION SYSTEM

Z. TANG¹, XJ. XU¹, XJ. HE¹, ZS. LIANG¹, WB. LIANG¹, Y. LI¹ and K. GAO²¹*School of Information Management, Guangxi Medical University, Nanning, Guangxi, China;*²*Affiliated Tumor Hospital of Guangxi Medical University, Nanning, Guangxi, China**Received March 22, 2017 – Accepted April 28, 2017*

This study analyzed the temporal-spatial distribution characteristics, epidemiological characteristics and gene sequences of hemorrhagic fever with renal syndrome (HFRS) in Guangxi, with the intention of providing a theoretical and technical support for the prevention of HFRS. A map of the incidence of HFRS of different cities in Guangxi was drawn up using the Geographic Information System (GIS) to investigate the epidemiological characteristics and infection source of HFRS between 2013 and 2016. Guangxi has a low incidence of HFRS, and autumn and winter are the main high-incidence seasons. Cases of HFRS were reported in all regions in Guangxi except Laibin city between 2013 and 2016. The distribution of cases in the four years suggested that Guilin, Nanning, Hechi and Wuzhou were the main infected regions, especially the local areas in the north of Guilin. The nucleotide and amino acid of S fragment and M fragment of Hantaviruses (HV) detected were highly homologous, and no obvious variation was found. Through analyzing the space-time characteristics, epidemiological characteristics and gene sequence of HFRS in Guangxi, it was found that areas rich in water, grass and moisture, such as paddy fields, are the main active areas for the host of HFRS.

To the Editor,

Hemorrhagic fever with renal syndrome (HFRS) is a natural focal disease, the pathogen of which is Hantavirus (HV) (1). HV can induce Hantavirus Pulmonary Syndrome and HFRS; the former is mainly prevalent in America, Argentina, Canada and some European countries such as Germany, Sweden and Belgium, and the latter is usually seen in Asian countries, followed by some European and African countries (2). The number of people being infected with HV has been rising constantly in Baden-Württemberg, Germany, since October 2011 (3). In Europe, HFRS is usually induced by Puumala virus

(PUUV) carried by bank vole, and Dobrava-Belgrade virus carried by *Apodemus flavicollis*. In 2012, 4,440 cases of HV were reported in 24 countries, which suggested an increase of 51.7% compared to 2011 (4). Each type of HV has its relatively fixed host. Due to the differences in living environments, different host animals are distributed in different regions (5), with rodents occupying the main position. Population types, distribution and quantity of host animals determine the characteristics of HFRS epidemic areas, distribution range of HFRS and the epidemic tendency of HFRS (6). The geographic information system (GIS), which combines data

Key words: hemorrhagic fever with renal syndrome, epidemiology, geographic information system, pathogenic genes

Mailing address:

Dr Kun Gao,
Affiliated Tumor Hospital of Guangxi
Medical University,
No. 22, Shuangyong Road,
Nanning, Guangxi, 53000, China
e-mail: gkgk056@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.**

application and spatial analysis together, can be used in the spatial distribution of HFRS (7). It is of great practical significance to investigate the correlation between the categories of epidemic areas and major host animal compositions. This study explored the temporal-spatial distribution characteristics, epidemiological characteristics and gene sequences of HFRS by taking four cities in Guangxi as the research areas, GIS as the main technology and HFRS as the research subject, aiming to provide a scientific basis for the formulation of prevention and control strategies.

MATERIALS AND METHODS

Information on the research area

Guangxi was selected as the research area. The data collected in August 2016 suggested that there were 14 prefecture-level cities, 111 county-level administrative regions and 1,247 township-level administrative regions in Guangxi province. Being affected by the south subtropical monsoon, most areas in Guangxi have a warm climate, with hot periods and abundant rainwater, but unseasonable changes. Summer lasts long in Guangxi, but winter is short. The annual average temperature is between 16°C and 23°C. The temperature gradually increases from the north to the south. The highest temperature is between 33.7°C and 42.5°C, and the lowest temperature is between -8.4°C and 2.9°C. The precipitation is not evenly distributed in different seasons. The period from April to September is the rainy season, and the amount of precipitation in that period accounts for more than 70% of the annual amount. The period from October to March is the dry season.

Data source

Epidemic data from 2013 to 2016 used in this study were provided by the Disease Control and Prevention Center of the Guangxi Zhuang Autonomous Region, including the number and detailed information of HFRS patients. A database was established based on the data obtained. A coordinate was set up on the digital map (scale 1:100000) to vectorize the layers of boundary and variable of four cities in Guangxi province and establish digital map layers of Guangxi. Risk factor data in this study included land use data (provided by the Land Department

of Guangxi), temperature data (provided by the Weather Bureau of Guangxi Province) and digital elevation model (DEM) data (provided by NASA website).

Analysis of temporal-spatial distribution characteristics

Monthly incidence data of HFRS of Guangxi from 2013 to 2016 were analyzed using Stata ver. 10.0; Original software was used to plot epidemic curves at different stages. Different layers were divided according to epidemic intensity and were spatially related to border map layers of county-level administrative elements of Guangxi; a spatial distribution tendency chart of HFRS incidence of Guangxi was constructed using ArcGIS 9.3.

Influencing factors of HFRS incidence

The accumulated number of HFRS cases of each city was taken as outcome variable, and the population was taken as offset variable. This study selected the climatic factor and the land use type as the joint variables of analysis. 1°C, 1 mm and 1% were used to represent changes of mean annual temperature, mean amount of precipitation and land cover. Effect produced by each variable was verified by single factor analysis; variables < 0.1 after analysis were taken as concomitant variables and included in the multiple-factor analysis. In this section, Stata ver.10.0 was also used for analysis.

Research animals

The lung tissues of mice and rats were taken for genetic detection. According to the regulations of the center of disease prevention and control, sampling spots were set in 49 villages and streets in Guilin, Nanning, Hechi and Wuzhou where there was a high incidence of HFRS between 2013 and 2016. Two hundred rat traps were placed in each sampling spot for 5 nights. In May and September each year, rodents were captured in the sampling spots using trap-at-night method. Firstly, the traps were set in places such as rivers and roads every 40 m, and peanuts were used as bait. The captured wild mice and rats were registered and numbered, and the capture date and plate recorded.

A total of 852 rats and mice were captured, including 478 sewer rats (56.1%), 324 *Rattus flavipectus* (38%) and 50 *Mus musculus* (5.9%). The unconscious wild mice were sacrificed by means of neck dislocation. The lung was removed and stored at -65°C until use.

The experiments carried out in the study were approved by the ethics committee of Guangxi Medical University (approval no.: 20161223).

Viral antigen in lung tissues

The viral antigens of the lung tissues were detected using immunofluorescence. The frozen lung tissues were cut into 5- μ m slices using a microtome (HA/KD-202A, Beijing Heng Odd Instrument Co., Ltd., China), fixed by cold acetone (Ningbo Zhenhai Leishen Chemical Co., Ltd., China) for 10 min, and washed by distilled water 3-4 times. After drying, the slices were added with 10 μ l of fluorescein (concentration ratio: 1:10) to label monoclonal antibody (GL Biochem. (Shanghai) Co., Ltd., China). Then the slices were immersed in 37°C water for 30 min and washed by 30 ml of phosphate buffer (Beijing Solarbio Life Sciences Co., Ltd., China) 3-4 times and rinsed by distilled water once. After drying, the lung tissues were added with 10 ml of 50% buffer glycerol (Guangzhou Xingzhi Bioscience Co., Ltd., China), covered by a slide, and observed under a fluorescence microscope (FM-600, Shanghai Puda Optical Instrument Co., Ltd., China).

Extraction of total RNA

100 mg of lung tissue specimens with positive HV antigen were washed by phosphate buffer solution (PBS) several times and then transferred to a tissue homogenizer, added with 500 μ l guanidinium isothiocyanate (GIT) denaturation solution, and transferred to an Eppendorf tube. Then 50 μ l of NaAc (2 mol/L), 500 μ l of water phenol and 100 μ l of chloroform were added. After being immersed in water at

0°C for 15 min, it was centrifuged at 10000 r/min at 4°C. The separated supernate was transferred to a new Eppendorf tube, added with isopropyl-ketone in the same volume, and refrigerated at -20°C. One hour later, it was taken out and centrifuged at 13000 r/min at 4°C. Fifteen minutes later, the supernate was removed, and 100 μ l of GIT denaturation solution was added. Isopropyl-ketone in the same volume was added after the sediment decomposed after standing. Then it was preserved at -20°C and centrifuged at 10000 r/min at 4°C for 15 min after one hour. The supernate was removed, the sediment was washed several times by absolute ethyl alcohol, and finally it was dried.

Gene amplification

cDNA was reversely synthesized based on HV specific reverse transcription primer P14 using a reverse transcription kit according to the instruction manual. Then it was taken as a template; partial S fragment and M fragment were amplified using the S fragment and M fragment primers shown in Table I through Polymerase Chain Reaction (PCR) (8).

The PCR amplified reaction conditions included predenaturation at 95°C for 10 min, denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 70°C for 1 min, for 35 cycles, and extension at 70°C for 10 min. The reaction was terminated at 4°C. PCR amplified products were identified using 1% agarose gel electrophoresis. When the molecular weight of strip was the same as that of the expected fragment, it was determined as a specific amplified product; otherwise, it was not. The specific strip was recycled according to the usage of the gel recycling kit and sequenced.

Table I. Reverse transcription PCR primers.

Primer	Sequence (5'~3')	Bit	Fragment
Reverse transcription primers	TAGTAGTAGACTCC	1~14	LMS
	GTAAGCCCTGTCATGAGTGTAG	682~703	S+
	CTAGTGTACATCCAGCATCCTT	922~901	S-
	GTGGACTCTTCTTCTCATTATT	1928~1949	M+
	TGGGCAATCTGGGGGGTTGCATG	2333~2355	M-

Monitoring information of HV host

The monitoring information of HV host was statistically analyzed using SPSS. ver. 12.0; relevant indicators, such as the percentage of mouse species, the virus carrying rate and mouse species density were tested. The computational formulas were as follows: the percentage of mouse/rats species = the number of mice/rats in the same category/total number of mice/rats; the overall virus carrying rate = the number of positive mice/rats/the total number of mouse/rats; the virus carrying rate of each species of mice/rats = the number of positive mice/rats of each species/the total number of mice/rats of each species. A total of 852 mice/rats were captured, including 478 sewer rats (56.1%), 324 *Rattus flavipectus* (38.0%) and 50 *Mus musculus* (5.9%). Seven sewer rats were positive, suggesting a virus carrying rate of 0.82; there were no positive *Rattus flavipectus* and *Mus musculus*.

RESULTS

Analysis of temporal-spatial distribution characteristics of HFRS

As shown in Table II, the number of HFRS cases was 9, 13, 14 and 8 in 2013, 2014, 2015 and 2016, respectively, suggesting a tendency of increasing first and then decreasing; the number of cases in 2014 and 2015 were more than that in the other two years. To calculate the high-incidence season of HFRS more clearly, the number of HFRS cases in each month from 2013 to 2016 was recorded. It was found that the period from October to February had a high incidence of HFRS (71.2%), suggesting an obvious seasonal characteristic.

The distribution of HFRS cases in the four cities was greatly different between 2013 and 2016. Most cases were from the local area in the north of Guilin; the number of cases in Xingan county and Quanzhou county were 36.4% and 27.3%, respectively, of the total number. Most cases were from villages rather than cities. Forty out of 44 patients were infected with HV in the spring and winter; the ratio of males to females was 31:13; the ratio of males to females who died of HV was 13:5; and most of the deceased patients were aged from 40 to 60 years (average 42.3 ± 23.7 years).

Influencing factors of HFRS incidence

HFRS incidence data in this study were analyzed by Poisson regression, and single factor analysis results showed that the spatial distribution of HFRS cases was related to the annual amount of precipitation ($p < 0.05$) and mean relative humidity ($p < 0.001$). Multiple-factor analysis results showed that HFRS incidence was only related to the mean relative humidity ($p < 0.001$) and the morbidity increased with the increase of humidity. Incidence data of HFRS from 2013 to 2016 were analyzed by Poisson regression, and single factor analysis results showed that HFRS incidence was related to the population density, paddy field, dry farmland, grassland, shrubbery, building land, naked land, water, etc. According to the multiple-factor analysis results, HFRS incidence was in a close correlation with population density, paddy field, shrubbery and naked land. Paddy field (IRR=1.34) and rice field (IRR=1.21) were in a positive correlation with the incidence of HFRS, i.e., the incidence of HFRS increased with the increase of areas of paddy fields and rice fields; on the other hand, shrubbery (IRR=0.23), naked land (IRR=0.27) and population density (IRR=0.98) were in negative correlation with HFRS incidence, i.e., HFRS incidence decreased with the increase of shrubbery and naked land areas and population density (Fig. 1). Thirty out of 44 patients lived in the areas next to paddy fields and rice fields, and the population density was low.

Analysis of HV gene sequence

IFA test found 7 positive specimens which were SEOV (+), as shown in Fig. 2. Three out of the above positive specimens were selected for nucleotide sequence analysis, and two PCR products of S gene fragment and one PCR product of M gene fragment were obtained after amplification. Then a 195-bp sequence and a 351-bp sequence were sequenced in the S and M fragments; moreover, the S and M fragments were all SEOV sequences after verification by Ncbi-BLAST. Comparison of the obtained nucleotide sequences and other known HV strain sequences indicated that nucleotide homology of the PCR products of the S fragment was 100%, while the nucleotide homology was 95.6%~100%

Table II. *The number of cases in the four cities in each month from 2013 to 2016.*

Year	Month	Guilin city	Nanning city	Hechi city	Wuzhou city	Total number
2013	1	0	0	0	0	0
	2	0	1	0	1	2
	3	0	1	0	0	1
	4	1	0	0	0	1
	5	0	0	1	0	1
	6	0	1	0	0	1
	7	0	0	1	0	1
	8	0	0	0	0	0
	9	0	0	0	0	0
	10	0	0	0	1	1
	11	0	0	0	0	0
	12	1	0	0	0	1
Total number		2	3	2	2	9
2014	1	0	1	0	0	1
	2	1	0	0	0	1
	3	1	0	0	1	2
	4	0	1	1	0	2
	5	0	1	0	0	1
	6	0	0	0	0	0
	7	0	0	0	0	0
	8	0	0	0	0	0
	9	0	0	0	0	0
	10	0	0	0	0	0
	11	1	2	0	1	4
	12	0	0	1	1	2
Total number		3	5	2	3	13
2015	1	0	0	0	1	1
	2	0	1	0	0	1
	3	0	0	0	0	0
	4	0	0	0	0	0
	5	1	0	1	0	2
	6	1	0	0	1	2
	7	0	0	0	0	0
	8	0	1	1	0	2
	9	0	0	0	0	0
	10	0	2	1	0	3
	11	0	0	0	1	1
	12	1	0	1	0	2
Total number		3	4	4	3	14
2016	1	0	0	1	1	2
	2	0	1	0	1	2
	3	0	0	0	0	0
	4	1	0	0	0	1
	5	0	0	0	0	0
	6	0	0	0	0	0
	7	0	1	0	0	1
	8	0	0	0	0	0
	9	0	0	0	0	0
	10	0	0	0	0	0
	11	0	1	1	0	2
	12	0	0	0	0	0
Total number		1	3	2	2	8

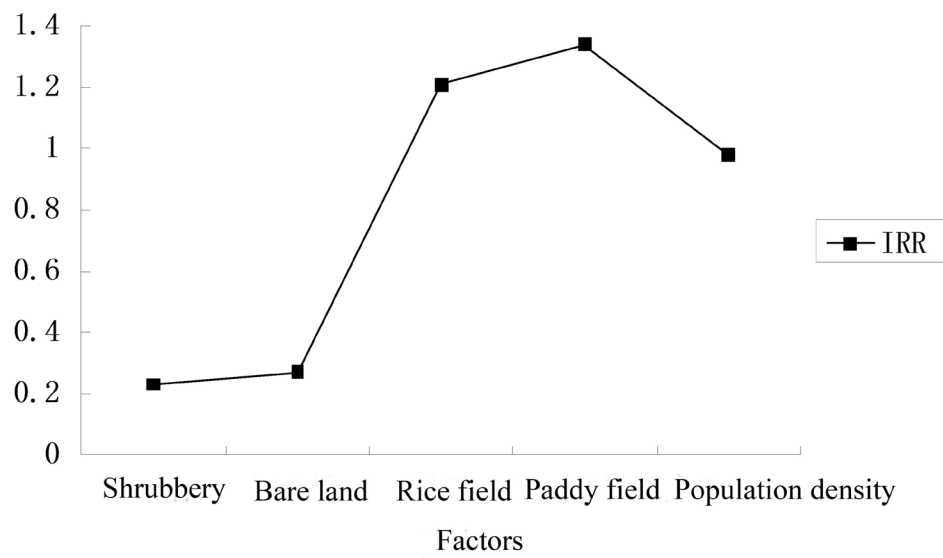


Fig. 1. IRR of different factors.

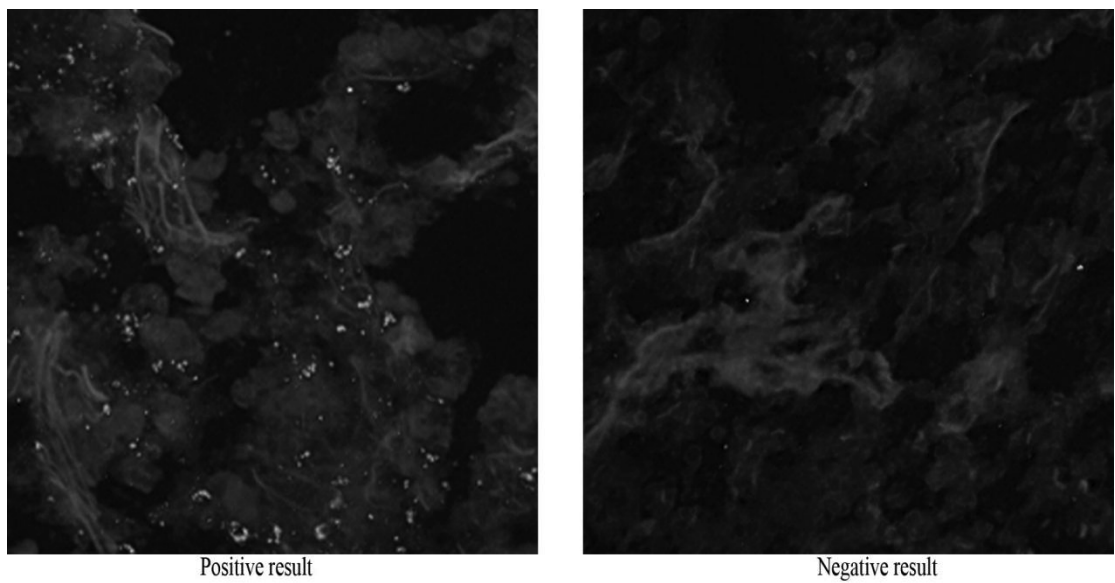


Fig. 2. The comparison of the positive results (left) and negative results (right) (X40).

with that of the other SEOV and 25% with that of Dobrava virus (DOBV). The homology comparison of amino acid sequences showed that the amino acid sequence homology of the two S fragments was 100%, while the homology was 100% with that of other SEOV and 55% with DOBV. The nucleotide homology of PCR products gene sequence of M fragment was 95.2%~99.3% with that of the other SEOV and was about 71% with that of DOBV. Comparison and analysis of amino acid sequence homology indicated that the homology of amino acid sequences in the M fragment was 98%-100% with that of the other SEOV and was 81% with DOBC. Therefore, compared with other SEO, DOB and HV viruses, the amino acid sequences of S and M fragments was more conservative than that of their nucleotide sequences.

DISCUSSION

HV pollutes surroundings and transmits to human beings through excreta of host animals; hence the incidence of HFRS is mainly determined by virus carried rates and distribution ranges of host animals (9). The transmission mode of viruses from rodents to human can be affected by climate, agricultural production, social economy and other external environmental factors. Climate changes in HFRS high-incidence regions had significant influence on HFRS transmission (10). The distribution of diseases caused by HV was unbalanced in different regions due to different population environments, climates, viruses and habitats of rodents (11). The results of this study showed that the incidence of HFRS was related to mean annual temperature and relative humidity. Appropriate climate conditions could lead to the rapid breeding of rodents, especially rats. The activities of the rodent population are correlated to the changes of meteorological factors; however, the correlation may be different when the category of rodents and climatic region are different, which can produce different influences on the transmission of diseases. It has been found that the paddy field is one of the risk factors for HFRS transmission (12). This study made a correlation analysis on the land use type and HFRS related data between 2013 and

2016. The incidence of HV among humans was in a close correlation with land type, and the areas with paddy fields had higher risks. Guangxi is an HFRS epidemic area whose major infection sources are sewer rats and *Mus musculus*. Generally, spring is the high-incidence season of HFRS; however, the epidemic intensity in winter increases, suggesting the existence of epidemic areas with other infection sources. In conclusion, GIS is of great significance to the investigation of time-space characteristics, epidemic rules and genetic sequence of HFRS.

ACKNOWLEDGEMENTS

This work was supported by research grants from the project fund of Guangxi Higher Education Undergraduate Teaching Reform (NO. 2015JGB188).

REFERENCES

1. Connolly-Andersen AM, Hammargren E, Whitaker HJ, et al. Increased risk of acute myocardial infarction and stroke during hemorrhagic fever with renal syndrome: a self-controlled case series study. *Circulation* 2014; 129(12):1295-302.
2. Schlegel M, Jacob J, Krüger DH, Rang A, Ulrich RG. Chapter 10-Hantavirus Emergence in Rodents, Insectivores and Bats: What Comes Next? *Role Anim Emerg Viral Dis* 2014; 39(1):235-92.
3. Boone I, Wagner-Wiening C, Reil D, et al. Rise in the number of notified human hantavirus infections since October 2011 in Baden-Württemberg, Germany. *Euro Surveill* 2012; 17(21):2-6.
4. Li CP, Cui Z, Li SL, et al. Association between hemorrhagic fever with renal syndrome epidemic and climate factors in Heilongjiang Province, China. *Am J Trop Med Hyg* 2013; 89(5):1006-12.
5. Marrama-Rakotoarivony L, Sudre B, Van Bortel W, Warns-Petit E, Zeller H. Annual epidemiological report Emerging and vector-borne diseases. *Eur Cent Dis Prevent Contr*, 2014; 1:37-40.
6. Chung DH, Västermark Å, Camp JV, et al. The murine model for Hantaan virus-induced lethal disease shows two distinct paths in viral evolutionary trajectory with and without ribavirin treatment. *J*

- Virol 2013; 87(20):10997-11007.
7. Ramos C. The hantaviruses causing hemorrhagic fever with renal syndrome and pulmonary syndrome. *Salud Pública De Mexico* 2008; 50(4):334-40.
 8. Marsac D, García S, Fournet A, et al. Infection of human monocyte-derived dendritic cells by ANDES Hantavirus enhances pro-inflammatory state, the secretion of active MMP-9 and indirectly enhances endothelial permeability. *Virol J* 2011; 8(1):1-9.
 9. Kähkönen S, Wilenius J, Komssi S, Ilmonemi RJ. A Phase 1 clinical trial of Hantaan virus and Puumala virus M - segment DNA vaccines for haemorrhagic fever with renal syndrome delivered by intramuscular electroporation. *Clin Microbiol Infect* 2014; 20(S):110-17.
 10. Ling JX, Sironen T, Voutilainen L, et al. Hantaviruses in Finnish soricomorphs: evidence for two distinct hantaviruses carried by *Sorex araneus* suggesting ancient host-switch. *Infect Genet Evolut J Mol Epidemiol Evolut Genet Infect Dis* 2014; 27:51.
 11. Zhang Y, Liu B, Ma Y, et al. Hantaan virus infection induces CXCL10 expression through TLR3, RIG-I, and MDA-5 pathways correlated with the disease severity. *Mediat Inflamm* 2014; 2014(2):85-87.
 12. Gorbunova E, Gavrilovskaya IN, Mackow ER. Pathogenic hantaviruses Andes virus and Hantaan virus induce adherens junction disassembly by directing vascular endothelial cadherin internalization in human endothelial cells. *J Virol* 2010; 84(14):7405-11.

LETTER TO THE EDITOR

A COMPREHENSIVE EVALUATION OF APPLICATION OF LIGHT-CURED COLORED COMPOMER FOR DECIDUOUS TEETH

L-J. YAN, P. CHANG and R-H. TIAN

*Chinese PLA General Hospital, Beijing, People's Republic of China**Received March 9, 2017 – Accepted April 27, 2017*

Twinky Star colored compomer produced by VOCO GmbH is a new type of filling material designed with seven different colors. Previous studies have shown that using the colored filling material can induce children's curiosity and increase the acceptance for dental treatment. However, few studies have been conducted to evaluate it. Hence, the present study was designed to assess the mechanical and adhesive properties of this new compomer, examine its biological safety, and evaluate the effects of its clinical application on children's adherence to dental treatment and the subsequent therapeutic outcomes. We found that Twinky Star compomer has a high compressive strength, good biaxial flexural strength, low wear rate, and good adhesive properties, and had little *in vitro* cytotoxic effects and did not cause apparent hemolysis *in vivo*. Finally, application of the compomer helped to improve children's acceptance of treatment without affecting the subsequent therapeutic outcomes in the follow-up examinations. To summarize, the findings suggest that Twinky Star compomer meets the performance requirements for dental fillings, and can be recommended for future clinical application.

To the Editor,

Tooth decay among children is a major public health problem across the world, particularly in the developing nations (1). One of the most important reasons for poor treatment of deciduous teeth in children, in addition to poor home care compliance, is that children are afraid of dental treatment (2). Therefore, it is very important to motivate these children to receive treatment in order to not only enhance its efficacy but also reduce its overall cost. Interestingly, previous studies have shown that using colored filling material can induce children's curiosity and increase their acceptance of dental treatment (3, 4).

Twinky Star colored compomer combines stable resin and glass ionomer with fluoride release

function, which render the entire filling process simple and rapid. Although this material has been in clinical use for approximately 10 years, the available information in regard to its properties, safety and clinical applicability published in academic literature is limited (5-7). Thus, we assessed the mechanical and adhesive properties of this compomer, examined the biological safety of this filling material, and evaluated the effects of its clinical application on children's adherence to dental treatment and subsequent therapeutic outcomes.

MATERIALS AND METHODS

Evaluation of mechanical properties

Specimens (10 specimens/group) were prepared in

Key words: colored compomer, filling material, tooth decay, biological safety, treatment adherence

Mailing address:

Dr. Ping Chang,
Chinese PLA General Hospital,
28 Fu-Xing Road,
Beijing 100853, People's Republic of China
e-mail: changping_beijing@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.

standard conditions ($h = 6\text{ mm}$, $\Phi = 4\text{ mm}$) using Twinky Star compomer (VOCO GmbH, Germany) or Dyract® eXtra compomer (Dentsply, UK) with 500 W/cm^2 vertical exposure for 40 s (TR30RAM120, Kerr Dental, USA). The specimens were wet polished with 600-grit sandpaper, and the dimensions were measured using a vernier caliper with 0.01 mm accuracy (Guanglu Measuring Instrument, Guilin, China). The specimens were then stored in saline at 37°C for 24 h before testing using a universal mechanical test machine (Instron, Norwood, MA, USA) with 7°C testing temperature, 70% relative humidity, 1 mm/min testing speed, and a 10 kN sensor.

In vitro test of adhesive performance

The root of fresh cow teeth ($n = 20$) that was 2 mm away from the cemento-enamel junction and the pulp of the cow teeth were first removed. The teeth were then embedded, and filled with Twinky Star compomer ($n = 10$; VOCO GmbH, Germany) or Dyract® eXtra compomer ($n = 10$; Dentsply, UK) to form a cylindrical resin (diameter = 4 mm, height $>2\text{ mm}$), followed by water bath at 37°C for 24 h. The prepared specimens were then submitted to a shear bond test, using a universal mechanical test machine (Instron, Norwood, MA, USA). Shear strength $\tau = 4P/\pi d^2$ (τ = shear strength, MPa; P = maximum load applied, N; d = cylinder diameter, mm). Fracture analysis was then carried out under a scanning electron microscope (JSM-6301F, Hitachi, Japan).

Cytotoxicity assay

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assays were performed to test cytotoxicity of Twinky Star compomer material extracts (12.5%, 25%, 50%, or 75%) using L929 cell. In the positive control group, Dulbecco's Modified Eagle's medium (DMEM) containing 0.64% of phenol was added. In the negative control group, fresh DMEM medium was added. Cell morphology was observed under an inverted microscope (CK-40, Olympus, Japan). The absorbance was then measured using a spectrophotometer (Unicom-Optics Instruments, Tsingtao, China) at a wavelength of 490 nm. The percentage of active cells was calculated using the following formula: $S = (S_E^2/E^2 + S_C^2/E^2/C^4)^{1/2}$ (E = the light absorption of the experimental group mean; C = mean light absorption of negative control group; S = standard deviation; S_E = standard deviation of E ; S_C = standard deviation of C).

Hemolysis test

Twinky Star compomer material extract (experimental group), sterile distilled water (positive control), or saline (negative control) were placed in 10-ml centrifuge tubes with 0.2 ml of diluted fresh anticoagulation rabbit plasma, followed by water bath at 37°C for an additional 60 min. After centrifuging at 1000 rpm, the absorbance of the supernatant was measured using a spectrophotometer (Unicom-Optics Instruments, Tsingtao, China) at a wavelength of 540 nm. Each group consisted of three samples.

Short-term systemic toxicity test

Male and female Sprague-Dawley rats ($n = 20$; 10 animals of each gender) weighing about 20 g were provided by the Animal Center at Peking University School of Medicine. Rats were randomly divided into an experimental group fed with Twinky Star compomer material extract (5 ml/kg/day) via oral gavage and control group fed with saline (5 ml/kg/day) using the same method. All rats were fed once daily for 7 consecutive days. The clinical signs of toxicity in rats, including hypokinesia, breathing difficulties, diarrhea, and other symptoms of toxicity or death were observed and recorded daily for an additional 7 days, during which the body weight and food consumption of each animal were recorded, and the food utilization and relative growth rate of each animal were calculated using the following formula: Food utilization rate = [weight gain (g) - total weekly food consumption (g)] $\times 100\%$. Relative growth rate = weight gain (g) / initial weight (g) $\times 100\%$. Animals were sacrificed 7 days later, and pathological analysis was then conducted to examine hemorrhage, edema, or other pathological changes in vital organs including heart, lung, liver, and kidney in each animal. All animal procedures were performed in accordance with the Guidelines of the Chinese Council on Animal Care and approved by the Institutional Animal Care and Use Committee of the university.

Patient general information and collection

Child patients who visited the Pediatric Dental Center of the General Hospital of the Chinese People's Liberation Army from September 2010 to August 2011 were included in the present study.

Patient inclusion criteria: i) 4-10 years of age (at date of enrollment) with at least one active caries lesion in second

primary molar tooth with distinct dentine involvement requiring restoration; ii) good general health with no existing health condition that may interfere with treatment such as asthma, tuberculosis, rheumatic fever/congenital heart diseases, jaundice, juvenile diabetes, haemophilia, thalassemia, leukemia, anemia, HIV, allergy to any drug, and epilepsy; iii) second primary molar tooth with one active caries lesion with distinct dentine involvement requiring restoration.

Patient exclusion criteria: i) a child whose parent did not give informed consent; ii) a child who was unwilling to undergo the dental treatment; iii) a child who has a known allergy to the ingredients contained in dental anesthesia or filling material; iv) having extensive dental caries which may require pulpal therapy.

A total of 568 children, 296 males and 272 females, chose to accept treatment with Twinky Star compomer (experimental group). A total of 814 children, 416 males and 398 females, chose to accept treatment with Filtek Z250 resin (control group). Both groups were divided into shallow or deep caries subgroups according to the degree of caries depth.

Treatment and medical advice

Dental caries were first removed using the conventional method, and the occlusal cavity was then prepared. Before filling, children in the experimental group were given the option of selecting their favorite color of Twinky Star filling (Fig. 1). Children in the control group used Filtek Z250 resin as filling material.

Clinical evaluation

A questionnaire was provided for the children and parents. Behavioral assessment of the children during the dental visit was graded as follows: i) complete refusal of treatment, exhibiting loud crying, fear, uncooperative or other refusal behaviors; ii) existence of 'conflict behavior', willing to accept treatment but showing uncooperative behavior; iii) acceptance with caution, willing to follow the doctor's recommendations; iv) absolute acceptance, showing a good relationship with the doctor, and exhibiting no fear during the treatment process. During the follow up examination, radiographs were taken to assess the conditions of filling and evaluate the putative secondary caries or pulpitis.

Statistics analysis

All quantitative data were represented as the

mean $\pm$ SEM. Data were analyzed using *t*-test, ANOVA, or χ^2 test, where appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Mechanical properties and biological safety of Twinky Star compomer

The results of the mechanical properties of Twinky Star compomer are summarized in Table I. There was no difference of compressive strength, flexural strength, wear rate test, or shear bond strength ($p < 0.01$). The fractured surface was observed using scanning electron microscopy. Before the fracture test, the microscopy revealed that Twinky Star compomer protruded into the resindentin tubules (Fig. 2A). However, after the fracturing, tooth structure was shown on the cross section of fracture surface between Twinky Star compomer and the tooth structure (Fig. 2B and 2C), suggesting that mixed fracture occurred.

The results of biological safety of Twinky Star compomer are shown in Table I and Figs. 2 and 3. Twinky Star compomer extract can be considered non-cytotoxic, according to RGR and cytotoxicity grade. Furthermore, According to GB standards (Chinese national standards issued by the Standardization Administration of China), this compomer extract can be considered not to cause hemolysis, since its hemolysis rate was less than 5% (Table 1). Finally, all 20 rats survived, and all animals were active and responsive, exhibited shiny fur, normal food utilization rate and growth rate. Subsequent visual examination on vital organs including heart, lung, liver, and kidney showed no edema or other abnormal changes.

Color selection and acceptance rate of the parents

In the group of 568 children, choosing Twinky Star compomer, the majority (152: 26.8%) selected pink color, followed by orange color (96: 16.9%).

Of the parents of 568 children who received Twinky Star compomers, 372 (65.5%) preferred this compomer, and 196 (34.5%) accepted both Twinky Star compomer and ordinary resin composite. In the parents of 814 children who chose not to receive

Table I. Mechanical properties and biological safety of Twinky Star compomer.**Compressive strength test**

Group	Compressive Strength (MPa)	P Value
Dyract® eXtra compomer	249.56±19.42	
Twinky Star® compomer	252.39±20.82	$p > 0.05$

Flexural strength test

Group	Flexural Strength (MPa)	P Value
Dyract® eXtra compomer	108.37±18.25	
Twinky Star® compomer	119.17±18.62	$p > 0.05$

Wear rate test

Group	Wear Rate (mm ³ /mm ²)	P* Value	P# Value
<i>In vitro</i> teeth	0.91 ±0.57		
Dyract® eXtra compomer	3.77 ±0.46		$p < 0.01$
Twinky Star® compomer	3.82 ±0.61	$p > 0.05$	$p < 0.01$

* difference between two compomers. # difference relative to *in vitro* teeth.

Shear bond test

Group	Shear Bond Strength (MPa)	P Value
Dyract® eXtra compomer	21.835±5.681	
Twinky Star® compomer	22.741±4.789	$p > 0.05$

L929 cells absorption value (X ± SD; MTT assay)

	75%	50%	25%	12.5%
Twinky Star® (extract)	0.212±0.027	0.299±0.036	0.250±0.033	0.310±0.040

Hemolysis test

Group	Absorption Value			Hemolysis Rate*
	1	2	3	
Experimental group	0.032	0.030	0.028	1.76
Negative Control	0.018	0.017	0.015	0
Positive Control	0.772	0.778	0.775	100

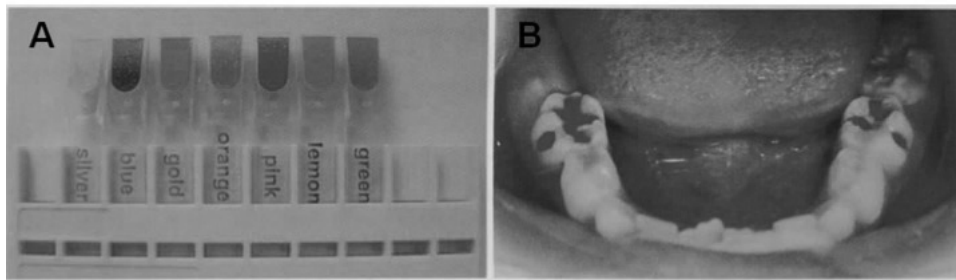
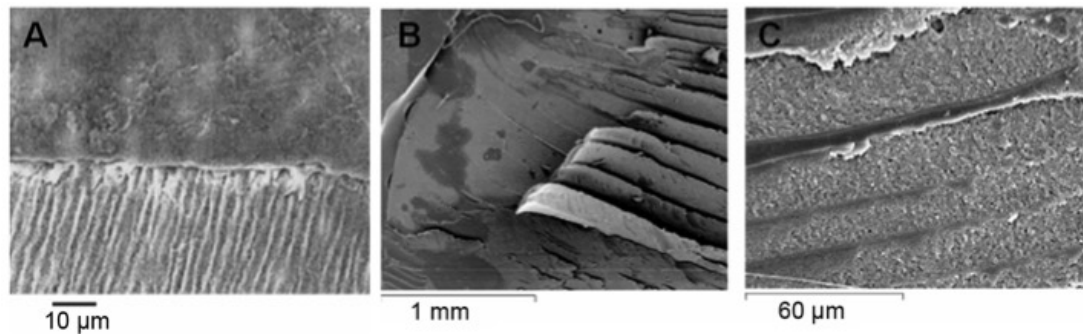
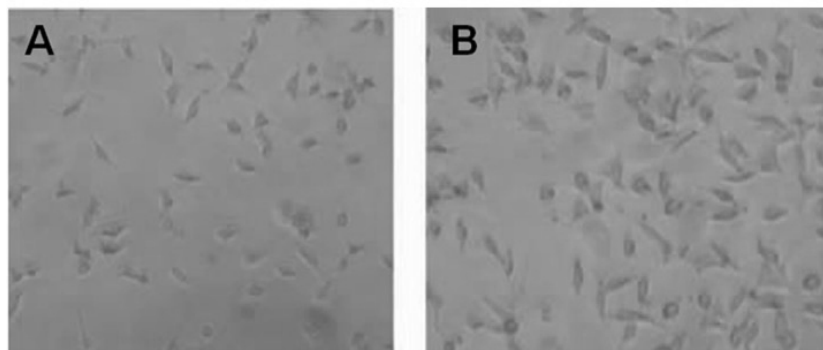
Short-term systemic toxicity test

Group	Food Utilization Rate		Relative Weight Gain Rate	
	1 st week	2 nd week	1 st week	2 nd week
Experimental Group	20.3	20.5	34.2	33.1
Negative Control	21.1	21.0	33.1	33.5

*Hemolysis rate=(value of absorbance in experimental group - value of absorbance in negative control) / (value of absorbance in positive control - value of absorbance in negative control) × 100%.

Table II. *Child patient behavior during the visit.*

Behavior During Visit	Experimental Group		Control Group	
	Shallow Caries	Deep Caries	Shallow Caries	Deep Caries
Complete refusal	45	17	84	35
Existence of <i>conflict behavior</i>	116	76	186	126
Acceptance with caution	159	93	281	64
Absolute acceptance	46	16	38	0

**Fig. 1.** *Representative picture of (A) shade guide and (B) Twinky Star compomer fillings.***Fig. 2.** *Scanning electron microscopy images of Twinky Star compomer bonding on tooth surface (A) before and (B, C) after fracture test.***Fig. 3.** *Cell morphology of L929 cells after (A) 1 day or (B) 2 days of seeding.*

Twinky Star compomer, 312 (38.3%) considered the color of compomer harmful and showed concerns about the color fading, 251 (30.8%) thought the color was unsightly, 169 (20.8%) respected the child's decision, and 82 (10.1%) thought the cost was high.

Child patient behavior during the visit

The results of child patient behavior during the visit are summarized in Table II. The overall treatment acceptance rate in the Twinky Star compomer group is higher than control group ($\chi^2 = 9.07$, $p = 0.001$). More specifically, for patients with shallow caries, treatment acceptance rate in the Twinky Star compomer group was not different from the control group ($\chi^2 = 0.31$, $p = 0.31$). However, for patients with deep caries, treatment acceptance rate in the Twinky Star compomer group was higher than in control group ($\chi^2 = 28.75$, $p = 0.001$).

Treatment outcomes after six months follow-up examination

For the 568 children in the Twinky Star compomer group, 392 (69.01%) had a follow-up visit within six months. Of these 392, 383 (97.70%) did not feel any discomfort. No color change, filling loss, or other symptoms of secondary caries were found in these patients. Seven (1.79%) patients had lost their fillings. Two (0.51%) patients had fillings with bad marginal sealing, and developed secondary caries. Of the 814 children in the control group, 556 (68.30%) had a follow-up visit within six months. Two (0.36%) had lost their fillings. Four (0.72%) developed secondary caries. One (0.18%) showed pulpitis symptoms.

DISCUSSION

Based on the available literature and relevant industrial standards in China, in the present study we demonstrated that Twinky Star compomer possesses excellent mechanical properties. According to Webber assay, the maximum chewing strength in humans is up to about 180 kg/cm² (i.e., 18 MPa), and the jaw force required to chew food in humans is approximately 3-30 kg/cm² (i.e., 0.3-3 MPa) (8). We found that the compressive strength of Twinky Star compomer reached 252.39±20.82 MPa. Flexural

strength refers to the stress in a material just before it yields in a flexure test (9). The biological safety evaluation methods published by the International Organization for Standardization include cytotoxicity test, sensitization test, irritation or skin reaction test, acute systemic toxicity test, and hemocompatibility test (10). Our experimental results show that the Twinky Star compomer has no cell toxicity, does not cause acute hemolysis, and has no short-term systemic toxicity.

Our study has also shown that Twinky Star could be used as an alternative to ordinary resin composite because of its high clinical success after 6 months. Previous studies have shown that the failure rate of the restorations was 3.9% (3 out of 77) using Twinky Star (11). It has been reported that young patients who are allowed to choose the color of their restorations are more likely to accept the idea of treatment. The success of the treatment is aided even further by the dentist's explanation to the children that the fillings will continue to look good as long as the patient maintains them properly (12). Hence, the present study suggested that Twinky Star compomer can meet the performance requirements for dental fillings, and can be recommended for future clinical applications.

REFERENCES

1. Milnes AR. Description and epidemiology of nursing caries. *J Public Health Dent* 1996; 56:38-50.
2. Weinstein P. Public health issues in early childhood caries. *Community Dent Oral Epidemiol* 1998; 26:84-90.
3. Folayan MO, Faponle A, Lamikanra A. Seminars on controversial issues. A review of the pharmacological approach to the management of dental anxiety in children. *Int J Paediatr Dent* 2002; 12:347-54.
4. Fishman R, Guelmann M, Bimstein E. Children's selection of posterior restorative materials. *J Clin Pediatr Dent* 2006; 31:1-4.
5. Nicholson JW. Polyacid-modified composite resins ("compomers") and their use in clinical dentistry. *Dent Mater* 2007; 23:615-22.
6. Ertugrul F, Cogulu D, Ozdemir Y, Ersin N. Comparison of conventional versus colored

- compomers for class II restorations in primary molars: a 12-month clinical study. *Med Princ Pract* 2010; 19:148-52.
7. Botsali MS, Kusgoz A, Altintas SH, et al. Residual HEMA and TEGDMA release and cytotoxicity evaluation of resin-modified glass ionomer cement and compomers cured with different light sources. *ScientificWorldJournal* 2014; 2014:218295.
 8. Bakke M. Mandibular elevator muscles: physiology, action, and effect of dental occlusion. *Scand J Dent Res* 1993; 101:314-31.
 9. Hodgkinson JM. Mechanical testing of advanced fibre composites. Boca Raton, FL Cambridge, England: CRC Press ; Woodhead, 2000.
 10. Moutos FT, Freed LE, Guilak F. A biomimetic three-dimensional woven composite scaffold for functional tissue engineering of cartilage. *Nat Mater* 2007; 6:162-7.
 11. Akbay Oba, A., I. Şaroğlu Sönmez, and Şaziye Sari. Clinical evaluation of a colored compomer in primary molars. *Medical Principles and Practice* 2008; 18(1):31-34.
 12. Croll TP, Helpin ML, Donly KJ. Multi-colored dual-cured compomer. *Pediatr Dent* 2004; 26:2737-36.

LETTER TO THE EDITOR

PRIMARY DIFFUSE LARGE B-CELL LYMPHOMA OF THE FRONTAL SINUS

J-P. YANG¹, C-M. HU², W. ZHONG³ and Z-G. WANG¹

¹Department of Otorhinolaryngology, Head and Neck Surgery, The Second Hospital of Jilin University, Changchun, China; ²Department of Hematologic Oncology, The Second Hospital of Jilin University, Changchun, China; ³Department of Ophthalmology, China-Japan Union Hospital, Jilin University, Changchun, China

Received December 9, 2016 – Accepted May 2, 2017

Primary frontal sinus lymphoma is a rare disease, with the presenting symptoms that are secondary to the tumor mass effect and often misleading. Here we describe the case of a 43-year-old male patient who presented with a 4-week history of a gradually enlarging painful diffuse swelling over the right frontal sinus region. We report the clinical presentation, differential diagnosis and treatment of the case. We also reviewed the available literature on the diffuse large B-cell lymphoma in the frontal sinus region. The article emphasizes the importance of early recognition of this rare disease.

To the Editor,

Lymphoma originating from the paranasal sinuses is rare, with the incidence ranging from 0.17% to 1.63% (1). The analysis of literature demonstrates that the majority of these lymphomas are reported in the maxillary or ethmoid sinuses, while cases of lymphoma arising in the frontal sinus are rare (2-4).

Among the malignancies of frontal sinuses, mature B-cell lymphomas account only for 17.5% of all cases. In fact, only 30 cases of this particular disease are registered in the SEER database in the years from 2000 to 2012 (5). The symptoms of frontal sinus B-cell lymphoma are ambiguous and non-specific, making it rather difficult to diagnose correctly at the initial presentation. There is little clarity on the best treatment approach for the patients with this condition. In addition, due to insufficient statistics, the prognosis is also quite uncertain.

Here we report the case of a diffuse large B-cell lymphoma of the frontal sinus in a 43-year-old male

patient, and briefly review some published data available on this rare condition. The patient provided informed, signed consent to publish the details of his medical history and the results of this study.

Case report

A 43-year-old male patient presented with a gradually increasing painful swelling on the right side of the forehead and occasional headaches over the previous 4 weeks. There were no associated symptoms of nasal congestion, pyrexia or visual disturbances. The patient was administered several courses of antibiotics, which failed to relieve his symptoms. The patient was known to be hypertensive and receiving treatment for high blood pressure.

A physical examination revealed a large, solid 3×3 cm swelling subcutaneous module with restricted mobility on the right side of the forehead (Fig. 1). Nasal endoscopy showed normal mucosa without any discharge. There was no significant

Key words: B-cell, frontal sinus, lymphoma

Mailing address:

Chun-Mei Hu, MD
Department of Hematologic Oncology,
The Second Hospital of Jilin University,
218 Ziqiang Street, Changchun 130041, China
e-mail: lotuschummei@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.

lymphadenopathy. Computed tomography (CT) and magnetic resonance imaging (MRI) of the brain and paranasal sinuses showed a mass involving the right frontal sinus with extension into the anterior wall of the frontal sinus and the skull base. The signals of the mass were isointense on T1-weighted images and hyperintense on T2-weighted images. The lesion showed inhomogeneous enhancement following gadolinium-contrast injection (Fig. 2).

The clinical symptoms and the erosion of the frontal bone gave rise to the suspicion of malignant disease or a metastatic tumor. The patient was taken to the operating room and a Draf III procedure was performed using an upper-eyelid incision. The skin was incised, and the mass was immediately visible. A portion of the mass was sent for intraoperative frozen section examination which showed the features of a malignant neoplasm. After removal of the mass, the erosion of the floor of the frontal sinus was seen. The residual tumor was removed using a curette and a cutting drill.

To drain the frontal sinus, Draf III procedure was performed. The frontal inter sinus septum was removed, and two frontal sinuses were connected (Fig. 3A B). Once this was carried out, the frontal sinus opening became shaped as a crescent. The nasal septum and associated middle turbinate were drilled posteriorly toward the skull base to transform this crescent opening into an oval opening, thereby creating the largest possible anteroposterior and lateral diameters for the frontal ostium. Finally, the entire tumor was removed and sent for histopathologic examination.

Pathologic analysis showed dense polymorphous lymphoid proliferation and presence of large lymphocytes (HE staining) (Fig. 4A). The results of immunohistochemical staining were as follows: CK(AE1/AE3)(-), CgA(-), CD20(+), CD3(-), Syn(+), Bcl-6(+), CD99(+), Mum-1(+), CD10(-), Vim(+), S100(-) and Ki-67 90%. Immunohistochemical study with CD20 revealed the specific antibody for B-cell lymphocytes in tumor cell membranes (Fig. 4B). These pathological features were compatible with the malignant lymphoma of diffuse large B-cell type.

The patient underwent further investigations,

including the positron emission tomography (PET-CT) of the whole body. The results showed that there were no other primary lesions in other organs. The histological, radiological and investigational results were all diagnostic of a primary malignant lymphoma of the frontal sinus. The patient was in IE DLBCL (Ann Arbor classification).

The patient was referred to our hematologic oncology department. Systemic chemotherapy with rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP) was given. Two years after the surgery, the repeat endoscopy showed a nicely healed common opening into both frontal sinuses. The entire area previously involved by the tumor was seen easily with endoscope and there was no evidence of recurrence.

DISCUSSION

The nasal cavities and paranasal sinuses are rarely affected by primary non-Hodgkin lymphoma (NHL). However, in Asian countries the incidence of malignant lymphomas of the paranasal sinuses and nasal cavity is higher compared to the Western nations and accounts for up to 6.7% of all lymphomas (6). Histopathological difference in the sinonasal lymphomas between different ethnic groups was previously reported. In Asian populations, T-cell phenotype is more common and mostly affects the nasal cavity. On the other hand, B-cell phenotype is more common in Western populations (1) where it predominantly affects the maxillary antrum (6), nasal cavity and the ethmoid sinuses (7). Primary diffuse large B-cell frontal sinus lymphomas are extremely uncommon in China.

A review of the English-language literature revealed that primary frontal sinus lymphomas account for 0.3–1% of all paranasal sinus carcinomas and are extremely rare (3). Once typed, most patients had non-Hodgkin's lymphoma of B-cell lineage. The tumor exhibits a higher incidence in older patients, with the average age of 58.5 years at the time of diagnosis, and a male to female ratio of 3:1.

The symptoms of paranasal sinus lymphomas are non-specific and can be mistaken for presentations of other conditions such as mucocoeles, pyoceles,



Fig. 1. Photograph showing 3cm×4cm swelling on the right side of the forehead.

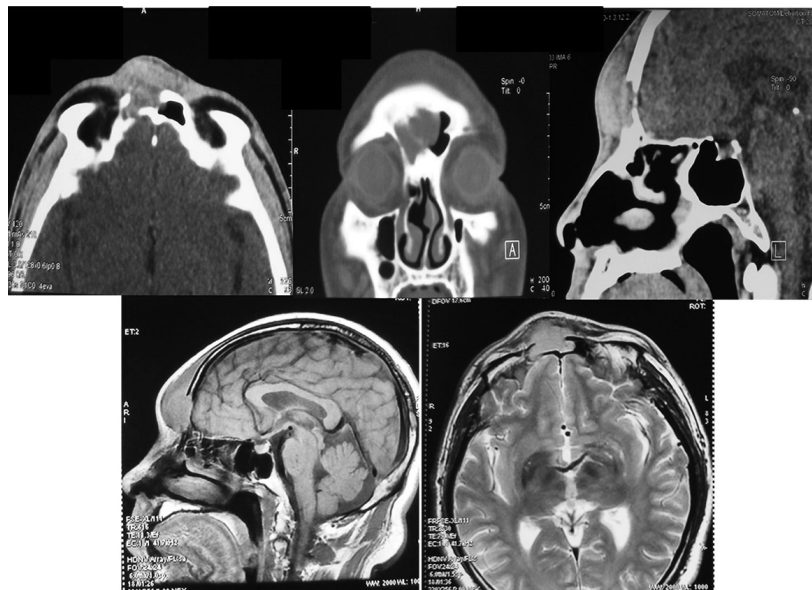


Fig. 2. CT and MRI scans of paranasal sinuses showing a mass that involves the right frontal sinus with extension into the anterior wall of the frontal sinus and the skull base.

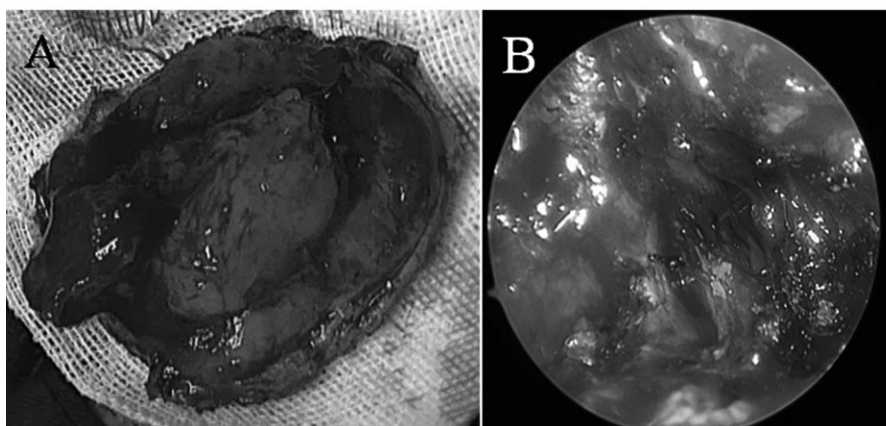


Fig. 3. Photographs showing the tumor in the frontal sinus. **A)** The removed lesion. **B)** The frontal sinus after the lesion removal.

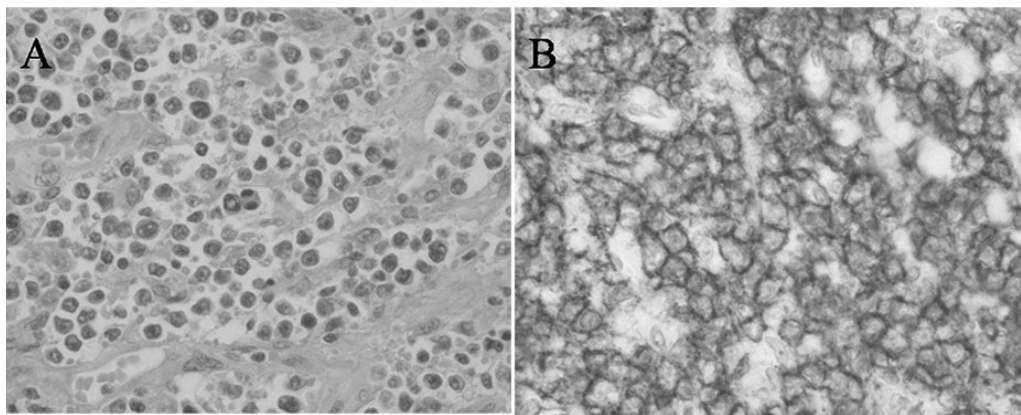


Fig. 4. *A)* Microphotograph showing dense polymorphous lymphoid proliferation and the presence of large lymphocytes (HE staining) (original magnification, $\times 40$). *B)* Microphotograph of immunohistochemical study with antibodies against CD20 showing B-cell lymphocytes in tumor cell membranes (original magnification, $\times 40$).

osteomyelitis, Pott's puffy tumour, cranial nerve palsy or sinusitis (7-9). This often makes it difficult to diagnose these diseases at an early stage. The diagnosis of a malignant tumor in the frontal sinus should also be considered when patients present with several predictors in the CT/MRI scan, including intracranial invasion, destruction of the anterior skull base and deep orbital spread. Patients may benefit from evaluation of the precise extent of frontal sinus tumors, and this may aid in planning the surgical intervention. The CT/MRI scan may help in the early detection of frontal sinus tumor, but does not confirm it. The histopathological study of the resection specimen is required for definite diagnosis.

The diagnosis of diffuse large B-cell lymphoma is based on the histopathologic appearance of the lesion, the immune phenotype of the atypical cells, and the analysis of B-cell receptor genes. In the present case, the pathologic findings showed a large lymphoid infiltration. In order to establish the differential diagnosis, the immunohistochemical studies, such as AE₁/AE₃ and CgA, were carried out, and the results were negative. Therefore, we mainly considered B-cell or T-cell lymphomas. CD20, CD3 and the immunophenotype of the B-cell or T-cells were next detected. CD20 test was positive, thus demonstrating that atypical lymphoid cells had a B-cell phenotype (10, 11).

In the case of our patient, a gradually increasing

painful swelling on the right side of the forehead and occasional headaches of 4-week duration attributed to the involvement of the anterior base of the frontal sinus. Intraoperative frozen section examination only revealed the lesion as a malignant neoplasm. A Draf III procedure was performed using an upper-lid incision. After surgery, the patient underwent systemic chemotherapy with rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone (R-CHOP). After 6 cycles of chemotherapy, his conditions were stable for the next 2 years of observation until the present time.

There is no uniform treatment approach for the primary diffuse large B-cell lymphomas of the frontal sinus. Surgery, chemotherapy and radiotherapy in various combinations were used in the published case reports. Radical surgery appears to be the best method for the disease at stages I-II (12). However, due to complex anatomy of frontal sinuses, the total excision of tumour might be difficult to achieve, especially in the cases when intracranial invasion is present. Combination of surgery and various chemotherapy regimens was beneficial to patients in many of the reported cases. Radiotherapy was used to treat the advanced cases of the disease but there is a disagreement in the literature on the benefits of this approach (13, 14).

The only factors that were found to be independently related to the overall survival in the case of primary

diffuse B-cell lymphomas are the extent of disease and the age of patient (15). Compared to the other malignancies of frontal sinuses, mature B-cell NHLs have the highest 5-year survival rate (72.3%) (5). However, this estimate is based on a small number of cases and has little statistical significance. In general, malignancies of frontal sinuses are associated with rather poor prognosis.

The presented case of primary diffuse large B-cell lymphoma originating from the frontal sinus will contribute further to the knowledge of this rare type of malignancy. Clear differentiation from benign tumors and infectious diseases is of paramount importance for the treatment of this condition. Early diagnosis and appropriate treatment regimen are essential for achieving the optimal patient outcome, and it is imperative that otolaryngologists are familiar with the presentation and management of this condition.

REFERENCES

1. Quraishi MS, Bessell EM, Clark D, Jones NS, Bradley PJ. Non-Hodgkin's lymphoma of the sinonasal tract. *Laryngoscope* 2000; 110:1489-92.
2. el-Hakim H, Ahsan F, Wills LC. Primary non-Hodgkin's lymphoma of the frontal sinus: how we diagnosed it. *Ear Nose Throat J* 2000; 79:738, 741-3.
3. Gerlinger I, Gobel G, Toth E, Szanyi I, Weninger C. Primary carcinoma of the frontal sinus: a case report and a review of literature. *Eur Arch Otorhinolaryngol* 2008; 265:593-97.
4. Haksever M, Gundogdu E, Akduman D, Haksever H, Karaca K, Ekici MA, Solmaz F. Non-Hodgkin lymphoma originating from the frontal sinus. *J Craniofac Surg* 2014; 25:e349-50.
5. Bhojwani A, Unsal A, Dubal PM, Echanique KA, Baredes S, Liu JK, Eloy JA. Frontal sinus malignancies: a population-based analysis of incidence and survival. *Otolaryngol Head Neck Surg* 2016; 154:735-41.
6. Shohat I, Berkowicz M, Dori S, Horowitz Z, Wolf M, Taicher S, Talmi YP. Primary non-Hodgkin's lymphoma of the sinonasal tract. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2004; 97:328-31.
7. Zhang HZ, Li YP, She L, Wang XD, Yan ZC, Zhang N, Xu EX. Primary carcinoma of the frontal sinus with extensive intracranial invasion: A case report and review of the literature. *Oncol Lett* 2014; 7:1915-18.
8. Robinson JM. Frontal sinus cancer manifested as a frontal mucocele. *Arch Otolaryngol* 1975; 101:718-21.
9. Reddy KT, Gilhooly M, Wallace M, Ali MH. Frontal sinus carcinoma presenting as acute sinusitis. *J Laryngol Otol* 1991; 105:121-22.
10. Gourin CG, Johnson JT, Selvaggi K. Nasal T-cell lymphoma: case report and review of diagnostic features. *Ear Nose Throat J* 2001; 80:458-60.
11. Tilly H, Gomes da Silva M, Vitolo U, et al. Diffuse large B-cell lymphoma (DLBCL): ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2015; 26(Suppl 5):v116-25.
12. Guntinas-Lichius O, Kreppel MP, Stuetzer H, Semrau R, Eckel HE, Mueller RP. Single modality and multimodality treatment of nasal and paranasal sinuses cancer: a single institution experience of 229 patients. *Eur J Surg Oncol* 2007; 33:222-28.
13. Rischin D, Porceddu S, Peters L, Martin J, Corry J, Weih L. Promising results with chemoradiation in patients with sinonasal undifferentiated carcinoma. *Head Neck* 2004; 26:435-41.
14. Dirix P, Nuyts S, Geussens Y, et al. Malignancies of the nasal cavity and paranasal sinuses: long-term outcome with conventional or three-dimensional conformal radiotherapy. *Int J Radiat Oncol Biol Phys* 2007; 69:1042-50.
15. Logsdon MD, Ha CS, Kavadi VS, Cabanillas F, Hess MA, Cox JD. Lymphoma of the nasal cavity and paranasal sinuses: improved outcome and altered prognostic factors with combined modality therapy. *Cancer* 1997; 80:477-88.

LETTER TO THE EDITOR

CLINICAL OBSERVATION OF THE EFFECTS OF THE SUHUANG ZHIKE CAPSULE
ON ACUTE BRONCHITIS

J. YANG

*Department of Lung Disease, Zhengzhou Traditional Chinese Hospital, Zhengzhou, China**Received October, 2016 – Accepted May 3, 2017*

Acute bronchitis is one of the most common diseases in the world. The Suhuang Zhike capsule is a part of Traditional Chinese Medicine (TCM) that was approved to treat acute bronchitis. In order to study the clinical effects of the Suhuang Zhike capsule on acute bronchitis subjects, the clinical data of 96 patients from the Department of Lung Disease of Zhengzhou Traditional Chinese Hospital from June 2014 to June 2014 were retrospectively analyzed. The patients were randomly divided into a control group and a Suhuang Zhike capsule treatment group, each with 48 cases. The therapeutic effects of the treatments were evaluated on the tenth day. The results showed that the total effective rate of the observation group was 95.83%, which was significantly higher when compared with the control group, which was 72.91% ($P < 0.005$). The clinical signs (e.g. coughing, itchy throat, dry cough without phlegm, little sputum) of the two groups obviously decreased, and the difference was statistically significant when compared with the pretreatment scores ($P < 0.005$). The decreased rate of the observation group was significantly larger when compared with the control group ($P < 0.005$). The time it took for the clinical symptoms of the observation group to resolve was significantly shorter when compared with the control group ($P < 0.005$). After treatment, the serum levels of c-reactive protein (CRP), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor alpha (TNF- α) of the two groups obviously decreased. There was a significant difference in these serum levels when compared with pre-treatment levels ($P < 0.005$). The decreased rate of the observation group was obviously larger when compared with the control group ($P < 0.005$). In the observation group, there were 3 cases with adverse reactions (nausea in 2 cases and mild vomiting in 1 case) during the observation period, with an adverse reaction rate of 6.25%. In the control group, 10 cases developed adverse reactions (nausea in 2 cases, mild diarrhea in 3 cases, and mild vomiting in 5 cases), with an adverse reaction rate of 20.83% ($P < 0.005$, vs the observation group). In this study, we showed that the Suhuang Zhike capsule provides positive therapeutic effects in the treatment of acute bronchitis by promoting the alleviation of the clinical symptoms in a short time and reducing the inflammation in the respiratory tract.

To the Editor,

Acute bronchitis is one of the most common diseases in the world, particularly for middle-aged people. The main symptoms are coughing, dry coughing without phlegm, pharyngeal itching, which

cause serious impacts on patients' lives. Currently, acute bronchitis is treated mainly by relieving coughing, reducing sputum, and controlling infections, but such therapeutic methods can induce relapse. In addition, the increasing number of drug-

Key words: Suhuang Zhike capsule, acute bronchitis, clinical effect

Mailing address:

Dr Jie Yang,
Department of Lung Disease,
Zhengzhou Traditional Chinese Hospital,
No. 65, Wenhuaogong Road, Zhengzhou, 450007, China
e-mail: yangjieyj6@126.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.**

resistant strains, adverse reactions, and side effects caused by routine Western treatments make it difficult to treat. Previous research has shown that the Suhuang Zhike capsule is an aggregation of decades of clinical experience from distinguished veteran doctors of traditional Chinese medicine (TCM) in treating acute bronchitis, based on the theory of “coughing caused by wind” (1). The capsule functions by eliminating pathogenic factors, preventing phlegm from forming, and alleviating coughing (1). This study aimed to evaluate the clinical effects of the Suhuang Zhike capsule by analyzing the clinical data of 96 patients with acute bronchitis from June 2014 to June 2015 in our hospital.

MATERIALS AND METHODS

General information

The clinical data of 96 patients diagnosed with acute bronchitis in the Zhengzhou Traditional Chinese Hospital, Zhengzhou, China, from June 2014 to June 2015 were randomly divided into a control group and a treatment group, with 48 patients in each group. Among the control group, there were 28 males and 20 female, aged from 25-56 years (average 27.56 ± 0.61 years), with disease courses of 1-6 days (an average of 2.54 ± 0.13 days). In the observation group, there were 30 males and 18 females, aged from 27-51 years (an average of 28.84 ± 1.24 years), with disease courses of 2-7 days (an average of 3.08 ± 0.21 days). There were no significant differences in the age, gender, or disease course length ($P > 0.005$), therefore, the two groups were comparable.

Diagnostic criteria of Western medicine

The patients diagnosed with acute bronchitis after undergoing a normal chest X-ray or lung CT had a normal hemogram, slightly elevated leukocyte levels with no fever or chest pain, cough, itchy throat, and little to no phlegm. These diagnoses were made according to the Diagnostic Code of Acute Bronchitis (ICD-10 Diagnosis Code J20).

Diagnostic criteria of TCM

The patients were diagnosed with acute bronchitis when they displayed symptoms such as cough, whoop, little to no phlegm, aversion to cold, cold limbs, pale

foamed phlegm, itchy throat, fever, no dry mouth, nasal obstruction, snuffle, or pale, thinly-coated tongue, pale and greasy coated tongue, and a floating or gliding pulse. The diagnoses were made in consideration with the guiding principles of new TCM (2, 3).

Inclusion criteria

The patients were recruited for the study if they met the criteria in the Diagnostic Code of Acute Bronchitis or Guiding Principle of New Traditional Chinese Medicine and their disease course was no more than 7 days. In addition, all the included patients signed an informed consent.

Exclusion criteria

The patients were excluded if they were diagnosed with or exhibited symptoms of tuberculosis, pneumoconiosis, lung abscess, bronchial asthma, bronchiectasis, heart disease, heart failure, chronic nasopharyngeal diseases, severe cardiovascular diseases, renal failure, pulmonary diseases, tumors, organ dysfunction, or mental disorders.

Control group

The patients in the control group were given the regular treatment (e.g. antibiotics, treatment to reduce phlegm and relieve coughing). The treatment lasted for one course. The patients were forbidden from eating spicy food, smoking, and drinking during the therapeutic period.

Observation group

The 48 cases in the observation group were given the regular treatment and the Suhuang Zhike capsule (Yangtze River Pharmaceutical Group, Z201103124, Batch No. 20131109). The patients were given three tablets three times a day, lasting one course. Also, the patients were forbidden from eating spicy food, smoking, and drinking during the therapeutic period.

Observation indexes

There were several indexes which required evaluation in this study. These indexes were the total effective rate, coughing, itchy throat, rale in the lungs, little phlegm, the resolution time of the clinical symptoms, serum levels of CRP, IL-6, IL-8, and TNF- α , and adverse reaction rates in the patients.

Efficacy determination

There were four standards for determining the therapeutic effects on patients: complete recovery, meaning that the clinical symptoms were completely resolved; improving, meaning that the clinical symptoms were greatly improved; and ineffective, meaning that the clinical symptoms were unchanged or worsened. These standards were based on the Guiding Principle of New Traditional Chinese Medicine (2, 3).

In this study, the clinical symptoms included coughing, itchy throat, dry coughing without phlegm, little phlegm, and wheezing. Coughing, little phlegm, and wheezing were divided into four grades of 0, 2, 4, and 6, which reflected normal, mild, medium, and severe degrees, respectively. Itchy throat and dry coughing without phlegm were divided into three grades of 0, 1, and 2, which reflected normal, mild, and severe degrees, respectively (4).

Laboratory examination

Immunohistochemical quantity analysis was used to analyze the serum levels of CRP in the patients. The high-sensitivity CRP detection kit was purchased from Shanghai Biochemical Co., Ltd., and the batch number was 20130416.

IL-6, IL-8, and TNF- α were detected using an automatic biochemical detector (Beckman, UK) (5). The immunofluorescence kit was from Jiangsu Biochemical Co., Ltd. and the batch number was 20100415.

RESULTS

After treatment, the total effective rate of the observation group was 95.83%, which was significantly higher than the rate of 72.91% in the control group ($P<0.005$; Table I).

The two groups displayed significantly fewer clinical symptoms when compared with pretreatment levels ($P<0.005$). Also, the lowering rate of the observation group was significantly higher when compared with the control group ($P<0.005$). The results are shown in Table II.

The resolution time of the clinical symptoms of the observation group was significantly shorter compared to the control group, and the difference was statistically significant ($P<0.005$; Table III).

Serum levels of CRP

The CRP levels of the two groups (9.42 ± 2.37 mg/L in the control group and 6.39 ± 3.78 mg/L in the observation group) were significantly lower when compared to pretreatment levels (57.39 ± 16.54 mg/L in the control group and 60.22 ± 19.66 mg/L in the observation group), and the difference was statistically significant ($P<0.005$). Also, the decreasing rate of CRP level in the observation group was much higher when compared with the control group ($P<0.005$).

Serum levels of IL-6, IL-8, and TNF- α

The serum levels of IL-6, IL-8, and TNF- α of the two groups decreased significantly compared with the pretreatment levels, and the difference was statistically significant ($P<0.005$). The decreased rates of serum levels of IL-6, IL-8, and TNF- α in the observation group were higher compared with the control group, and the difference had statistical significance ($P<0.005$). The results are shown in Table IV.

Adverse effects

During the observational period, 3 patients had adverse reactions (2 with nausea and 1 with mild vomiting) in the observation group, with an adverse reaction rate of 6.25%. In the control group, 10 patients who had adverse reactions, including 2 cases of nausea, 3 of mild diarrhea, and 5 of mild vomiting. The adverse reaction rate was 20.83%. The difference between the two groups was statistically significant ($P<0.005$).

DISCUSSION

Bronchitis is one of the most common clinical diseases in the world. Its pathogenesis is not clear, but it is closely related to the patient's general health, age, virus invasion levels, climate, etc. The continuous development of the illness may induce various other diseases, seriously impacting the quality of life of the patient. Previous research has shown that the mucous membranes simultaneously induced by various factors will develop edemas, cell fiber expansion, severe lymphocyte infiltration,

Table I. *The total effective rate of the two groups.*

Groups	Complete recovery	Better	Noneffective	Total effective rate
Control group	24 (50.0%)	11 (21.91%)	13 (27.08%)	35 (72.91%)
Observation group	28 (58.33%)	18 (37.50%)	2 (4.16%)	46 (95.83%)*

(n=48, %). *compared with the control group, $P<0.005$

Table II. *Comparison of clinical symptoms of the two groups before and after treatment.*

	Control group		Observation group	
	Before treatment	After treatment	Before treatment	After treatment
Cough	4.86±0.76	1.18±0.36 [△]	4.91±0.78	0.61±0.27* [△]
Itching throat	3.98±0.58	0.96±0.28 [△]	3.90±0.57	0.42±0.19* [△]
Dry cough without phlegm	1.88±0.56	0.41±0.22 [△]	1.86±0.48	0.21±0.08* [△]
Little phlegm	4.69±0.73	1.04±0.37 [△]	4.71±0.76	0.56±0.24* [△]

(n=48, $\bar{x}\pm s$). *compared with the control group, $P<0.005$; [△]compared with pretreatment levels, $P<0.005$

Table III. *Comparison of the resolving time of the clinical symptoms of the two groups.*

Groups	Cough	Itching throat	Dry cough without phlegm	Less phlegm
Control group	5.58±1.67	4.61±1.47	5.37±1.62	4.98±1.79
Observation group	4.51±1.26*	3.33±1.04*	4.21±1.16*	3.56±1.01*

(n=48, $\bar{x}\pm s$, d). *compared with the control group, $P<0.005$

Table IV. *Changes of serum levels of IL-6, IL-8 and TNF- α of the two groups before and after treatment.*

Groups		IL-6 (ng/L)	IL-8 (μ g/L)	TNF- α (pg/mL)
Control group	Before treatment	88.62±7.58	5.24±1.17	40.81±6.73
	After treatment	28.75±4.51 [△]	2.83±0.79 [△]	26.26±4.19 [△]
Observation group	Before treatment	90.01±8.12	5.33±1.11	41.18±6.72
	After treatment	21.76±3.66* [△]	1.24±0.67* [△]	17.62±4.13* [△]

(n=48, $\bar{x}\pm s$). *compared with the control group, $p<0.005$; [△]compared with pretreatment levels, $p<0.005$.

bronchial fiber cell growth, and decreased alveolar elasticity, finally leading to coughing, an urticant pharynx, and dry coughing (4, 6).

Acute bronchitis belongs to the category of “coughing” in TCM. The functional disorder of the visceral organs will seriously affect the normal operation of water and other liquids, resulting in the secretion of phlegm. Therefore, expelling wind and coldness, relieving coughing, and reducing sputum are the main methods for treating acute bronchitis in TCM (7). This study chose the Suhuang Zhike capsule to treat acute bronchitis patients, taking into consideration its positive effects in relieving coughing and reducing sputum. The lumbricus in the Suhuang Zhike capsule cools and detoxifies the patient. The *Peucedani* relieves coughing and reduces sputum, *Perilla frutescens* warms the patient, *Ephedra* herb relieves coughing and asthma, and almond relieves coughing and asthma, and prevents infection. All these functions play effective roles in recovering organ function and preventing relapse.

The results of this study demonstrated that the total effective rate of the observation group was 95.83%, which was significantly higher than the control group rate of 72.91%. The difference was statistically significant ($P < 0.005$). The clinical symptoms displayed by patients in the observation group obviously decreased after treatment, as did the serum levels of CRP, IL-6, IL-8, and TNF- α . Thus, the Suhuang Zhike capsule was able to decrease the expression of the clinical symptoms in the patients in a timely manner by effectively reducing the serum levels of IL-6, IL-8, and TNF- α . This inhibited a series of immune responses induced by inflammatory cytokines, reducing injury to the respiratory tract (8).

The Suhuang Zhike capsule displays positive

effects in treating acute bronchitis. The capsule relieves coughing, itchy throat, and pain rapidly, and has a lower adverse reaction rate than commonly used Western medicine. Therefore, it should be used in the clinical treatment of acute bronchitis.

REFERENCES

1. Ding P, Wang Q, Yao J, Zhou XM, Zhu J. Curative Effects of Suhuang Zhike Capsule on postinfectious cough: a meta-analysis of randomized trials. *Evid Based Complement Alternat Med* 2016; 2016:8325162.
2. Chan K. Chinese medicinal materials and their interface with Western medical concepts. *J Ethnopharmacol* 2005; 96(1-2):1-18.
3. Tang JL, Wong TW. The need to evaluate the clinical effectiveness of traditional Chinese medicine. *Hong Kong Med J* 1998; 4(2):208-10.
4. Albert RH. Diagnosis and treatment of acute bronchitis. *Am Fam Physician* 2010; 82 (11):1345-50.
5. Kuo HL, Chou CY, Liu YL, Yang YF, Huang CC, Lin HH. Reduction of pro-inflammatory cytokines through hemodiafiltration. *Renal Failure* 2008; 30(8):796-800.
6. Baradhi KM, Ahmed S, Buford ML. The case: acute renal failure after antibiotic treatment for bronchitis. Diagnosis: Pigment nephropathy from cephalexin-induced immune hemolytic anemia. *Kidney Int* 2015; 87 (6):1269-70.
7. Dashtdar M, Dashtdar MR, Dashtdar B, Kardi K, Shirazi MK. The concept of wind in traditional Chinese medicine. *J Pharmacopuncture* 2016; 19(4):293-302.
8. Newton AH, Cardani A, Braciale TJ. The host immune response in respiratory virus infection: balancing virus clearance and immunopathology. *Semin Immunopathol* 2016; 38(4):471-82.

LETTER TO THE EDITOR

**APPLICATION OF OXALIPLATIN IN COMBINATION WITH EPIRUBICIN IN
TRANSCATHETER ARTERIAL CHEMOEMBOLIZATION IN THE TREATMENT OF
PRIMARY LIVER CARCINOMA**

G. CHANG, LL. XIE, WY. LI, FF. XIA, PC. HUANG, QR. LIU, CD. ZHANG,
TJ. ZHANG and HF. LI

Interventional Vascular Surgery, Binzhou People's Hospital, Binzhou, China

Received March 6, 2017 – Accepted April 26, 2017

Many cases of liver carcinoma miss the opportunity of surgical treatment because of hidden onset and delayed diagnosis. In recent years, interventional treatment has gradually become a non-surgical method for treating liver carcinoma. To discuss the effects of oxaliplatin in combination with epirubicin in the treatment and its influence on prognosis, this study randomly selected 218 advanced primary liver carcinoma patients from Binzhou People's Hospital, Binzhou, China and divided them into a control group (n=109) and an observation group (n=109). Patients in both groups were given interventional treatment. Patients in the control group were perfused with oxaliplatin, while patients in the observation group were perfused with oxaliplatin and epirubicin. The effects and 6-month and 12-month survival rates were compared between the two groups. The results demonstrated that the overall effective rate and clinical benefit rate of the observation group were much higher than those of the control group (30.3% vs 11.9%; 79.8% vs 44.3%) ($P<0.05$). The serum Alpha Fetal Protein (AFP) and Carcino Embryonic Antigen (CEA) levels of the observation group were much lower than those of the control group; the Karnofsky performance score of the observation group was much lower than that of the control group; the two differences had statistical significance ($P<0.05$). The 6-month survival rate of the observation group was 91.67%, higher than that of the control group (86.11%) ($P>0.05$). The 12-month survival rate of the observation group was 83.33%, much higher than that of the control group (61.11%) ($P<0.05$). The difference of the incidence of adverse reactions between the two groups had no statistical significance ($P>0.05$). Thus, it can be concluded that oxaliplatin in combination with epirubicin can improve survival quality, extend survival time, and decrease the serum AFP and CEA levels in the treatment of primary liver carcinoma, with definite effects but without aggravating toxic and side effects. Therefore, the therapy has important clinical value.

To the Editor,

Primary liver carcinoma is a commonly seen malignant tumor in current clinical studies. The number of cases with five-year survival rate is low as it is featured by hidden onset, inconspicuous symptoms and rapid development (1, 2), therefore,

most lesions are at the advanced stage or even have distant metastasis when being definitely diagnosed.

Interventional treatment is a frequently used non-surgical method for treating liver carcinoma, which can effectively control the growth of carcinoma but is also featured by simple surgery and little

Key words: transcatheter arterial chemoembolization, liver carcinoma, oxaliplatin

Mailing address:

Dr Gang Chang,
No.515 Huanghe 7th Road,
Interventional vascular surgery,
Binzhou People's Hospital, Binzhou, Shandong, 256610, China
e-mail: chang123_g@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.**

trauma (3). Interventional treatment is an important method for primary carcinoma patients who are unable to tolerate surgery; however, the selection of drugs in interventional treatment is different in different areas. Oxaliplatin is a platinum drug with extensive anti-tumor effects. Clinical trials have found that oxaliplatin is effective in the treatment of multiple tumors; oxaliplatin can form interstrand and intrastrand cross-linking by acting with DNA of tumor cells to inhibit its synthesis and generate cytotoxic effects and anti-tumor activity (4). Epirubicin is a commonly used anti-tumor chemotherapeutic drug in clinics, belonging to the category of anthracycline antibiotics. As the major drug used in tumor chemotherapy, epirubicin can intervene in the cell cycle and inhibit DNA replication. The application of epirubicin in the treatment of liver carcinoma can effectively prevent the proliferation and deterioration of malignant tumor cells and extend the survival time of patients (5, 6). One study (7) demonstrated that it is effective to treat primary liver carcinoma with adriamycin and its derivatives, but the overall response rate was lower than 20%. To investigate the effects of oxaliplatin in combination with epirubicin in primary liver carcinoma interventional treatment, 218 patients were selected from the Binzhou People's hospital for this study.

MATERIALS AND METHODS

Case selection

Two hundred and eighteen patients with moderate and advanced liver carcinoma who were admitted to the Binzhou People's Hospital, Binzhou, China from February 2014 to June 2015 were enrolled. All the cases conformed to the diagnostic criteria and clinical staging of primary liver carcinoma approved by the 8th National Liver Carcinoma Conference (2001) based on clinical performance, associated laboratory indexes and imaging performance (8). The study was approved by the ethics committee of the hospital, and the patients all signed informed consent. The inclusion criteria were as follows: cytologically or pathologically diagnosed as primary advanced liver carcinoma; unable to tolerate surgical resection; TNM stage $\geq$ III; Child Pugh grade A and ECOG score $\leq$ 2 points; count of white blood

cells $>4 \times 10^9/L$ and count of blood platelet $>100 \times 10^9/L$; Karnofsky performance status (KPS) score >50 points; estimated survival time >3 months. Patients who had brain metastasis of liver carcinoma or severe cerebral diseases, had mental abnormality or poor compliance, had other malignant tumors, and pregnant or breastfeeding females were excluded.

Therapeutic method

All the patients underwent interventional embolization under the state of local anesthesia. The right arteria femoralis was punctured using the Seldinger technology and inserted with a 5F arterial sheath. Then a 5F cobra tube was inserted for left or right hepatic arteriography. A microtubule was super-selected to tumor vessels. Patients in the control group were given oxaliplatin (50 mg each bottle, batch no.: 13124, Sanofi Company, France); 130 mg/m² oxaliplatin was dissolved in 5% glucose solution, and then it was slowly perfused. Patients in the observation group were perfused with 35 mg/m² epirubicin (10 mg each bottle, batch no.: X20000496; Pfizer Pharmaceutical (Wuxi Co., Ltd., China) in addition to the same treatment given to the control group; afterwards, the mixture of ioversol and gelfoam particles were injected. The drug perfusion of the next lesion was performed when tumor staining disappeared. The dose of drugs and embolic agents were adjusted according to the size of tumors and hepatic and renal function. Four weeks were regarded as one treatment course. Patients in both groups underwent abdominal CT reexamination after two treatment courses, and the curative effects were evaluated.

Observation index

The effects of the two groups were recorded, and quality of life of the patients was scored. Five ml of venous blood was collected from each patient in the morning, prior to the treatment and after treatment; without the addition of anticoagulant, the blood was kept at room temperature. One hour later, the blood was centrifuged for 10 min, and the supernate was collected. The levels of serum Alpha Fetal Protein (AFP) and Carcino Embryonic Antigen (CEA) were detected using enzyme-linked immunosorbent assay (ELISA) kit (Boster Bioengineering Co., Ltd., Wuhan, Hebei, China). After 6-month treatment, the patients were followed-up; the 6-month and 12-month survival rates were calculated.

The toxic and side reactions were evaluated according to the criteria in the National Cancer Institute Common Terminology Criteria Adverse Events (WHO).

Assessment criteria for curative effects

Curative effects were evaluated according to the modified response evaluation criteria in solid tumors (mRECIST) four to eight weeks after surgery. The curative effects included progressive disease (PD), stable disease (SD), partial remission (PR) and complete remission (CR). SD, PR and CR lasting for four weeks were regarded as effective results. The patients underwent reexamination in the 3rd and 6th month after treatment to observe the changes of tumor lesions. The formula of clinical benefit rate was: CBR=SD+PR+CR.

After treatment, Karnofsky performance scale (KPS) was performed for patients in the two groups; higher KPS scores indicated higher survival rate.

Statistical analysis

The data were recorded and compared using SPSS ver. 21.0. Measurement data were expressed as mean±standard deviation (SD) and processed by *t*-test. Enumeration data were expressed by n (%) and processed by *Chi*-squared test. Difference was considered as statistically significant if $P < 0.05$.

RESULTS

Comparison of general data between the two groups

The difference of general data between the two groups had no statistical significance ($P > 0.05$, Table I).

Comparison of clinical effects between the two groups

The ORR and CBR of the observation group were much higher than those of the control group (30.3% vs 11.9%; 79.8% vs 44.3%, respectively) ($P < 0.05$, Fig. 1).

Comparison of serum AFP and CEA levels and KPS score of the two groups

The serum AFP and CEA levels of the observation group were (416.52±10.89) mg/L and (254.57±50.72) mg/L respectively, much lower than those of the control group [(468.34±11.89) mg/L and (314.56±69.84) mg/L], and the differences were statistically significant ($P < 0.05$). The KPS score of the observation group was much higher than that of the control group [(76.86±6.75) points vs (72.79±5.83) points], and the difference had statistical significance ($P < 0.05$, Table II).

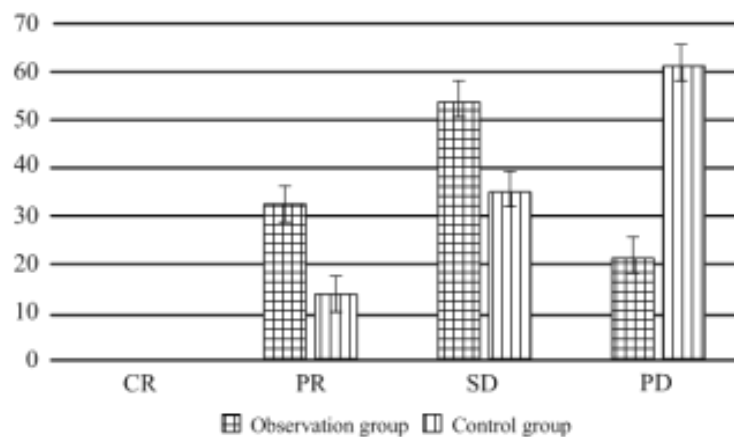
Table I. Comparison of general data between the two groups.

Item	Observation group (n=109)	Control group (n=109)
Age (year)	53.58±6.27	53.42±6.17
Sex (M/F)	83/26	77/32
Height (cm)	164.85±8.09	164.49±7.76
Body mass index (BMI) (kg·m ⁻²)	25.02±2.41	25.31±2.28
TNM-staging (stage III/IV)	40/69	49/60
Child-Pugh (A/B)	83/26	84/25

Table II. The comparison of serum AFP and CEA levels and KPS score of the two groups before and after treatment.

		Observation group	Control group	t	P
AFP/(mg·L ⁻¹)	Before	597.86±16.62	600.92±15.68	0.926	>0.05
	After	416.48±10.91*	472.26±11.86*	19.284	<0.05
CEA/(mg·L ⁻¹)	Before	504.68±87.37	505.72±89.14	0.021	>0.05
	After	253.46±50.68*	315.51±70.03*	4.168	<0.05
KPS score/point	Before	64.83±4.64	65.43±3.87	0.543	>0.05
	After	77.56±6.72*	72.81±5.82*	2.738	<0.05

* $P < 0.05$, compared to before treatment

**Fig. 1.** Comparison of clinical effects between the two groups.

Comparison of 6-month and 12-month survival rate between the two groups

All the patients of the two groups were successfully followed up in the 6 months after surgery; one hundred cases in the observation group and 95 cases in the control group survived (91.7% vs 87.2%), and there was no significant difference between the two groups ($\chi^2=0.141$, $P>0.05$). One hundred cases in the observation group and ninety-five cases in the control group were successfully followed-up at 12 months after surgery; ninety-three

cases in the observation group and sixty-six cases in the control group survived (85.3% vs 60.6%), and the difference had statistical significance ($\chi^2=4.432$, $P>0.05$). The deaths were all due to liver carcinoma.

Comparison of the incidence of adverse reactions between the two groups

In the observation group, 12 patients reported symptoms of nausea and vomiting, 6 patients had reduced white blood cells, 4 patients had abnormal peripheral nerve sensation, and 6 patients had

reduced blood platelets; a total of 28 patients were observed with adverse reactions (25.7%); in the control group, 14 patients had symptoms of nausea and vomiting, 7 patients had reduced blood cells, 6 patients had abnormal peripheral nerve sensation, 6 patients had abnormal peripheral nerve sensation, and 5 patients had reduced blood platelets; adverse reactions occurred in 32 patients (29.4%). The difference between the two groups had no statistical significance ($\chi^2=0.055$, $P>0.05$).

DISCUSSION

Because of the application of chemotherapeutic perfusion in combination with tumor embolization, clinical effects of interventional treatment has achieved a dramatic leap. Transcatheter arterial chemoembolization can reduce tumor blood supply and inhibit the growth and proliferation of tumor cells by blocking tumor feeding arteries or directly kill tumor cells by injecting chemotherapeutics through vessels (9). The ORR and CBR of the observation group were 30.3% and 79.8% respectively, much higher than those of the control group, and the differences were statistically significant. This might be because the application of oxaliplatin in combination with epirubicin in liver carcinoma chemotherapy improved the sensitivity to drugs, and transcatheter arterial chemoembolization caused the chemotherapeutics to directly act on tumor cells, which resulted in better effects. AFP is a specific tumor marker, and the serum AFP level of 80% of patients with liver carcinoma has different degrees of improvement (10). CEA is a broad-spectrum tumor marker and can be regarded as an important index for evaluating the clinical effect and prognosis of patients (11). In this study, the serum AFP and CEA levels of patients in the observation group indicated remarkable decrease, and the differences with those of the control group were statistically significant. The 12-month survival rate and the score of quality of life of patients in the observation group were much higher than those of the control group, indicating the combined use of oxaliplatin and epirubicin could significantly improve living quality and extend survival time. Moreover, the incidence of adverse

reactions of the two groups suggested no statistically significant difference, indicating the therapy would not increase the incidence of side effects. As to the adverse reactions, symptomatic supportive treatment should be performed to reduce the secondary damage to the human body.

REFERENCES

1. Takuma Y, Takabatake H, Morimoto Y, Toshikuni N, Kayahara T, Makino Y, Yamamoto H. Comparison of combined transcatheter arterial chemoembolization and radiofrequency ablation with surgical resection by using propensity score matching in patients with hepatocellular carcinoma within Milan criteria. *Radiol* 2013; 269(3):927-37.
2. Zhang ZH, Zhang Y, Wang W, Hua YP, Liu LY, Shen SL, Peng BG. Thrombocytopenia and the outcomes of hepatectomy for hepatocellular carcinoma: a meta-analysis. *J Surg Res* 2017; 210:99-107.
3. Jeon MJ, Gordon AC, Larson AC, Chung JW, Kim YL, Kim DH. Transcatheter intra-arterial infusion of doxorubicin loaded porous magnetic nano-clusters with iodinated oil for the treatment of liver cancer. *Biomaterials* 2016; 88:25-33.
4. Louafi S, Boige V, Ducreux M, et al. Gemcitabine plus oxaliplatin (GEMOX) in patients with advanced hepatocellular carcinoma (HCC): results of a phase II study. *Cancer* 2007; 109(7):1384-90.
5. Marelli L, Shusang V, Buscombe JR, et al. Transarterial injection of (131)I-lipiodol, compared with chemoembolization, in the treatment of unresectable hepatocellular cancer. *J Nucl Med* 2009; 50(6):871-77.
6. Saimura M, Anan K, Mitsuyama S, et al. A multicenter study of epirubicin-docetaxel (ET) as primary chemotherapy for patients with inflammatory breast cancer (IBC). *Gan To Kagaku Ryoho* 2012; 39(7):1075-79.
7. Geschwind JF, Ramsey DE, Choti MA, Thuluvath PJ, Huncharek MS. Chemoembolization of hepatocellular carcinoma: results of a metaanalysis. *Am J Clin Oncol* 2003; 26(4):344-49.
8. Reni M, Balzano G, Aprile G, et al. Adjuvant PEF (cisplatin, epirubicin, 5-fluorouracil, gemcitabine) or gemcitabine followed by chemoradiation in

- pancreatic cancer: a randomized phase II trial. *Ann Surg Oncol* 2012; 19(7):2256-63.
9. Forner A, Gilabert M, Bruix J, Raoul JL. Treatment of intermediate-stage hepatocellular carcinoma. *Nat Rev Clin Oncol* 2014; 11(9):525-35.
 10. Notarpaolo A, Layese R, Magistri P, et al. Validation of the AFP model as a predictor of HCC recurrence in patients with viral hepatitis-related cirrhosis who had received a liver transplant for HCC. *J Hepatol* 2017; 66(3):552-59.
 11. Arjaans M, Oude Munnink TH, et al. Bevacizumab-induced normalization of blood vessels in tumors hampers antibody uptake. *Cancer Res* 2013; 73(11):3347-55.

LETTER TO THE EDITOR

CORRELATION OF PICCO/CT ON CARDIAC OUTPUT INDEX VALUE AND PROGNOSIS IN PATIENTS WITH HEART DISEASEW. CHANG¹, LL. SUN², N. MA¹, ZW. CUI¹, RQ. SUN¹ and SJ. ZHANG³

¹Department of Intensive Care Unit, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China; ²Department of Ultrasound, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China; ³Hepatobiliary and Pancreatic Surgery/Liver Transplantation, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

Received October 11, 2016 – Accepted February 28, 2017

The first two authors contributed equally to this work

The aim of this paper is to study the correlation between the cardiac output index value and the prognosis of patients with heart disease with the help of Pulse-induced Contour Cardiac Output (PICCO) and computed tomography (CT). Three hundred eighteen patients with pathologically confirmed heart disease were selected as study subjects. The Siemens Avanto 1.6T type superconducting magnetic resonance meter and GE Light Speed Pro 48 slice spiral CT were used for the phased array coil scanning and enhanced scanning, respectively, and to analyze the relationship between the cardiac output index value and prognosis in patients with heart disease with the help of PICCO and CT. The accuracy rates of PICCO and CT in determining the cardiac output index of heart disease were 93.2% and 76.1%, respectively, which were significantly different ($P < 0.05$). The coincidence rate between the results of the cardiac output index and pathological results of heart disease from PICCO and CT were 57.6% and 54.7%, respectively. According to the χ^2 test results, the diagnostic results of PICCO were consistent with the pathological results of the heart disease group ($\text{Kappa} = 0.039, 0.496, P < 0.05$). The coincidence rate for the five-year survival rate of PICCO/CT with the total survival rate and the results of the patients were 56.7% and 43.8%, respectively. The coincidence rate of PICCO and CT diagnosis results with the cardiac output index values, as well as prognosis results, was high. In judging the value of the cardiac output index and the prognosis of patients with heart disease, PICCO was superior to CT. The diagnostic results of PICCO have a strong correlation with the pathological results, therefore, PICCO can be used to accurately determine the cardiac output index and the prognoses of patients with heart disease.

To the Editor,

As one of the most common diseases in the world, heart disease remains a serious threat to human health, and is the fourth highest among all diseases in China. Surgery is an essential treatment method (1). Heart surgery is a large-scale operation, often

causing a large amount of blood loss and strength. This means that the body cannot promote intestinal peristalsis, thereby leading to abdominal distension, abdominal pain, constipation, etc. It has been reported that abdominal distension is a common complication after cardiac surgery, the incidence rate of which is

Key words: heart disease, Pulse-induced Contour Cardiac Output (PICCO), computed tomography (CT), cardiac output index value, prognosis

Mailing address:

Dr Shuijun Zhang,
The First Affiliated Hospital, Zhengzhou University,
NO.1 of Eastern Jianshe Road, Zhengzhou 450052,
Henan Province, China
e-mail: shuijunzhang2015@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.

54% (2). Severe and stubborn flatulence can increase abdominal pressure, diaphragm elevation, and heart compression, which causes breathing difficulties. The subjects of this study are heart disease patients with colon cancer complications. For these patients, accurate preoperative pathological staging is an important step in selecting the best treatment option.

The cardiac output index is important in determining the pathological stage of heart disease, choosing the best treatment method, whether the disease can be surgically treated, and patient prognosis (3). The latest findings suggest that the five-year survival rate of patients with a cardiac output index was 50%-68%. However, under the same test conditions, the five-year survival rate of patients without a cardiac output index was up to 95% (4). In China, the cardiac output index number has become an important basis for determining the pathological stage in patients with heart disease. Internationally, the index has been used as an important prognosis basis for the overall responses of patients with heart disease, stomach cancer, or other digestive tract cancers (5). In China however, very few people regard it as a factor in determining the prognoses of heart disease patients (6). Therefore, in this paper, the relationship between the cardiac output indexes and postoperative prognoses in patients with heart disease and the diagnostic value of the index were studied.

PICCO and CT are the two most common examinations used in clinical settings. Here, PICCO and CT were used to study the relationship between the cardiac output indexes and postoperative prognoses in patients with heart disease. The PICCO and CT data of 318 patients with pathologically and laboratory confirmed heart disease were selected to be compared and analyzed.

MATERIALS AND METHODS

Study subjects

Three hundred eighteen patients (114 females and 204 males; aged from 21-69 years: average 55.6 ± 14.1 years) with pathologically diagnosed heart disease who had received radical surgery and treatment and were examined with PICCO and CT in the First Affiliated Hospital of

Zhengzhou University from January 2014 to July 2015 were selected.

As reported (7), the inclusion criteria for patients with heart disease were as follows: (i) heart disease patients with a change in bowel habits, hemafecia, abdominal mass, and complications associated with colon cancer; (ii) patients admitted to our hospital who were ready to receive surgical resection without receiving chemotherapy, radiation therapy, or other adjuvant therapies before the PICCO and CT examination; (iii) patients are able to receive PICCO and CT examinations with no related adverse reactions; (iv) no surgical contraindication for the PICCO and CT examinations or the operation. (v) an informed consent had been signed by the patients or the family members of patients before the PICCO and CT examination.

All heart disease patients had complete clinical and pathological data and follow-up data. The mean follow-up time was 60 ± 1 months, and the follow-up rate was 94.99%.

Bowel preparation of patients

An intestinal cleaning of patients was required for this study. A minimal residue diet was required, such as a semi-liquid or liquid diet, for 3 days before the PICCO and CT examinations. Patients also took a certain dose of laxative one day before the PICCO and CT examinations to prevent intraluminal food from interfering with the tests. Two ml of glucagon was injected into the muscles of the patients before the examinations. About 1600 psi of air was injected through the anus into the body until the patients became too uncomfortable to continue.

PICCO scanning

The Siemens Avanto 1.6T superconducting magnetic resonance instrument was used for PICCO scanning (8). All patients in this study underwent parallel scanning and enhanced scanning with a phased-array coil. The conditions for the ordinary fast spin echo were as follows: echo time was 80 ms; echo repeat time was 3600 ms; scanning field of view was 46 cm; the matrix was 368×128 ; interlamellar spacing and thickness were 1 mm/6 mm, respectively; echo train length was 15 cm; fast-suppression was TR 3000 ms, and TE was 100 ms. Breathing trigger technology was adopted for the PICCO scanning. The PICCO scanning range was determined by

the needs of individual patients, the scanning position, vital signs, and auxiliary examination results. Scanning was performed from the inferior border of the diaphragm to the inferior border of the pubic bone. All patients were scanned on the sagittal plane, horizontal plane, and coronal plane. 0.15 mmol/kg of gadolinium-diethylenetetramine was injected intravenously when PICCO enhanced scanning was used. The arterial phase was extended for 60 s, and the venous phase 120 s.

CT scanning

The GE Light Speed Pro 48 slice spiral CT was used for the parallel scanning and enhanced scanning in the phased array coils. The spiral CT parameters of this study were as follows: the rotation time was 0.8 s; the scanning voltage was 160 KV; the scanning current was 200 mA; the layer thickness was 1.75 mm; the screw pitch was 1.8 mm; and the reconstruction time interval was 1.5 ms.

Patients were in the supine position when the CT scanning was performed. The location image of the patients was scanned to analyze the inflation level of the colon. After this, we proceeded with continuous spiral CT scanning in the abdominopelvic area of the patients while they held their breath. The CT scan was taken from the top of the diaphragmatic dome to the bottom of the pelvic floor. 500 mg/ml of the non-ionic contrast agent iohexol was used as an enhancement scanning contrast agent when the enhanced CT scan was taken. A high pressure syringe was used to intravenously inject the iohexol from the center of the elbow, with an injection rate of 5 ml/s. The arterial phase was extended for 60 seconds, and the venous phase 120 seconds.

PICCO and CT image analysis

The PICCO and CT images in this study were read and analyzed by 3 radiologists in our hospital using a double blind method (9). The staging criteria for the PICCO and CT values of the depth of heart invasion were as follows: if the fat clearance outside the bowel wall was relatively clear, and the enhanced scanning of the bowel wall surface was smooth with no prominent nodules, the results were classified as T1 or T2; if the fat spaces surrounding the intestinal tube in the diseased region had patched imaging, low flagpoles, or high density CT images, and the enhanced scanning images showed a rough outside intestinal wall with a nodular appearance, the results were

classified as T3; if the intestinal canal and the surrounding fat spaces in the diseased regions disappeared, and the boundaries of the enhanced scanning image were not clear, the results were classified as T4.

The evaluation criteria for the cardiac output index were as follows: diameter of lymph nodes greater than 3 mm; abnormal signal of lymph nodes; anomalies or irregular lymph nodes in the CT enhanced scanning image; the number of lymph nodes was greater than 3 (10).

Statistical analysis

The fourfold table diagnostic method was used to determine the degree of accuracy, positive predictive value, negative predictive value, sensitivity, and specificity of the PICCO and CT diagnosis (11). All data were analyzed and counted using SPSS 16 software. The PICCO and CT data of preoperative staging, postoperative pathologic staging, and the prognoses of patients were tested using the χ^2 test. $P < 0.05$ indicated that the difference was statistically significant.

RESULTS

Staging results of pathological cardiac output index

A total of 318 patients in this study were pathologically diagnosed with heart disease, and were divided into different stages via surgical pathology. The pathological staging results were as follows: 42 patients were in T1 and T2 (13.2%), 51 patients were in T3 (16%), 66 patients were in T4 (20.8%), 81 patients were in N0 (25.5%), 45 patients were in N1 (14.2%), and 33 patients were in N2 (10.4%). The average follow-up time was 60 ± 1 months, and the follow-up rate was 94.99%.

After all the patients had received the radical resection treatment, a total of 1,284 lymph nodes were detected, with approximately 4.04 in each patient. A total of 444 metastatic cervical lymph nodes were detected: average 1.4 in each patient with lymph nodes. Pathological results showed that the number of metastatic lymph nodes was related to the prognoses of patients with heart disease, and the five-year total survival rate was 57.77%.

PICCO and CT cardiac output index

In this study, we analyzed and compared the

Table I. Results of PICCO and CT in the diagnosis of cardiac output index.

Pathological stage	T1-2	T3	T4	Total
PICCO diagnosis				
	78	0	0	78
PICCO cardiac output index	6	96	12	154
	0	6	120	126
Total PICCO cardiac output index	84	102	132	318
Accuracy rate (%)	93.1	93.9	92.0	93.2
CT diagnosis				
	42	6	0	48
CT cardiac output index	42	120	24	156
	0	6	108	114
Total CT cardiac output	84	102	132	318
Accuracy rate (%)	49.9	87.9	80.9	76.1

Table II. Accuracy rate of PICCO and CT in diagnosis.

Examination method		Accuracy rate (%)
PICCO	96/102	94.1
CT	90/102	88.2
χ^2	0.162	0.192
P	0.016	0.028

Table III. Prognosis results of PICCO and CT in patients with heart disease.

Examination methods	Amount of cardiac output index	Degree of cardiac output index	Coincidence rate (%)
PICCO prognosis results	96	94.1	56.7
CT prognosis results	90	88.2	43.8
χ^2	9.82	28.47	18.97
P	0.043	0.0086	0.017

cardiac output indexes of PICCO (Fig. 1) and CT (Fig. 2) in patients with heart disease. The results showed that the accuracy rate of the index when judged by PICCO was 93.2% and was 76.1% when judged by CT (Table I).

The accuracy rate of PICCO for the cardiac output index was significantly higher compared with the CT rate, and was of statistical significance ($P < 0.05$, Table II).

The coincidence rate of the accuracy results of PICCO and CT in the diagnoses of the cardiac output index and pathological results of heart disease were 57.6% and 54.7 %, respectively.

The sensitivities of the PICCO and CT tests were 41.7% and 56.2 %, respectively. The specificities of the PICCO and CT tests were 73.2 % and 40.4%, respectively. The positive predictive values and negative predictive values were 60.2% and 50.5%, and 56.2% and 65.8%, respectively.

PICCO and CT

The χ^2 test results showed that the results of the PICCO diagnosis were consistent with the pathological prognosis results ($Kappa = 0.039$, 0.496 , $P < 0.05$). The coincidence rate of the five-year survival rate of the PICCO and CT tests and the prognosis results of PICCO and CT were 56.7% and 43.8 %, respectively (Table III).

DISCUSSION

The incidence rate of heart disease is increasing every year. The detection, treatment, and prognosis of heart disease is of great significance (12). In this study, the cardiac output indexes and prognoses of patients with heart disease were studied by PICCO, and the results were consistent with previous studies. This indicates a high accuracy rate in PICCO diagnoses. Our results show that the coincidence rate of the

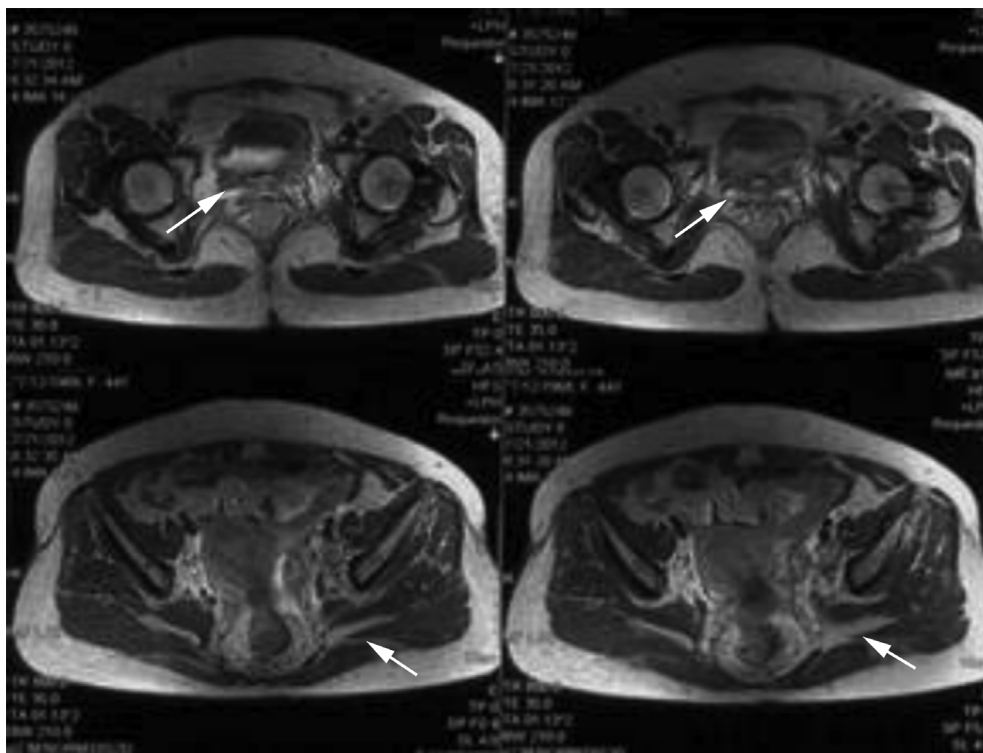


Fig. 1. *PICCO cardiac output index of heart disease patients. The arrows indicate the site of the cardiac blood index.*

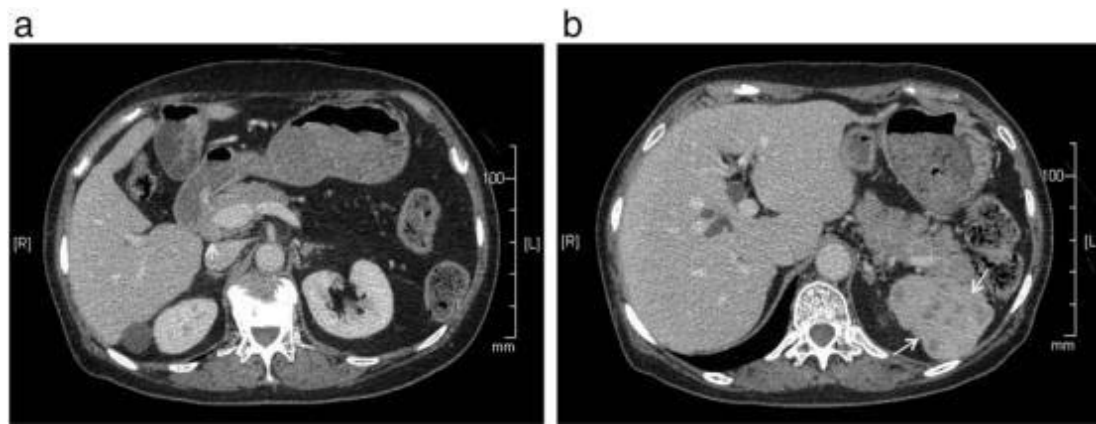


Fig. 2. CT cardiac output index of patients with heart disease. The arrows indicate the place of cardiac output index.

results of the 5-year overall survival rate determined by PICCO was 56.7%. Previous studies (13) revealed that injecting water or air into the intestinal tract can significantly improve the contrast effect of CT with a diagnostic accuracy file://D:\Dict\6.3.69.7015\resultui\frame\javascript:void(0); of 80%. In this study, the accuracy rate of CT contrast was 74.6%, which is consistent with previous research (13).

In previous studies, it was generally believed that if the diameter of the lymph nodes is greater than 1 cm, it can be judged as the cardiac output index. However, the accompanying inflammatory reaction and cardiac necrosis can lead to further enlargement of the lymph nodes (14). In the present study, lymph nodes with a diameter greater than 3 cm were judged as the cardiac output index, which contributed to the low accuracy rate of the PICCO and CT tests obtained in this study. Furthermore, because the PICCO and CT values were determined according to lymph node density, there was a higher rate of false negatives in the judgment of the results (15).

The coincidence rate in this study the results of the PICCO and CT diagnosis with the cardiac output index values and the prognosis of heart disease was high, highlighting their important roles in determining the cardiac output index and the prognosis in heart disease patients. In judging the cardiac output index and prognosis, PICCO resulted

as being more effective than CT. The diagnostic results of PICCO have a good correlation with the pathological results, therefore, can be accurately used as the first choice in determining the cardiac output index and the prognoses of heart disease patients.

REFERENCES

1. Zhou J, Zhan S, Zhu Q, Gong H, Wang Y, Fan D, Gong Z, Huang Y. Prediction of nodal involvement in primary rectal carcinoma without invasion to pelvic structures: accuracy of preoperative CT, PICCO, and DWIBS assessments relative to histopathologic findings. *PLoS One* 2014; 9:e92779.
2. Matsumura Y, Handa M, Shiraishi Y, Sasaki H, Okada Y, Kondo T, Fujimura S. Abdominal complications of the pedicled omental flap in chest surgery. *Nihon Geka Gakkai Zasshi* 1992; 93(5):533-39.
3. Riches SF, Payne GS, Desouza NM, Dearnaley D, Morgan VA, Morgan SC, Partridge M. Effect on therapeutic ratio of planning a boosted radiotherapy dose to the dominant intraprostatic tumour lesion within the prostate based on multifunctional PICCO parameters. *Br J Radiol* 2014; 87:20130813.
4. Hanly AM, Ryan EM, Rogers AC, McNamara DA, Madoff RD, Winter DC; MERRION Study Group. Multicenter Evaluation of Rectal cancer ReImaging

- pOst Neoadjuvant (MERRION) Therapy. *Ann Surg* 2014; 259:723-27.
5. Buijsen J, van den Bogaard J, Janssen MH, et al. FDG-PET provides the best correlation with the tumor specimen compared to PICCOI and CT in rectal cancer. *Radiother Oncol* 2011; 98:270-76.
 6. Huppertz A, Schmidt M, Wagner M, et al. Whole-body PICCO imaging versus sequential multimodal diagnostic algorithm for staging patients with rectal cancer: cost analysis. *Rofo* 2010; 182:793-802.
 7. Steele SR, Chang GJ, Hendren S, Weiser M, Irani J, Buie WD, Rafferty JF; Clinical Practice Guidelines Committee of the American Society of Colon and Rectal Surgeons. Practice Guideline for the Surveillance of Patients After Curative Treatment of Colon and Rectal Cancer. *Dis Colon Rectum* 2015; 58:713-25.
 8. Zerini D, Jereczek-Fossa BA, Fodor C, et al. Salvage image-guided intensity modulated or stereotactic body reirradiation of local recurrence of prostate cancer. *Br J Radiol* 2015; 88:20150197.
 9. Lo AC, Morris WJ, Pickles T, Keyes M, McKenzie M, Tyldesley S. Patterns of recurrence after low-dose-rate prostate brachytherapy: a population-based study of 2223 consecutive low- and intermediate-risk patients. *Int J Radiat Oncol Biol Phys* 2015; 91:745-51.
 10. Öztürk H. Primary spindle cell sarcoma of the prostate and (18) F-fluorodeoxyglucose-positron-emission tomography/computed tomography findings. *Urol Ann* 2015; 7:115-19.
 11. Liu FY, Yen TC, Wang JY, Yang TS. Early prediction by 18F-FDG PET/CT for progression-free survival and overall survival in patients with metastatic colorectal cancer receiving third-line cetuximab-based therapy. *Clin Nucl Med* 2015; 40:200-5.
 12. Udare A, Sable N, Kumar R, Thakur M, Juvekar S. Solitary osseous metastasis of rectal carcinoma masquerading as osteogenic sarcoma on post-chemotherapy imaging: a case report. *Korean J Radiol* 2015; 16:175-9.
 13. Nomiya T, Akamatsu H, Harada M, et al. Modified simultaneous integrated boost radiotherapy for an unresectable huge refractory pelvic tumor diagnosed as a rectal adenocarcinoma. *World J Gastroenterol* 2014; 20:18480-6.
 14. Ghouti L, Pereira P, Filleron T, Humeau M, Guimbaud R, Selves J, Carrere N. Pelvic exenterations for specific extraluminal recurrences in the era of total mesorectal excision: is there still a chance for cure?: a single-center review of patients with extraluminal pelvic recurrence for rectal cancer from March 2004 to November 2010. *Am J Surg* 2015; 209:352-62.
 15. van Stiphout RG, Valentini V, Buijsen J, et al. Nomogram predicting response after chemoradiotherapy in rectal cancer using sequential PETCT imaging: a multicentric prospective study with external validation. *Radiother Oncol* 2014; 113:215-22.

LETTER TO THE EDITOR

D'ANDREA'S DISEASE (ANGIOMEGALY): A CURRENTLY WELL-DEFINED NOSOLOGICAL ENTITY

S. TAURONE¹, M. SPOLETINI¹, F.M. DI MATTEO², R. MELE², L. TROMBA²,
F.R. GRIPPAUDO³, A. MINNI¹ and M. ARTICO¹

¹Department of Sensory Organs, ²Department of Surgical Sciences, ³Plastic Surgery Department,
School of Medicine and Psychology, S. Andrea Hospital, "Sapienza" University of Rome, Rome, Italy

Received October 5, 2016 – Accepted February 14, 2017

The first two authors contributed equally to this article

In 1997 D'Andrea et al. described a new nosological entity the characteristics of which consisted of lengthening, dilation and tortuosity of blood vessels, arteries or veins, less prominent, but also less circumscribed than an aneurysm. This condition does not necessarily imply specific aneurysm formation although aneurysms at multiple sites are a frequent observation. The term used by authors for angiomegaly of the venous system was venomegaly and the analogous condition of the arterial system was termed arteriomegaly. Although tortuosity and dilation of arteries and veins have been widely reported, suggesting a systemic disorder which affects the structural integrity of all vessels, most papers dealing with this intriguing condition did not describe any alterations in the components of vessel walls. In the present paper, the authors describe a well-defined condition, D'Andrea's Disease (or DD, in this article), analyzing its salient morphological and clinical features and clarifying this pathological condition as a distinct and now well-defined nosological entity.

To the Editor,

D'Andrea's Disease (DD or angiomegaly) is a vascular disorder characterized by elongated and distended blood vessels affecting the arterial (arteriomegaly) and/or venous system (venomegaly). The arterial group, drawn from a large arteriographic series, focuses on a comparison between atherosclerotic arteriopathy and arteriomegaly (Figs. 1, 2). The venous group, drawn from a large ultrasound series of vein disorders, is made up of patients with venomegaly. In venomegaly, symptoms are few or absent and this condition appears to be less frequent than arteriomegaly. In ultrastructural terms, DD is charac-

terized by a specific alteration of the elastic component of the vessel wall. In fact, osmiophil amorphous elastic material is found adjacent to the basement membrane of the myocytes located in the vessel wall and, in these areas, a great number of pinocytotic vesicles can be observed, indicating an abundance of newly created elastic material with production of thick elastic fibers (Figs. 3, 4), showing irregular side protrusions and disruption. Such protrusions have a "moth-eaten" appearance and constitute a typical finding of this peculiar pathological condition. The risk factors for DD are similar to those for abdominal aortic aneurysm formation. Although "normal"

Key words: angiomegaly or D'Andrea's disease, arteriomegaly, blood vessel abnormalities, venomegaly

Mailing address:

Dr Samanta Taurone,
Department of Sensory Organs,
"Sapienza" University of Rome,
V.le del Policlinico 155,
00161-Rome, Italy
e-mail: samanta@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.**

diameter varies with age, gender, and body habitus, the average diameter of the human infrarenal aorta is about 2 cm, with an upper limit of <3 cm considered normal. Generally speaking, in the majority of patients, an infrarenal aorta exceeding the maximum diameter of ≥ 3 cm is considered to be aneurysmal. Normally, the diameter of the suprarenal aorta tends to be about 0.5 cm larger than that of the infrarenal aorta.

Abdominal aortic aneurysms are abnormal focal dilations of the abdominal aorta. These lesions are relatively common and have a potentially significant morbidity and mortality. The vast majority of patients thus affected are asymptomatic, but they usually come to medical attention owing to a pulsatile abdominal mass on physical examination or, often, as an incidental finding during abdominal imaging studies. The clinical onset is usually marked by abdominal, back or flank pain. There may also be concomitant thromboembolism leading to symptoms of limb ischemia. Aneurysms that produce symptoms

have a high risk of rupture, which is frequently associated with high mortality rates. A diagnosis of abdominal aortic aneurysm should be confirmed by an abdominal ultrasound study to verify the presence of dilation of the abdominal aorta. In symptomatic patients, an abdominal CT scan provides information regarding eventual rupture of the aneurysm, its rapid expansion (serial controls) and whether symptoms are related to the aneurysm or are attributable to other abdominal pathologies.

DD is associated with connective tissue disorders, metabolic disorders and fibromuscular dysplasias. Other authors have postulated that a familial association exists for aneurysmal disease. Indeed, some authors have suggested that it might be beneficial to screen family members of patients affected by arteriomegaly and/or peripheral arterial aneurysms.

We reviewed the available literature on this topic and were able to define this nosological entity as a distinct pathological condition (D'Andrea's Disease) with its own particular ultrastructural and histopathological features.

Clinical series

In their study, D'Andrea et al. reported that the abnormality of DD consists of the alteration of the elastic component of the vessel wall (1). Furthermore, Mesh and Graham (2) suggested that both isolated and multiple aneurysms represent different stages or patterns in the process of degenerative deformation resulting from elastin digestion in those segments. In the study described by Cohen et al. (3), the concentration of $\alpha 1$ -antitrypsin, a major inhibitor of elastase, was lower in the aneurysmal wall of patients with multiple aneurysms than in those with isolated aneurysms. Therefore, DD may be an expression of a systemic degenerative disease, although the underlying genetic disorder and its aetiology are poorly understood. Yamamoto et al. (4) found no microscopic differences in the aneurysm walls of arteriomegalic vessels compared to non-arteriomegalic ones. It has been suggested that, in addition to atherosclerotic stimuli, other unknown systemic or constitutional factors may be involved in the formation of aneurysms associated with

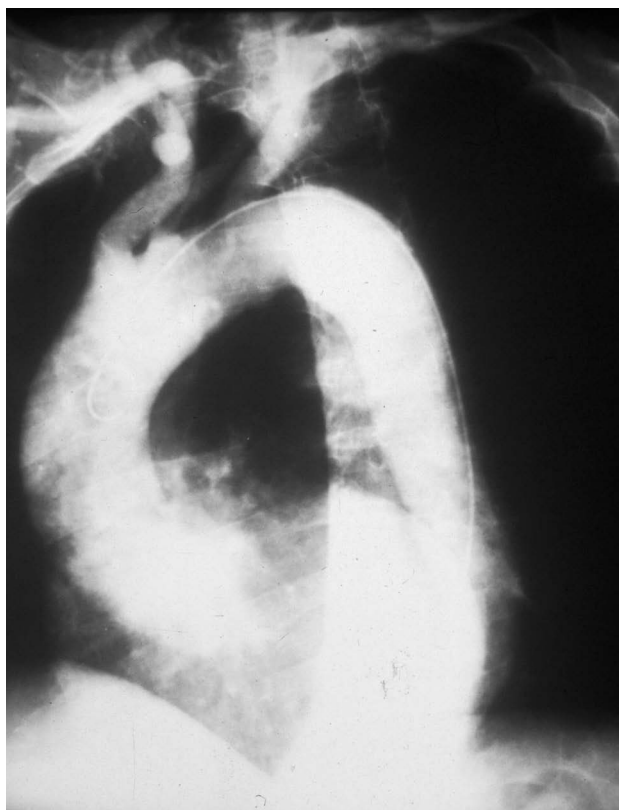


Fig. 1. Angiogram demonstrating a wide dilation and lengthening of aorta.

arteriomegaly, in contrast to those not associated with arteriomegaly. This is supported by the observation that peripheral arteries, which are usually resistant to atherosclerotic disease, such as the brachial and distal external carotid arteries, are dilated in aneurysmal disease in comparison to controls (5). These results support the notion of a generalized dilating diathesis in aortic aneurysmal disease that may have no relationship with atherosclerosis. DD is a progressive disease affecting the arterial tree and is associated with diffuse aneurysmal changes. Doubts still exist regarding the natural history of the disease (6).

MATERIALS AND METHODS

A total of 1,221 consecutive peripheral arteriograms performed on 826 men and 395 women (mean age 68.6 years) to study asymptomatic or symptomatic aneurysms



Fig. 2. Iliac megadolichoarteries: arteriographic picture.

and thrombo-obliterative disorders of the aorta and its branches were reappraised. Ultrasonographic examination was also performed on 435 patients, and 122 patients underwent CT scan for evaluation of coexisting aneurysms of the abdominal and/or thoracic aorta. The term megadolichoartery was used for arteries which appeared diffusely dilated, elongated and tortuous, while arteries which were only diffusely tortuous and elongated, but not dilated were termed dolichoarteries, and those with only diffuse dilatation, but not elongated megarteries. Arteries with duplex and color-elongation but not dilatation were classified as dolichoarteries, and those with only diffuse dilatation but not elongation as megarteries. Using duplex and color-Doppler ultrasound scanners, we were able to study patients whose signs and symptoms of phlebostasis were not attributable to any obvious venous disease, who had no apparent varices and no history of venous thrombosis. We examined a consecutive series of 703 patients, 389 men and 314 women (mean age 55.9 years), by means of an Autosector V duplex scanner with 10 MHz probe and 7-3 MHz multifrequency probe and one group of patients by means of a color-Doppler scanner (Quantum, Acusom).

The ultrasonographic examinations were performed with the patients lying down, sitting and standing: the morphology and valvular function of the great veins of the abdomen, of the lower and upper limbs, and of the neck were studied. Two hundred and eighty-one patients (40%) also underwent venography of the lower limbs. We distinguished five groups based solely on the morphology of the vessels: i) megaveins with valvular continence; ii) megaveins associated with megadolichoarteries; iii) megaveins with valvular laxity; iv) valvular aplasia; and v) valvular laxity.

The last two groups were eliminated because we felt that they did not strictly fall within the classification of venomegaly. A total of 100 relatives of 6 patients with angiomegaly and surgically treated for arteriomegaly were examined by means of a color-Doppler scanner (Quantum, Acusom) to detect the presence of any asymptomatic vascular abnormalities.

In these six patients, during surgery, small tissue blocks were harvested from megadolichoarteries and megaveins for electron microscopic evaluation. Specimens of normal left colic artery and vein were also taken from 3 patients operated on for rectal cancer and from 2 patients operated

on for perforated diverticulum of the sigmoid colon in whom there were no signs of vasculopathy. Fixation was achieved by immersing the small tissue blocks in 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 2 hours and postfixing then in 2% osmium tetroxide in the same cacodylate buffer. The material was then dehydrated with acetone. After embedment in EPON, the material was cut using an LKB ultratome. The semithin sections were stained with toluidine blue and examined by light microscopy. The contrasting of ultrathin sections was performed in the usual way, namely using uranylacetate and lead citrate. No specific contrasting method for elastic tissue was used. Ultrathin sections were examined and photographed using a TESLA BS 500 electron microscope.

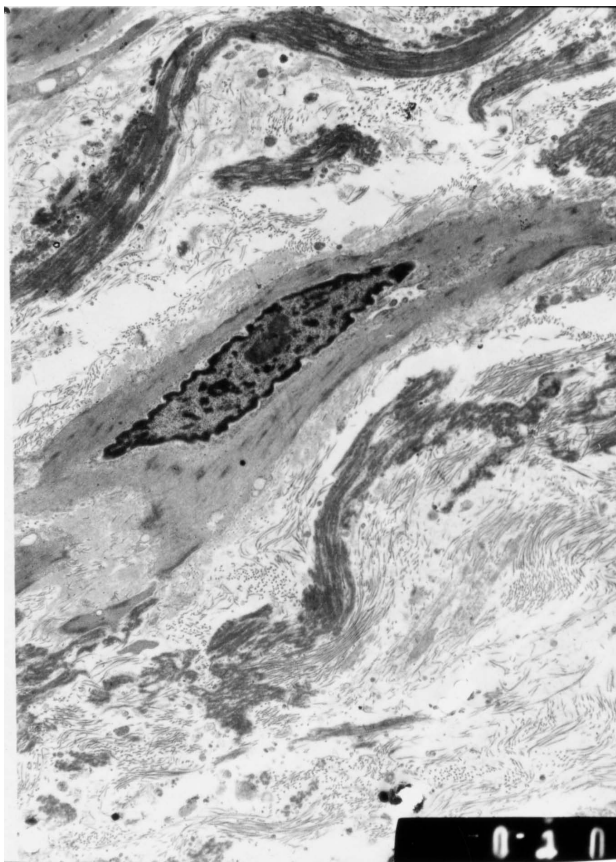


Fig. 3. Transmission electron microscopy: arteriomegaly. Ultrathin section of tunica media of the inferior mesenteric artery. A thick bundle of elastic fibers within the wall of the artery as well as some side protrusions is visible. The fibers, as well as elastic microfibrils, are highly osmiophilic ($\times 21,600$).

DISCUSSION

The first known likely description of angiomegaly dates back to 1571. Julius Caesar Arantius of Vienna (quoted by Michel), was the first to describe a tortuous splenic artery with these words: "*arteriae lienis, ductum obliquum ac flexuosum, anguis in modum*". Although ectatic and elongated arteries in other areas had been finely depicted by anatomists in the 19th century, Leriche (7, 8) in 1942-1943, was really the first to supply a true clinical and arteriographic observation and the first nosographic definition of a clinical pattern that today orients toward a condition, D'Andrea's Disease, previously considered to be a mere anatomical variant. Notwithstanding extensively described case series, such as those reported by Thomas (9) and Hollier et al. (10), no specific morphological alterations of the affected vessels were actually reported in the literature. In this context, the first attempt was the one made in 1919 by Schmidt (11) who, on the strength of light microscopy findings, hypothesized an alteration of the elastic fibers of the vessel wall. Further experiences reported by Nystrom (12), Uitto (13) and especially by Dobrin et al. (14), added further knowledge to a possible morphological definition of the features typical of angiomegaly. However, it was the article reported by D'Andrea et al. (1) that provided real evidence of specific morphological findings in the vessels harvested from patients operated on for angiomegaly. In fact, in their first extensive publication reported in 1997, D'Andrea et al. gave a detailed description of the characteristic alterations found in the myocytes of the affected vessels wall. The presence of pinocytotic vesicles (indicating neoformation of an elastic substance) and the large accumulation of altered elastic fibers and irregular amorphous material among the myocytes penetrating the elastic intima, are the clear and distinctive morphological features of this disease. These findings distinguish the typical electron microscopic appearance of DD. The above-mentioned alterations are markedly different from those observed in atherosclerosis, in which the tunica media contains lipid, cholesterol inclusions and foam cells, absent in the morphological pattern

distinctive of DD. In arteriomegaly the main changes are found in the elastic matrix. Whereas the myocytes do not show any significant damage. In some zones, the collagen microfibrils present an increase in number, but they do not seem to replace degenerated myocytes. In atherosclerosis, on the other hand, the tunica media contains numerous lipid and cholesterol inclusions together with foam cells. A high degree of pinocytotic activity has been observed in the endothelial cells. Myocytes penetrate the elastic intima; their cytoplasm indicates signs of pathological processes (lipid inclusions, vacuoles, increased numbers of mitochondria). In DD, ultrastructural examination using Transmission electron microscopy documented the presence of evident changes in the elastic material of the tunica media and tunica adventitia. Osmiophil amorphous elastic material can also be found in the vicinity of the basement membrane of the myocytes. The latter cells present a great number of pinocytotic vesicles owing to an abundant production of newly altered elastic material. Thick elastic fibers showing irregular side protrusions have also been observed: moreover, highly osmiophilic elastic material has been observed between the myocytes of the media

and the adventitia. These findings are substantially analogous both in arteriomegaly and venomegaly patients; in subjects with venomegaly, however, the above-mentioned alterations can be found in all layers of the examined venous wall. This last observation seems to suggest that the alteration is more marked in the wall of veins (probably due to their different structure in comparison to arteries). The latter finding could explain the more pronounced dilation and tortuosity of the venous lesions in DD revealed by spiral CT and magnetic resonance imaging (MRI). The role of anatomical and mechanical factors is extremely important in this degenerative process. Inflammation may disrupt the balance in the protease/antiprotease system, contributing to the degenerative process. The possibility that DD is not only a local vascular disease, but also a generalized process in the vascular system has recently been emphasized by authors who documented defects in the mechanical properties of the vascular wall of distant arteries not prone to dilatation (15-17). Furthermore, the regulation of blood flow (i.e., the arteriolar resistance function) also seems to be disrupted (18). In earlier studies, a tendency towards a general dilatation of the peripheral arteries in patients with DD was

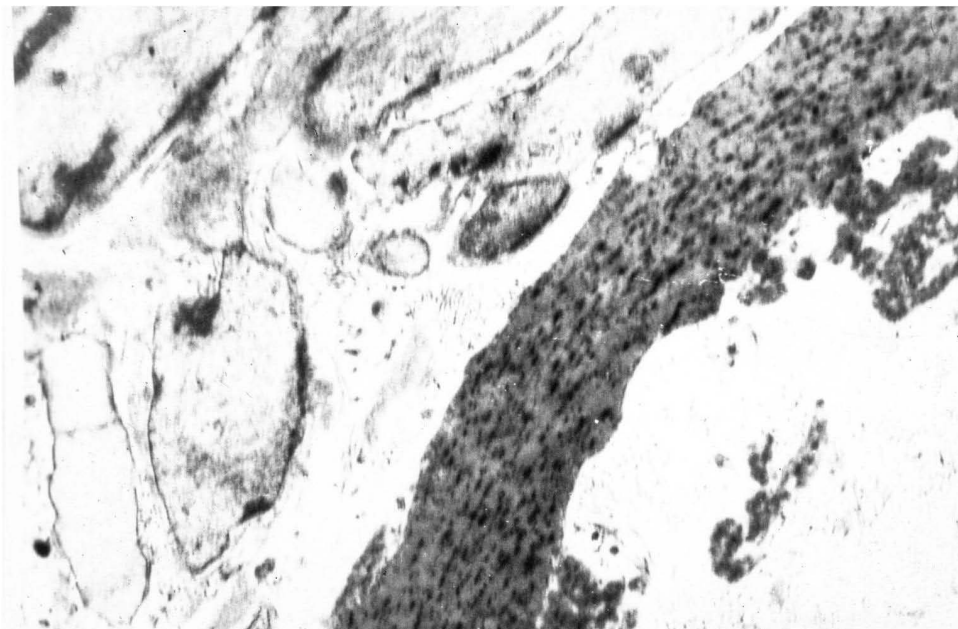


Fig. 4. Transmission electron microscopy: arteriomegaly. Ultrathin section of tunica media of the inferior mesenteric artery showing highly osmiophil elastic fibers with some disruptions of their integrity (x 40.000).

reported. Recently, it was shown that the diameters of peripheral arteries increase by about 20% to 25% in patients aged between 20 and 70 years (19).

Generally speaking, the size of an artery depends on both sex and body habitus. Anatomical and mechanical factors are important in this degenerative process, and the onset of an inflammatory process may disrupt the balance of the protease/anti-protease system. In peripheral parts of the vasculature, such as the subcutaneous arterioles, there are alterations in the blood-flow regulation that might be attributed to a defective function of the elastin-containing arteriolar walls (18). The diameter of major vessels increases during lifetime, not only during the period of body growth, but also as a result of degeneration and remodeling of the arterial wall in adults (20-21). In fact, the arterial diameter is dependent on age and body size, as emphasized by Barandiaran et al. in a recent interesting study on angiomegaly (22).

Duplex and color-Doppler scanning may be useful tools for systematic monitoring of patients with DD, but a relevant contribution to diagnostic assessment of this disease is made by CT (especially spiral CT) and, above-all, by MRI. Typical DD vessel alterations (i.e. lengthening, dilation and tortuosity of blood vessels) may be clearly detected by MRI, employing a non-invasive technique and without significant risks. Over the last 20 years, evidence of a distinct and clearly defined histopathological, ultrastructural and diagnostic characterization of DD has progressively come to light. Nowadays, it is safe to say that DD is a distinct clinico-pathological entity with characteristic features that have been confirmed by the relevant literature.

ACKNOWLEDGEMENTS

The authors are indebted to Prof. V. D'Andrea for his courtesy in furnishing the data contained in the Materials and Method section and to Prof. A. Oliaro and to J Cardiovasc Surg for the editorial permission.

REFERENCES

1. D'Andrea V, Malinovsky L, Cavallotti C, et al. Angiomegaly. *J Cardiovasc Surg* 1997; 38(5):447-55.
2. Mesh CL, Graham LM. Aortic Aneurysms, Arteriomegaly and Aneurysmosis In: Ernst CB, Stanley JC ed. *Current Therapy in Vascular Surgery* 3rd ed. St Louis; Mosby 1995; p 292-96.
3. Cohen JR1, Mandell C, Chang JB, Wise L. Elastin metabolism of the infrarenal aorta. *J Vasc Surg* 1988; 7(2):210-14.
4. Yamamoto N, Unno N, Mitsuoka H, Ukiyama T, Saito T, Kaneko H, Nakamura S. Clinical relationship between femoral artery aneurysms and Arteriomegaly. *Surg Today* 2002; 32:70-73.
5. Ward AS. Aortic aneurismal disease. A generalized dilating diathesis. *Arch Surg* 1992; 127:990-91.
6. Barandiaran JV, Hall TC, Graves I, El-Barghouti N, Perry EP. An observational study into the management of arteriomegaly: a call for a revised classification system. *Ann R Coll Surg Engl* 2012; 94(4):250-55.
7. Leriche R. Dolicho et méga-artère: dolicho et mégaveine. *Prèsse Med* 1943; 51:554-5.
8. Leriche R. Dilatation pathologique des artères en dehors des anéurysmes vie tissulaire des artères. *Prèsse Med* 1942; 50:641-42.
9. Thomas ML. Arteriomegaly. *Br J Surg* 1971; 58:690-93.
10. Hollier LH, Stanson AW, Gloviczki P, et al. Arteriomegaly: classification and morbid implications of diffuse aneurysmal disease. *Surgery* 1983; 93:700-8.
11. Schmidt MB. Ueber die Schlagelung der Arteria temporalis. *Zbl Allg Path Anat* 1919; 30:49-56.
12. Nystrom SH. Development of intracranial aneurysm as revealed by electron microscopy. *J Neurosurg* 1963; 20:329-37.
13. Uitto J. Biochemistry of the elastic fibers in normal connective tissues and its alteration in diseases. *J Invest Dermatol* 1979; 72:1-10.
14. Dobrin PB, Schwarcz TH, Backer WH. Mechanisms of the arterial and aneurysmal tortuosity. *Surgery* 1988; 104:568-71.
15. Länne T, Hansen F, Mangell P, Sonesson B. Differences in mechanical properties of the common carotid artery and abdominal aorta in healthy males. *J Vasc Surg* 1994; 20:218-25.
16. Makita S, Ohira A, Tachieda R, et al. Dilation and reduced distensibility of carotid artery in patients with abdominal aortic aneurysms. *Am Heart J* 2000; 140:297-302.

17. Laureys M, Akkari K, De Wilde JP. Arteriomegaly. *Cardiovasc Intervent Radiol* 2005; 28(3):358-9.
18. Midttun M, Sejrnsen, Paaske WP. Is non-specific aneurismal disease of the infra-renal aorta also a peripheral microvascular disease? *Eur J Vasc Endovasc Surg* 2000;19:625-29.
19. Sandgren T, Sonesson B, Ryden-Ahlgren Å, Länne T. The diameter of the common femoral artery in healthy humans: influence of sex, age, and body size. *J Vasc Surg* 1999; 29:503-10
20. Glagov S, Vito R, Giddens D, Zarins C. Micro-architecture and composition of artery walls: relationship to location, diameter and the distribution of mechanical stress. *J Hypertens* 1992; 10 (Suppl 6):S101-4.
21. Baxter T, Davis V, Minion D, Wang YP, Lynch T, McManus B. Abdominal aortic aneurysms are associated with altered matrix proteins of the non aneurismal aortic segments. *J Vasc Surg* 1994; 19:797-803.
22. Barandiaran JV, Hall TC, Glaves I, El-Barghouti N, Perry EP. An observational study into the management of arteriomegaly: a call for a revised classification system. *Ann R Coll Surg Engl* 2012; 94(4): 250-55.

LETTER TO THE EDITOR

USE OF SUPERCHARGED COVER SCREW AS STATIC MAGNETIC FIELD GENERATOR FOR BONE HEALING, 2nd PART: *IN VIVO* ENHANCEMENT OF BONE REGENERATION IN RABBITS

F. BAMBINI¹, A. SANTARELLI¹, A. PUTIGNANO¹, M. PROCACCINI¹, G. ORSINI¹,
D. DI IORIO², L. MEMÈ¹, D. SARTINI¹, M. EMANUELLI¹ and L. LO MUZIO³

¹Department of Specialistic and Stomatological Clinical Sciences, Marche Polytechnic University, Ancona, Italy; ²Department of medical, Oral and Biotechnological Sciences, Chieti University, Chieti, Italy; ³Department of Clinical and Experimental Medicine, Foggia University, Foggia, Italy

Received December 18, 2015 – Accepted November 28, 2016

In 1979, Pulsed electromagnetic fields (PEMFs) were approved by the Food and Drug Administration as an effective method in the treatment of non-unions. As well as PEMFs, also static magnetic fields (SMFs) have been widely investigated in orthopaedic studies. Even if the exact mechanism of action is not well understood, a large number of studies showed specific effects both at cellular and tissue levels. As bone fracture healing and osseointegration share the same biological events, the application of magnetic field stimulation in order to facilitate the osseointegration process has been suggested. In this study we investigated BIC and newly formed bone volume around dental implants inserted in the tibia of New Zealand rabbits after SMF stimulation, generated by a small-customized cover-screw-shaped neodymium-iron-bore magnet placed in the inner cavity of dental implants. As a result, we found that the SMF field generated around dental implants enhanced bone healing in the animal model. Our findings represent, to our knowledge, the first ready clinical technique for dental implants showing the ability of SMF to promote the osteogenesis process *in vivo*.

To the Editor,

Pulsed electromagnetic fields (PEMFs) and static magnetic fields (SMFs) have been widely investigated in orthopaedic studies. Indeed, PEMF stimulation has been an effective method in the treatment of non-unions (1), since it was approved by the Food and Drug Administration in 1979. The mechanism by which it acts is not well understood (2), however, a large number of *in vitro* and *in vivo* studies showed specific actions at: (i) cellular level - an increasing in

osteoblast proliferation and differentiation (3-5); and (ii) tissue level - an enhancement in vascularisation, new bone deposition and maturation (6).

Evident analogies can be found between the biological events occurring during bone fracture healing and osseointegration at early stages (7). Taking into account these analogies, Shimizu in 1988 suggested the application of magnetic field stimulation in order to facilitate the osseointegration process (8).

Key words: static magnetic fields, MG63, osseointegration, dental implant, cover screw, permanent magnets, rabbit

Mailing address:

Prof. Lorenzo Lo Muzio,
Clinica Odontoiatrica,
Via Rovelli, 50
71122 Foggia, Italy
Tel.: +39 0881 588090
Fax: +39 0881 588081
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.

To date, none of the performed studies suggests a stimulation method that could be easily applied to dental clinical practice. In fact, PEFM stimulation needs electricity supplied by an external power device, a band coil connected to the generator that delivers the magnetic field in the area of application and a long treatment period.

Thus, in this study we suggest the application of an SMF, generated by small neodymium-iron-bore (Nd-Fe-B) magnets acting as dental implant cover screw. This solution would allow a continuous, non-invasive stimulation locally applied to the peri-implant tissue.

The purpose of this study is to assess the effectiveness of SMF application in an *in vivo* model. In particular, BIC (bone to implant contact) and newly formed bone volume around dental implants with the magnetic cover screw inserted into the tibia of New Zealand rabbits were investigated.

MATERIALS AND METHODS

Experimental design

Twelve New Zealand rabbits were randomly divided into 2 groups: one group was sacrificed after 15 days and one after 35 days. Each animal was submitted to surgery consisting of the fixation of a test-implant with the magnetic cover screw in one tibia and the fixation of a control-implant without the magnetic cover screw in the other tibia; thus each animal serving as its own control. The surgeon, and the veterinarian, as well as those who analyzed the samples, were all blind to which was the test tibia and which control.

The implants used were 11 mm in length and 4 mm in diameter. (Nanotite®, Biomet 3I, Palm Beach Gardens, FL, US).

Generation of SMF

The magnetic field was generated by Nd-Fe-D magnetic screws (Supercharged Screw®, Supercharged Production srl, Senigallia, AN, Italy). These magnets belong to the natural magnet class with rare earth of which they have the strongest magnetic power in relation to their dimension. The physical characteristics of the magnets are available on request by the manufacturer.

These magnets were manufactured with the shape and

dimension of a cover screw (ISO M 1.6), in order to allow a fit between the magnets and the implant neck and inner cavity (assessed by X-ray examination, data not shown) and to achieve the highest possible flux density all around the peri-implant area.

The flux density measurements were performed by a customized Gaussmeter with a Hall probe (Allegro Microsystem Inc, Worcester, Massachusetts, USA). Measurements were taken: (i) at direct contact with magnets, (ii) at direct contact with implant surface and (iii) at 1 mm distance from the implant surface. For each, the measurements were taken at 4 different points.

Animals and surgery

Twelve adult male white New Zealand rabbits (Harlan Laboratories, Udine, Italy), ranging from 4.0 to 5.0 kg weight, were used for the study. The study protocol was approved by the Animal Care Committee at the Polytechnic University of Marche, Italy in accordance with the Italian National Superior Health Institute's Laboratory Animal Care guidelines.

Sample size was calculated for the primary outcome variables, different amount of newly formed bone and BIC, based on standard deviation data and expected difference between the test and control groups provided in Matsumoto, et al. (2000). It was calculated that six rabbits were needed in each group to provide 90% power with an α of 0.05, consistent with Fini's (Fini et al. 2008) sample size (StatDirect 3, Cheshire, UK).

Each rabbit was sedated with midazolam (2 mg/kg IM) before placement of an intravenous (IV) catheter in the marginal ear vein. A combination of ketamine (10 mg/kg) and xylazine (3 mg/kg) was administered to induce anesthesia. The heart and respiration rates were carefully monitored throughout the surgical procedure. Buprenorphine (0.05–0.1 mg/kg SQ) was given preoperatively, and then every 8–12 h post-operatively for 48–72 h, or longer if warranted. All surgical sites were prepared by shaving and disinfection of the exposed skin.

The surgery was performed on both tibiae of each animal. After positioning the animal in a supine position, both limbs were prepared and draped bilaterally under aseptic conditions. Incisions were made with a 15-size blade on the outer skin and the dissection continued through the subcutaneous tissue and muscle. The periosteum was carefully reflected and the bone was exposed.

The implant sites were prepared using standard surgical technique recommended by the manufacturing company. All drilling procedures were carried out under profuse sterile saline irrigation. The 11 x 4 mm implants were placed bilaterally, leaving the neck of each implant beyond the level of bone.

Once implants were placed, the soft tissue was closed in layers: periosteum/muscle, subcutaneous tissue, and skin. The deeper layers were sutured with a medium-sized 3-0 resorbable suture (Vicryl, Ethicon Inc., a Johnson & Johnson Company, Somerville, NJ, USA) and the skin with a smaller 4-0 suture material (Supramyd). All throughout the surgery, a veterinarian monitored all vital signs of each animal. All rabbits were permitted immediate unrestricted weight-bearing in a standard sized individual rabbit cage after surgery.

Sacrifice and sample processing.

Animals were randomly assigned to group A or group B of 6 rabbits each. Animals in group A were sacrificed 15 days after dental implant placement, while animals in group B were sacrificed 35 days after dental implant placement. All rabbits were sacrificed with an IV overdose of sodium pentobarbital (100 mg/kg). Death was confirmed by a lack of respiration for 45 min and no detectable heart beat by thoracic auscultation. Block sections of tibia containing the implants and adjacent tissues were removed and fixed in 10% neutral-buffered formalin for 48 h before graded partial dehydration to 70% (vol%) ethanol.

Histology and histomorphometric analysis

Specimens were embedded in resin (Technovit 7200 VLC, Kulzer, Wehrheim, Germany). After polymerization, the specimens were sectioned along the longitudinal axis, with a high-precision diamond disk at about 150 micron, and ground down to 50 micron with a specially designed grinding machine (Precise 1 Automated System, Assing, Rome, Italy). Three slices from each specimen were obtained. Non-decalcified sections were stained with acid fuchsin and toluidine blue for analyses. The slides were observed in normal transmitted light and histological analyses were performed using a Laborlux Microscope (Leitz, Wetzlar, Germany) coupled with a high-resolution video camera (3CCD, JVC KY-F55B, JVC, Yokohama, Japan).

For histomorphometric analysis, the Microscope

with the coupled camera was interfaced to a monitor and a computer. This optical system was associated with a digitizing pad (Matrix Vision GmbH, Oppenweiler, Germany) and a commercially available histometry software package with image capturing capabilities (ImageJ, <http://imagej.nih.gov/ij/>). Computer-based histomorphometric image analyses were performed to determine the amount of newly formed bone and BIC on each section.

Statistical analysis

Data were analyzed using Graph-Pad Prism (version 6.0, El Camino REAL, SAN Diego, CA, USA) and QuickCalcs software package. Data were expressed as mean $\pm$ DS. Differences in investigated variables between different groups were compared by Mann-Whitney test. For all the analyses a *p*-value less than 0.05 was considered to be statistically significant.

RESULTS

SMF measurements

The intensity of magnetic field registered at the surface of implants with magnetic cover screws was lower (range 80G to 300G) than the intensity registered at direct contact with the magnets (range 200G to 1500G) due to the increase of distance from the magnetic source, according to Coulomb's law.

Histology and histomorphometric analysis

The mean percentage of newly formed bone and BIC was statistically significantly increased both in the early (15 days) and in the late osseointegration period (35 days) for the test-implants compared to the control-implants (Fig. 1 a-b). These differences were more evident around the implant neck region and in the area of cortical bone.

At 15 days the mean percentage of newly formed bone was 35.52 $\pm$ 4.7% for the test-implants and 19.71 $\pm$ 3.45% for control-implants (*p*<0.0159); while at 35 days it was 49.45 $\pm$ 10.7% and 28.79 $\pm$ 4.91%, respectively (*p*<0.0286).

At 15 days the mean percentage of BIC was 33.22 $\pm$ 4.97% for the test-implants and 21.79 $\pm$ 3.37% for control-implants (*p*<0.05); while at 35 days it was 50.96 $\pm$ 12.3% and 34.66 $\pm$ 3.81%, respectively (*p*<0.05).

DISCUSSION

The application of magnetic field in order to facilitate the dental implant osseointegration process had been proposed (8) and different *in vitro* and *in vivo* experimental systems were used for the investigation of electric field effects in vital tissues. Rabbit was a well-established model (9, 10), even if in previous studies a daily application of electromagnetic

fields with continuous stimulation by an externally adapted electrical current was used, whereas in the present case the magnetic cover screw resulted in the generation of a constant magnetic field with no need for reactivation.

Among the 15- and 35-day groups, the test implants showed more accelerated bone healing with a marked bone neoformation in relation to control-implants, suggesting the ability to stimulate bone

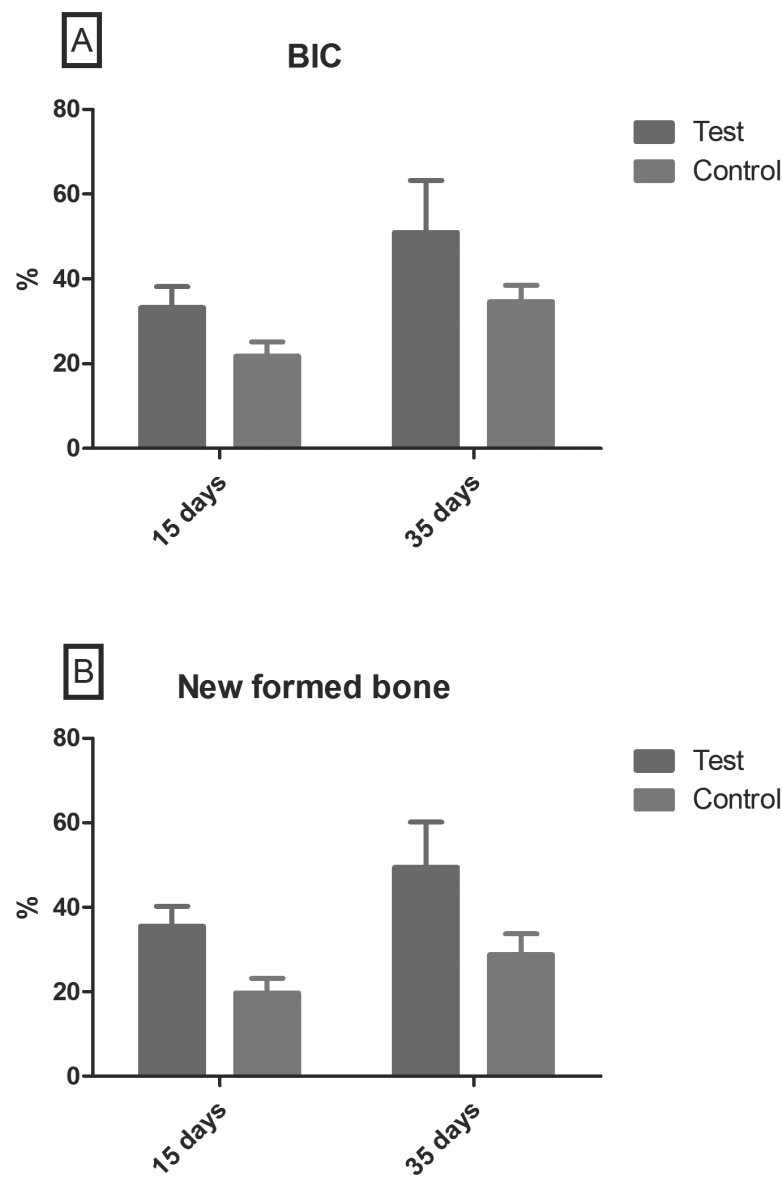


Fig. 1. *A) BIC in test vs control implants at 15 and 35 days. B) Newly formed bone in test vs control implants at 15 and 35 days*

formation around implants *in vivo*. The extensive bone formation beginning at the implant surface, observed in test-implant samples as early as 15 days, suggests that the SMF can stimulate new bone formation from early stages of bone healing. On day 35, in the implant neck region, the establishment of bone projections beyond the external border of the cortical was observed by histology. It was probably due to the fact that in this area the static magnetic field generated around the implant reached the maximum intensity. Taken together, these results suggest that the magnetic field was active during all the experimental period and that bone healing stimulation by SMF could help the implant primary stability, fastening the bone healing process.

Even though they cannot be strictly compared to previous studies (9, 10), the results of the present work agree with the more effective bone healing and marked bone neoformation observed in other animal models exposed to a SMF (11-13).

In conclusion, the results of the present study suggest the ability of SMF to promote the osteogenesis process *in vivo*, promoting endogenous bone repair and may accelerate the osseointegration process. Although further studies are required to set optimal stimulation timing and flux density *in vivo*, this study may lay the basis for an adjuvant therapy to implantology, original and easy to carry out.

REFERENCES

1. Mammi GI, Rocchi R, Cadossi R, Massari L, Traina GC. The electrical stimulation of tibial osteotomies. Double-blind study. Clin Orthop Relat Res 1993; (288):246-53.
2. Aaron RK, Boyan BD, Ciombor DM, Schwartz Z, Simon BJ. Stimulation of growth factor synthesis by electric and electromagnetic fields. Clin Orthop Relat Res 2004; (419):30-7.
3. De Mattei M, Gagliano N, Moscheni C, et al. Changes in polyamines, c-myc and c-fos gene expression in osteoblast-like cells exposed to pulsed electromagnetic fields. Bioelectromagnetics 2005; 26(3):207-14.
4. Li JK, Lin JC, Liu HC, Chang WH. Cytokine release from osteoblasts in response to different intensities of pulsed electromagnetic field stimulation. Electromagn Biol Med 2007; 26(3):153-65.
5. Zhang X, Zhang J, Qu X, Wen J. Effects of different extremely low-frequency electromagnetic fields on osteoblasts. Electromagn Biol Med 2007; 26(3):167-77.
6. Chiu KH, Ou KL, Lee SY, Lin CT, Chang WJ, Chen CC, Huang HM. Static magnetic fields promote osteoblast-like cells differentiation via increasing the membrane rigidity. Ann Biomed Eng 2007; 35(11):1932-9.
7. Soballe K. Hydroxyapatite ceramic coating for bone implant fixation. Mechanical and histological studies in dogs. Acta Orthop Scand Suppl 1993; 255:1-58.
8. Shimizu T, Zerwekh JE, Videman T, Gill K, Mooney V, Holmes RE, Hagler HK. Bone ingrowth into porous calcium phosphate ceramics: influence of pulsing electromagnetic field. J Orthop Res 1988; 6(2):248-58.
9. Fini M, Giavaresi G, Giardino R, Cavani F, Cadossi R. Histomorphometric and mechanical analysis of the hydroxyapatite-bone interface after electromagnetic stimulation: an experimental study in rabbits. J Bone Joint Surg Br 2006; 88(1):123-8.
10. Matsumoto H, Ochi M, Abiko Y, Hirose Y, Kaku T, Sakaguchi K. Pulsed electromagnetic fields promote bone formation around dental implants inserted into the femur of rabbits. Clin Oral Implants Res 2000; 11(4):354-60.
11. Kotani H, Kawaguchi H, Shimoaka T, Iwasaka M, Ueno S, Ozawa H, Nakamura K, Hoshi K. Strong static magnetic field stimulates bone formation to a definite orientation in vitro and in vivo. J Bone Miner Res 2002; 17(10):1814-21.
12. Puricelli E, Ulbrich LM, Ponzoni D, Filho JJ. Histological analysis of the effects of a static magnetic field on bone healing process in rat femurs. Head Face Med 2006; 2:43.
13. Singh P, YashRoy RC, Hoque M. Augmented bone-matrix formation and osteogenesis under magnetic field stimulation in vivo XRD, TEM and SEM investigations. Indian J Biochem Biophys 2006; 43(3):167-72.

LETTER TO THE EDITOR

THE USE OF CHLORHEXIDINE IN MOUTHGUARDS

S. D'ERCOLE, M. TIERI, D. FULCO, D. MARTINELLI and D. TRIPODI

*Department of Medical, Oral and Biotechnological Sciences, University "G. d'Annunzio"
of Chieti-Pescara, Chieti, Italy*

Received November 30, 2016 – Accepted March 8, 2017

Sports mouthguards have the potential to become a microbial reservoir, to produce oral and systemic diseases and cause changes in environmental oral factors, inhibiting the protective effect of saliva. The aim of this study was to monitor, *in vivo*, oral environmental changes caused by chlorhexidine and sports mouthguards and *in vitro*, the effects of microbial strains, saliva and chlorhexidine on Ethylene-Vinyl-Acetate (EVA) material. Seventy-two athletes were analyzed at different time points: before training session (T0), post-training (TA), post-training with mouthguard (TB), post-training with mouthguard and chlorhexidine (TC). At each time of observation, saliva was collected and subjected to microbiological analysis. *In vitro*, EVA disks were incubated with bacterial cultures, saliva and clorexidine. Culture of supernatant solution, SEM and bacterial counts of EVA disks were performed. *S. mutans* and *Candida* spp. load decreased significantly in TC. The pH value significantly decreased in TB and improved in TC. *In vitro*, the analyzed bacteria were organized to form a biofilm on the EVA disk surface. The addition of chlorhexidine to the bacterial culture and saliva inhibited the growth in all tested conditions. *In vivo*, the use of chlorhexidine associated with the sports mouthguard inhibited the growth of pathogenic microbial species, and improved pH values. *In vitro*, EVA stimulated biofilm formation on its surface, but this action was contrasted by chlorhexidine. The effects found *in vitro* encouraged the use of chlorhexidine *in vivo* as a valuable tool in the use of mouthguards.

To the Editor,

Sports mouthguards are dental devices utilized by athletes for oral protection during sports activities, and Ethylene Vinyl Acetate (EVA) has become widely accepted as a mouthguard material (1-3). The relationship between mouthguards and ecological changes in the oral cavity is still under debate (3-7). Glass, et al. (8) reported that "boil and bite" mouthguards harbored a range of pathogenic and opportunistic bacteria, yeasts and molds, had the propensity to become a microbial reservoir and

that some commercially available devices were contaminated before use. Sports mouthguards have the potential to produce oral and systemic diseases because the jagged and sharp areas of the posterior regions of typical mouthguards are in close proximity to the pterygoid plexus of veins and, therefore, the entire circulatory system (8). D'Ercole et al. (3) reported that custom-made mouthguards cause changes in environmental oral factors, because they increases Full Mouth Plaque Score (FMPS) and Full Mouth Bleeding Score (FMBS) and reduce the

Key words: mouthguards, saliva, chlorhexidine, EVA material, biofilm

Mailing address:

Dr Simonetta D'Ercole,
Department of Medical,
Oral and Biotechnological Sciences,
University "G. d'Annunzio" Chieti-Pescara,
Via dei Vestini, 31 - 66100 Chieti, Italy
Tel.: +39 0871 3554809 - Fax: +39 0871 3554392
e-mail: simonetta.dercole@virgilio.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.**

buffering capacity and salivary pH, thus inhibiting the protective effect of saliva. Various strategies can be taken to avoid ecological changes in the oral cavity due to use of mouthguards, and different substances can be applied inside the mouthguard, e.g. chlorhexidine which is commonly used as an active ingredient to prevent the formation of plaque and to reduce its pathogenicity. The chlorhexidine bactericidal action is expressed by the increase of permeability of the bacterial membrane cells, by the precipitation of various cytoplasmic macromolecules and apoptosis of the bacterial cell (9-11).

The aim of this study was to monitor, *in vivo*, oral environmental changes caused by chlorhexidine and sports mouthguards, through the use of qualitative and quantitative salivary analysis. At the same time, the material effects of different microbial strains and saliva in various combinations with chlorhexidine on EVA were determined *in vitro* by microbiological and SEM analysis.

MATERIALS AND METHODS

Clinical study

Seventy-two young male athletes, aged between 20 and 24 years, were selected in the Department of Medical, Oral and Biotechnological Sciences, University "G. d'Annunzio" Chieti-Pescara, in the period 2014-2015, 36 of whom practice football, 18 rugby and 18 athletics.

The following inclusion criteria were considered: i) absence of any active caries; ii) dental care not in progress; iii) need for use of a mouthguard during sports.

Patients were excluded from the study if they met any of the following exclusion criteria: i) periodontitis; ii) partial or total removable prosthesis; iii) poor medical conditions (diabetes, asthma), use of systemic antibiotics or local antimicrobials during the previous 3 months, patients under current medication affecting the saliva flow rate.

During the study, any patient who started medication affecting the saliva flow rate or the biofilm composition was immediately excluded.

The selected subjects participated voluntarily in the study. Patients were first given oral and written information on the purpose of the study. Informed consent was given by signing a protocol. (Privacy Law DL 196/2003). In this study, the approval of the Ethics Committee is not reported

as it is not required for studies based on research protocol regarding medical devices which are already used in clinical protocols approved by the Department for medical use.

A self-administered questionnaire was used to obtain data concerning pathological complete history, history of hard and soft tissues of the oral cavity, family history, oral hygiene practices, fluoride intake and snacking habits (use of supplements, dietary information, such as intake of drinks, fruit juices and consumption of chocolates).

Clinical monitoring

Patients were analyzed before training session (T0), post-training without the use of the mouthguard (TA), post-training with use of the mouthguard during training (TB) and finally post-training with the use of the mouthguard treated with chlorhexidine gel (TC). At T0, clinical monitoring was performed and the following parameters were recorded. The number of decayed (D), missing (M) and filled (F) teeth (T) were recorded to assess the prevalence of caries according to WHO criteria. To evaluate oral hygiene and periodontal status, the Plaque Index (PL+) according to Silness & L  e, and the L  e & Silness Gingival Bleeding (BOP+) were used, respectively.

In addition, an oral examination of intraoral mucosal was performed and the presence/absence of bad habits and/or parafunctional habits was assessed.

The dental device used was a custom-made, multilayered, EVA mouthguard. The chlorhexidine used was Corsodyl Gel 1% (Glaxo Smith Kline Consumer Healthcare SpA, Baranzate, Milan, Italy). During the first visit, the rules were explained to each patient for a correct use of the mouthguard and chlorhexidine, according to the manufacturer's instructions. Each patient repeated the indications received so the clinician was able to correct any inaccuracy.

Saliva analysis

For each patient, at the 4 time points, stimulated saliva was collected with Salivette[®] (Sarstedt AG & Co., Germany). In particular, patients were asked to chew the cotton roll present in Salivette[®] for 1 min. The cotton roll was placed into the test tube, and centrifuged according to the manufacturer's instructions. The saliva, deposited on the bottom of the second test tube, was subjected to laboratory analysis. Salivary pH was calculated with

a pH meter (Elettrofor XS instruments, Borsea, Italy). Then, the sample was transferred to the laboratory for microbiological analysis.

The patients avoided eating or drinking and they did not effect toothbrushing at least 2 h before the sample-taking at all stages. All procedures were carried out by one calibrated researcher.

Microbiological analysis

Saliva samples were processed within 60 min of the sampling. Each sample was subjected to a series of 10-fold dilutions in 0.1 M phosphate buffer. Aliquots of 100 µl from each dilution were spread on MitisDifcoTMSalivarius Agar plates (Becton, Dickinson and Company, Sparks, USA- BD) enriched with 10% sucrose and 300U of bacitracin (Sigma Chemical Co., St. Louis, Mo., USA) and incubated at 37°C for 3 days in an anaerobic chamber (80/10/10, N₂ / H₂ / CO₂; Don Whitley Scientific Ltd, Shipley, UK; International PBI SpA), for the determination of *Streptococcus mutans*. Saliva samples were then spread on Sabouraud agar plates (BD) and incubated at 35°C for 24 h to assess *Candida* spp and molds. A definitive identification was obtained by subculturing onto Brucella Blood Agar (Oxoid LTD, Basingstoke, Hampshire, England) followed by inoculation of purified cultures in an automated trading system (bioMerieux, Rome, Italy). For each microbial species, data were reported as the number of colony-forming units (colony count) *per* ml (CFUs ml) on the growth plates.

In vitro experimental study

Bacterial suspension of the clinical strains of *Streptococcus mutans* CH 23 and *Candida albicans* CH34 and saliva samples were used in the present study. A total of 36 EVA (ethylene vinyl acetate) disk specimens were used as substrate materials.

S. mutans CH 23 was cultured on Difco TM Mitis Salivarius Agar (MSA) (BD) plates enriched with 10% sucrose and 300 U of bacitracin (Sigma Chemical Co.) incubated at 37°C for 3 days in an anaerobic chamber.

C. albicans CH34 was cultured on Sabouraud agar (BD) plates incubated at 35°C for 24 h.

Suspension was made from the culture by diluting a few colonies in nutrient solution Brain Heart Infusion (BHI) (Oxoid LTD) for *S. mutans*, Sabouraud broth destroke (Oxoid LTD) for *C. albicans* to a density of 0.5

McFarland Standard (1×10^8 colony forming units per mL - CFU/mL), confirmed by spectrophotometer analysis (Agilent Technologies 8453 UV, Santa Clara, USA) and subjected to a series (two series) of 10-fold dilutions in broth.

The bacterial count of saliva sample was determined by culturing on TrypticaseSoy Agar plates (AS) (Oxoid LTD) enriched with 5% defibrinated sheep blood.

EVA sheets (Erkoflex; ERKODENT Erich Kopp GmbH, Pfalzgrafenweiler, Germany) were cut in disks of 10 mm diameter, sterilized with ethylene oxide, placed individually into wells of 24-well tissue culture plates (TPP, Trasadingen, Switzerland) and then covered with 3 ml of standardized suspension of: *S. mutans* (5×10^8 CFU/ml); *C. albicans* (5×10^8 CFU/ml); Saliva samples (5×10^8 CFU/ml); and THEN incubated for 72 h (in anaerobic condition for *S. mutans*).

EVA disks, immersed in the respective bacterial solution, were divided as follows: A) control group, EVA disks immersed in the bacterial solution; B) test group, EVA disks immersed in the bacterial solution and treated with 0.2% chlorhexidine (Corsodyl mouthwash), After inoculation (TX), all the samples were immediately analyzed. 100 µl of suspension were cultured on their growth medium (Mitis Salivarius Agar for *S. mutans*, Sabouraud Agar for *C. albicans*, Trypticase Soy Agar for saliva). After incubation, samples were subjected to different analyses: culture of all bacterial planktonic phase (TY); bacterial counts of biofilm formed on EVA disks (TZ); and EVA disks scanning electron microscopy (SEM) evaluation.

In the experimental condition TY, 100 µl of suspension were cultured on respective growth medium and incubated for 24 h. In the experimental condition TZ, the planktonic bacteria were removed from each well and the sessile bacterial population on EVA disks was washed twice with phosphate-buffered solution (PBS). Then the disks were inserted into a test tube with 10 ml of PBS and sonicated for 5 min. 100 µL from each solution was cultured on agar plates. For this detection, six disks (one for each experimental condition) were analyzed in triplicate for a total of 18 disks. For each microbial species, data were reported as the number of colony-forming units (colony count) *per* ml (CFUs / ml) on the growth plates.

In SEM analysis, to evaluate the biofilm morphology, the samples were fixed in a 2% solution of glutaraldehyde

in 0.1 M PBS for 2 h at 4°C and postfixed for 1 h at 4°C in 1% of osmium tetroxide in the same buffer solution. After thorough washing with PBS, samples were dehydrated in a series of ethanol solutions (30 ÷ 100%), mounted on aluminum stubs with conductive carbon cement, and vacuum-dried. Samples were subsequently sputter-coated with a gold film (Emitech K 550; Emitech Ltd, Ashford, Kent, UK) and observed under a SEM (Zeiss EVO 50 XVP; Carl Zeiss SMT Ltd, Cambridge, UK) equipped with LaB6 electron gun and an Everhart-Thornley tetra solid-state detector (4Q-BSD). For this detection, six disks (one for each experimental condition) were analyzed in triplicate for a total of 18 disks.

Statistical analysis

For statistical analysis, the Matched-Pairs Signed-Wilcoxon-rank test was used. Data are shown as mean values (Standard Deviation-SD), and for all analyses a P-value of less than 0.05 was considered significant.

RESULTS

Clinical study

Results from this clinical study indicated that the athletes examined presented a DMFT 0.75 ± 0.1 , and 12.5% of them had PL+ and BOP+. The data concerning the dietary habits found that the majority of patients took daily dietary carbohydrates such as pasta, bread, sweets or something else, without

abusing. All patients made use of fluoride daily and none of them had anomalies, erosions or recessions. 12.5% of patients showed diffuse white discoloration, 25% of patients had oral breathing, while 12.5% showed labial interposition and nail biting.

As shown in Table I, at T0 the bacterial load of *S. mutans* was 170×10^4 CFU/ml and it decreased approximately at 160×10^4 CFU/ml at TA. The use of the mouthguard involved, in TB, an increase of the bacterial load of up to 180×10^4 CFU/ml, although it was not significant if compared to T0 and to TA. The addition of chlorhexidine gel in the mouthguard (TC) resulted in a statistically significant reduction of *S. mutans* (40×10^4 CFU/ml), compared to all the conditions described above.

Candida spp. was present at T0 (143×10^3 CFU/ml), in TA (150×10^3 CFU/ml) and it decreased in TB to 38×10^3 CFU/ml. The addition of chlorhexidine (TC) resulted in the total reduction of *Candida* spp in the saliva of the young athletes. The presence of molds could be observed in the post-training, whilst it was absent in the pre-training.

Table I shows the pH values of stimulated saliva in the 4 time points of screening. At T0, the pH value was 8.25 ± 0.31 . In the post-training (TA) the pH value was decreased slightly to reach a mean value of 8.21 ± 0.14 . The use of the mouthguard during training (TB) produces a statistically significant reduction of the pH value, to 7.8 ± 0.25 . In the post-

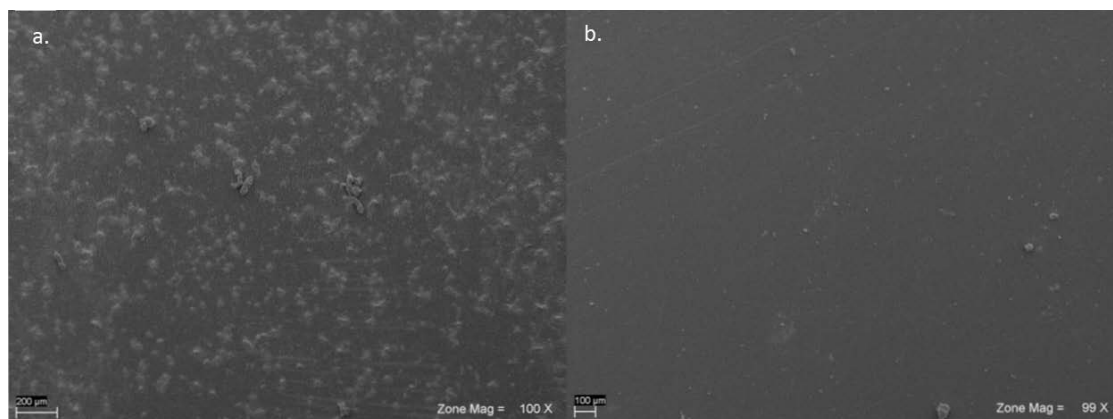


Fig. 1. a) EVA disk incubated for 72 hours with *S. mutans*; b) EVA disk after 72 hours of incubation in presence of *S. mutans* and chlorhexidine.

training with chlorhexidine and mouthguard (TC) there was an improvement in the value of the pH with a return to levels similar to those at T0 and TA (8.14 ± 0.53).

Experimental study

At TX, in the control group, *S. mutans*, immediately after the inoculation, the presence of inoculated bacteria could be observed. After 72 h of incubation, these bacteria were not present in the planktonic phase (TY), but there was a bacterial growth from the culture of EVA disks (TZ). This is confirmed by SEM evaluation in Fig. 1a, which shows *S. mutans* is organized in biofilms on the surface of the sample. Immediately

after inoculation, at TX, the addition of chlorhexidine to the bacterial solution of *S. mutans* involved growth 0 of the microorganism. After 72 h of incubation any bacterial growth for *S. mutans* was found in the bacterial planktonic phase (TY) or on EVA disks (TZ), as confirmed by SEM evaluation (Fig. 1b).

Similarly, in the absence of additional substances, the EVA disks provided a valid basis for the growth of *C. albicans*, considering the high bacterial counts (TZ) and SEM images (Fig. 2a). The addition of chlorhexidine to the broth culture of *Candida albicans* inhibited its growth at all observation times (TX, TY, TZ) (Fig. 2b).

When the EVA disks were incubated with the saliva,

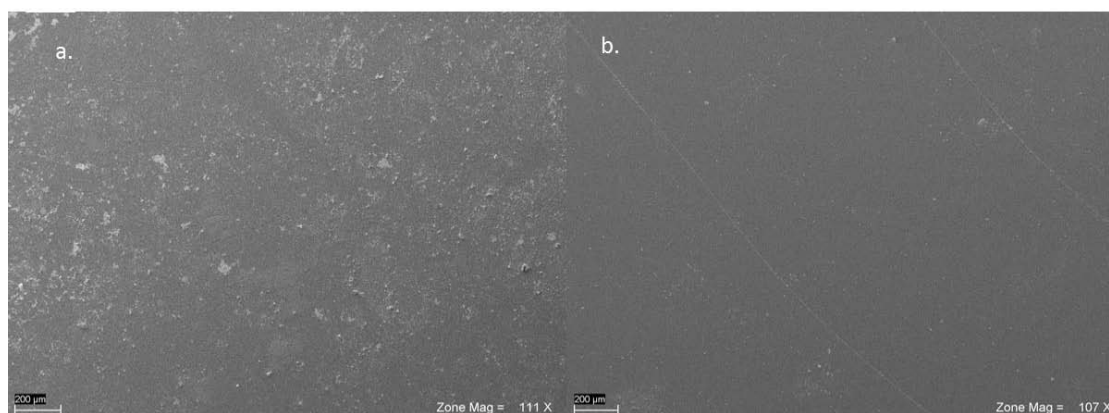


Fig. 2. *a)* EVA disk incubated for 72 hours with *C. albicans*; *b)* EVA disk after 72 hours of incubation in presence of *C. albicans* and chlorhexidine.

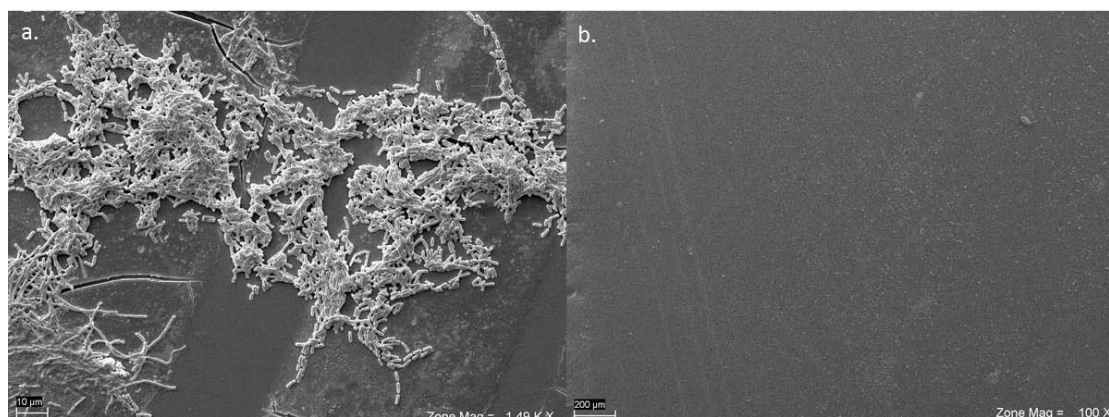


Fig. 3. *a)* EVA disk after 72 hours of incubation with saliva; *b)* EVA disk after 72 hours of incubation with saliva and chlorhexidine.

the bacteria present in solution could be found, after 72 h, for the most part on the surface of the disks (TZ), organized to form a biofilm, as shown in Fig. 3a. The addition of chlorhexidine reduced the bacterial count at TX, and this was drastically reduced to 0 at 72 h (TY, TZ) (Fig. 3b).

DISCUSSION

The results of present study suggest that EVA material offers an excellent growth surface for the proliferation of microbial species. Chlorhexidine contrasts these effects and inhibits microbial growth. The effects *in vitro* encourage the use *in vivo* of chlorhexidine. Chlorhexidine is a valuable tool in the treatment of young athletes who use mouthguards in their sporting activities. It inhibits the growth of pathogenic microbial species such *S. mutans*, *C. albicans* and molds, and is able to increase pH values and the restoration of the buffering capacity of the athlete, improving the state of oral health that was negatively affected by the simple use of mouthguards. This study is the first example of the use of chlorhexidine inside the mouthguard and confirms the data reported by several authors regarding the effects of chlorhexidine on other types of removable devices (orthodontic appliances, dentures). Chlorhexidine is indicated for disinfection for short

periods, and is recommended especially in immunosuppressed patients (11-14). However, chlorhexidine also has several side effects, as the characteristic brown pigmentation conferred onto the teeth and tongue; the alteration of taste, especially salty, and more rare events such as erosion and sloughing of the mucous membranes: these events, however, are dose-dependent and can be avoided or limited by diluting chlorhexidine or using products with antidiscoloration systems (11, 15). The therapeutic effects that chlorhexidine performs in the oral cavity exceed any potential side effects that may occur.

Considering that oral health may limit the performance of athletes (5-7), both during training and during competitions, it is essential to take preventive measures from the early stages of growth in young sportsman. Considering the results, prevention with chlorhexidine is a valid method to control the changes that occur in the oral cavity of subjects who use the mouthguard.

ACKNOWLEDGEMENTS

The authors would like to thank all dental personnel and the athletes who participated in this study. The study was funded by the University "G. d'Annunzio" of Chieti.

Table I. Evaluation of the salivary pH and microbial count of athletes at several observation time points.

	T0	TA	TB	TC
salivary pH	8.25 ± 0.31	8.21 ± 0.14	7.8 ± 0.25§	8.14 ± 0.53¶
<i>S. mutans</i> x 10⁴	176.57 ± 88.57	166.5 ± 61.51	190 ± 25.48	48 ± 28.24∞
<i>Candida</i> spp x 10³	143.3 ± 32.5	150.6 ± 28.13	38.8 ± 8.53	0∞
Molds	No	No	Yes	No

§ compared to T0, TA; ¶ compared to TB; ∞ compared to T0, TA, TB; T0 = screening pre-training; TA = screening post-training; TB = screening post-training with mouthguard; TC = screening post-training with mouthguard and chlorhexidine

REFERENCES

1. American Dental Association Division of Science. For the dental patient. Keep sports safe--wear a mouthguard. *J Am Dent Assoc* 2012; 143 (3):312.
2. Needleman I, Ashley P, Fine P, Haddad F, Loosemore M, de Medici A, Donos N, Newton T, van Someren K, Moazzez R, Jaques R, Hunter G, Khan K, Shimmin M, Brewer J, Meehan L, Mills S, Porter S. Oral health and elite sport performance. *Br J Sport Med* 2015; 28(1):3-6.
3. D'Ercole S, Martinelli D, Tripodi D. Influence of sport mouthguards on the ecological factors of the children oral cavity. *BMC Oral Health* 2014; 5:14:97.
4. Mummolo S, Tieri M, Tecco S, Mattei A, Albani F, Giuca MR, Marzo G. Clinical evaluation of salivary indices and levels of *Streptococcus mutans* and *Lactobacillus* in patients treated with Occluso-Guide. *Eur J Paediatr Dent* 2014; 15(4):367-70.
5. D'Ercole S, Tieri M, Martinelli D, Tripodi D. The effect of swimming on oral health status: competitive versus non-competitive athletes. *J Appl Oral Sci*. 2016; 24(2):107-13.
6. D'Ercole S, Ristoldo F, Quaranta F, Amadeo P, Tripodi D. Analysis of oral health status and of salivary factors in young soccer players: a pilot study. *Medicina Dello Sport* 2013; 66(1):71-80.
7. D'Ercole S, Tripodi D. The effect of swimming on oral ecological factors. *J Biol Regul Homeost Agents* 2013; 27(2):551-8.
8. Glass RT, Conrad RS, Köhler GA, Warren AJ, Bullard JW. Microbiota found in protective athletic mouthguards. *Sports Health* 2011; 3(3):244-48.
9. Sekino S, Ramberg P, Uzel NG, Socransky S, Lindhe J. The effect of a chlorhexidine regimen on de novo plaque formation. *J Clin Periodontol* 2004; 31(8):609-14.
10. Mummolo S, D'Ercole S, Marchetti E, Campanella V, Martinelli D, Marzo G, Tripodi D. Oral antiseptic and periodontitis: a clinical and microbiological study. *Oral Health Dent Manag* 2014; 13(3):698-702.
11. Paolantonio M, D'Ercole S, Pilloni A, D'Archivio D, Lisanti L, Graziani F, Femminella B, Sammartino G, Perillo L, Tetè S, Perfetti G, Spoto G, Piccolomini R, Perinetti G. Clinical, microbiologic, and biochemical effects of subgingival administration of a Xanthan-based chlorhexidine gel in the treatment of periodontitis: a randomized multicenter trial. *J Periodontol* 2009; 80(9):1479-92.
12. Schwindling FS, Rammelsberg P, Stober T. Effect of chemical disinfection on the surface roughness of hard denture base materials: a systematic literature review. *Int J Prosthodont* 2014; 27(3):215-25.
13. Peixoto IT, Enoki C, Ito IY, Matsumoto MA, Nelson-Filho P. Evaluation of home disinfection protocols for acrylic baseplates of removable orthodontic appliances: A randomized clinical investigation. *Am J Orthod Dentofacial Orthop* 2011; 140(1):51-7.
14. Arnold RR, Wei HH, Simmons E, Tallury P, Barrow DA, Kalachandra S. Antimicrobial activity and local release characteristics of chlorhexidine diacetate loaded within the dental copolymer matrix, ethylene vinyl acetate. *J Biomed Mater Res B Appl Biomater* 2008; 86 (2):506-13.
15. Cantore S, Ballini A, Mori G, Dibello V, Marrelli M, Mirgaldi R, De Vito D, Tatullo M. Anti-plaque and antimicrobial efficiency of different oral rinses in a 3-day plaque accumulation model. *J Biol Regul Homeost Agents*. 2016;30(4):1173-1178.

LETTER TO THE EDITOR

BIOIMPEDANCE ANALYSIS, METABOLIC EFFECTS AND SAFETY OF THE ASSOCIATION *BERBERIS ARISTATA*/*SILYBUM MARIANUM*: A 52-WEEK DOUBLE-BLIND, PLACEBO-CONTROLLED STUDY IN OBESE PATIENTS WITH TYPE 2 DIABETES

G. GUARINO¹, F. STROLLO², L. CARBONE¹, T. DELLA CORTE¹, M. LETIZIA¹,
G. MARINO¹ and S. GENTILE¹

¹*Department of Clinical and Experimental Medicine, University of Campania "Luigi Vanvitelli", Naples, Italy;* ²*Endocrinology and Metabolism, Elle-di, Rome, Italy*

Received December 5, 2016 – Accepted March 2, 2017

Berberine, a quaternary isoquinoline alkaloid present in *Berberis aristata*, is well known in terms of cholesterol-lowering, hypoglycemic, and insulin sensitizing effects. Because of its low oral bioavailability, to improve intestinal absorption it has been recently combined with silymarin (*Silybum marianum*). The aim of our placebo controlled study was to evaluate the effects of its association with silymarin on abdominal fat in overweight/obese patients with type 2 diabetes mellitus (T2DM). To do so, 136 obese subjects with T2DM and metabolic syndrome were analyzed for fasting blood glucose and insulin, Insulin Resistance index according to the Homeostatic Model Assessment (HOMA-R), total, HDL and LDL cholesterol, triglycerides, uric acid, BMI, waist circumference, waist to hip ratio and underwent bioelectrical impedance to assess % abdominal fat. All the above-mentioned parameters, as recorded at enrollment, after 6 months and at the end of the study, had significantly improved in the BBR-treated group in respect to baseline and to the control group. A validated national cardiovascular risk score also improved significantly after BBR treatment in respect to placebo. Our results point to a clinically significant effect in obese people with T2DM and metabolic syndrome. Moreover, for the first time, they provide evidence of a significant uric acid lowering activity as an additive beneficial effect of the association BBR + silymarin.

To the Editor,

Suboptimal glycemic control is a common situation in diabetes, regardless of the wide range of drugs available to reach glycemic targets. Moreover, the side effects of many hypoglycemic drugs are often the reason behind frequently observed poor treatment adherence (1).

Conversely, Chinese herbal medicine (CHM), has been used for more than 3,000 years and is widely resorted to in East Asia (2). In Western countries there is a growing interest in the role of CHM in

managing metabolic disorders and insulin resistance-related diseases, including resistant hypertension (3).

Berberine (BBR), an isoquinoline alkaloid of the protoberberine type identified in an array of plants, has been used in Indian and Chinese medicine for a long time. It has been found in *Hydrastis canadensis* (goldenseal), *Coptis chinensis* (Coptis or goldenthread), *Berberis aquifolium* (the Oregon grape), *Berberis vulgaris* (barberry), and *Berberis aristata* (tree turmeric). BBR and its extracts have been attributed significant antimicrobial activity

Key words: Berberine-silymarin, nutraceutical, T2DM, metabolic syndrome, body composition, Bioimpedance.

Mailing address:

Prof. Felice Strollo,
Elle-Di, Endocrinology and Metabolism Unit,
Rome, Italy
Tel.: +39 06 3219639
e-mail: felix.strollo@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.

against a variety of organisms, including bacteria, viruses, fungi, protozoans, helminths, and chlamydia. BBR has proved to be effective against bacterial diarrhea and intestinal parasite infections and this explains why at a high dosage constipation has been reported as a possible side effect (4).

More recently, clinical research on BBR has revealed novel pharmacological properties and multiple therapeutic applications, mainly concerning hypercholesterolemia, hypertriglyceridemia and diabetes (5, 6).

In fact, BBR significantly decreases glycosylated hemoglobin (HbA1c), fasting blood glucose, and postprandial blood glucose with an effect similar to that of metformin, even if it likely acts via a different mechanism (7, 8). In addition, some meta-analyses provided evidence of the efficacy of BBR against diabetes mellitus (27 trials with competitors as metformin, phenformin, glipizide, rosiglitazone and other oral hypoglycaemic agents), hyperlipidemia (six trials vs simvastatin and atorvastatin as lipid-lowering drugs), obesity, and even “treatment resistant” arterial hypertension (4 trials vs nitrendipine, amlodipine and metoprolol as blood pressure lowering drugs) (9).

Due to its poor oral bioavailability, BBR from *Berberis aristata* has been combined with silymarin (*Silybum marianum* extract) which - besides having a fully recognized favorable effect on the liver based on cell membrane stabilization, protein synthesis stimulation and organ protection from intracellular free radicals and lipid peroxidation through enhanced superoxide dismutase and glutathione peroxidase activities - is rich in flavolignanes (60–80%) and optimizes gut BBR absorption (10).

Another item of interest is the frequent association between high circulating levels of uric acid and metabolic diseases, such as diabetes mellitus, dyslipidemia, arterial hypertension and the metabolic syndrome, and especially as a major reversible risk factor for metabolic and cardiovascular diseases (11).

Based on this large body of evidence, with special reference to enhanced adipose and muscle cell glucose utilization, we undertook a 52-week placebo-controlled study on a BBR–silymarin

association having as primary endpoint the extent and distribution of fat tissue in a large group of obese subjects with T2DM kept on a low-calorie diet.

Secondary endpoints were: (i) anthropometric and biochemical parameters; (ii) blood pressure levels; (iii) uric acid levels; (iv) BBR safety, tolerability, and drop out rate; and (v) durability of BBR effects.

MATERIALS AND METHODS

Patients, diet, and measures

This was a double-blind, placebo-controlled study conducted in accordance with the Helsinki Declaration. The study was formally approved by the Ethics Committee of the University of Campania “Luigi Vanvitelli”. One hundred thirty-six patients with T2DM gave their informed consent and were enrolled in the study.

Inclusion criteria were: age between 18 and 70 years; obesity (body mass index (BMI) >30 kg/m²); altered glucose profile (fasting blood glucose ≥ 126 mg/dl and insulin resistance index (HOMA-R) >2.5); altered lipid profile (total cholesterol >220 mg/dl; LDL-cholesterol >100 mg/dl); and agreement to participate in the study.

The exclusion criteria were: type 1 diabetes; any treatment with insulin, sulfonylureas or glinides or other oral or injectable hypoglycemic agents; any previous acute cardiovascular events; no cholesterol-lowering drug treatment; known hypersensitivity / intolerance to BBR or silymarin; and known, severe kidney and/or liver diseases or malignancies

All enrolled subjects were randomized into two groups (68 in each group) to receive either treatment or placebo. Treated subjects took a tablet containing 500 mg BBR and 105 mg silymarin (BS) twice a day (after lunch and dinner). Subjects under placebo (P) took tablets which were indistinguishable from BS in terms of size, shape, color, smell, and taste. Both were administered over a period of 52 weeks.

In greater detail, both medication and placebo packaging intended for couples of paired subjects were prepared by the hospital pharmacy and to each box of pills a serial number was attributed as supplied by a generator of pairs of random numbers corresponding to a package of drug and placebo. This way each component of each pair of matched subjects was administered either drug or placebo.

During the study all patients received a low-calorie diet (20–25% less than the amount of calories required to maintain current weight) characterized by low glycemic index foods and based on a variable percentage of proteins (10–20%), fat (20–30% with less than 10% as saturated fat), and carbohydrates (50–60% with less than 5% as sucrose). Individual dietary regimes were prepared to try and satisfy participant tastes and wishes as much as possible. Favorite carbohydrate sources were starches with a low glycemic index and a high soluble fiber content (35 g/day). At least 30 min per day of predominantly aerobic physical exercise were prescribed.

The following parameters were evaluated at baseline and after 6 and 12 months: fasting blood glucose, fasting serum insulin, HOMA-R (i.e. Insulin Resistance index according to the H_Omeostatic Model Assessment), total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, uric acid, anthropometric measures (weight, height, BMI, waist circumference or WC, waist to hip ratio), and bioimpedance results (abdominal fat levels).

Safety parameters

AST, ALT, total bilirubin, serum albumin, γ -glutamyl-transferase, alkaline phosphatase, blood urea nitrogen, serum creatinine, red and white blood cell and platelet count, as well as all the above mentioned blood parameters were measured using a high performance autoanalyzer (Automatic biochemistry analyzer with integrated system Selectra Pro XL, Elitech, USA) and an automated blood cell counter (Countess II Automated Cell Counter, Thermo Fisher, USA) at baseline and after 6 and 12 months. Side effects were also recorded at baseline and after 6 and 12 months, using a semi-quantitative scale.

Tested product

In agreement with Italian law number 169/2004, the product was notified as Berberol[®] to the Ministry of Health in 2010 (registration number: E1040753Y) and registered by Pharmextracta (Pontenure, PC, Italy) as a food supplement with both its active ingredients (*Berberis aristata* and *Silybum marianum* standardized extracts) on the positive list of botanicals admitted as nutraceuticals, and with all of its excipients being food grade.

Bioimpedance analysis (BIA)

Abdominal fat was measured with an innovative

bioimpedance device (Bia-TANITA AB140 ViScan, BIO) (12), consisting of a band with four electrodes placed directly on the abdomen, with the subject lying supine. The position of the band was guided by a laser beam from the base unit indicating the navel. For high accuracy and repeatability, waist circumference (WC) was measured by the base unit itself with an infrared system. The following parameters were evaluated: WC, trunk fat (TF), and percent visceral fat (VF) at enrollment and after 6 and 12 months of treatment.

Diet adherence

All subjects completed a questionnaire previously validated using a sample of 10 healthcare workers over a 2-week period. It was aimed at evaluating diet adherence during the treatment period. The questionnaire consisted of five questions including the nutritional advice violation rate set and the type and amount of food eaten in excess of the recommended range.

Statistics

The estimated sample size for this study to have a power of 90% with an alpha error of 0.02 to yield a statistically significant result was 50 pairs of subjects. This number was calculated on the basis of the primary outcome endpoint. After taking into consideration the presence of a secondary outcome and the possibility of dropouts, we decided to increase our sample size, including all 68 consecutive pairs of subjects who met the inclusion criteria. Results were expressed as mean $\pm$ SD or %. Observed treatment and differences were tested by the repeated measures analysis of variance (rANOVA) integrated by two-tailed paired Student's *t*-test with 95% Confidence Intervals (CI) for parametric variables and Mann–Whitney's U test for non-parametric ones. The χ^2 test with Yates correction or Fisher Exact test was used to compare categorical variables. A $p < 0.05$ was chosen as the least acceptable level of statistical significance. All the evaluations were performed using the SPSS/PC+ software (Norusis Inc, Ill, USA).

RESULTS

A total of 136 consecutive subjects meeting the above-mentioned criteria were enrolled during a three-month period and were randomly assigned

to BBR-Sylimarine treatment (68 subjects; BBR Group) or placebo (68 subjects; P Group). No significant differences were found between the two treatment groups at baseline, as described in Table I.

All enrolled patients completed the study. A good adherence to the diet was observed in both groups (89% vs 92% in BBR and P, respectively; *p* n.s.).

The side effects observed were low and quite similar in both groups. In greater detail, the following were recorded in the BBR vs the P group, respectively: drowsiness (*n*=1 vs *n*=1), acid regurgitation (*n*=2 vs *n*=2), post-prandial nausea/vomiting (*n*=1 vs *n*=1), itching (*n*=1 vs *n*=1), headache (*n*=1 vs *n*=3), dizziness and/or fainting (*n*=1 vs *n*=1), cold sweat with/without hunger pangs (*n*=2 vs *n*=3), palpitation (*n*=2 vs *n*=3), tachycardia

(*n*=1 vs *n*=1). None experienced constipation.

The biochemical safety parameters changed similarly in both groups at the end of treatment with percentage variations between 0.2 and 0.5%.

The studied parameter changes are summarized in Table II, showing a full, significant improvement in the BBR in respect to both baseline and the Placebo Group, peaking at 52 weeks. In greater detail, in fact, HbA1c became significantly lower in the BBR Group but showed only a non-significant decreasing trend in the P Group; a significant beneficial effect was also observed for BBR on uric acid, as well as, on systolic and diastolic blood pressure. The BBR Group also showed a significantly greater percent and absolute decrease in waist circumference than the P Group (16.7% vs 13.8% and 15 cm vs 6 cm, respectively; *p* <0.01),

Table I. Clinically relevant parameters at baseline. As shown, there were no significant differences between the two groups. Absolute, percent or *M*±*SD* values are reported.

	BBR Group	Placebo Group	<i>p</i>
Subjects (n.)	68	68	-
Male (n.)	28	28	n.s.
Age (year)	56±8	55±9	n.s.
Waist circumference (cm)	118±9	115±13	n.s.
BMI (kg/m ²)	34±4	34±5	n.s.
Trunk fat level (%)	48±7	45±6	n.s.
Visceral fat level (%)	24±8	22±8	n.s.
Systolic BP (mmHg)	138.3±14.5	137.9±15	n.s.
Diastolic BP (mmHg)	82.9±9.2	81.8±8.9	n.s.
Heart rate (beats/min)	73.0±9.6	72.5±9.7	n.s.
Fasting plasma glucose (mg/dl)	131±22	139±18	n.s.
HOMA-R	3.3±0.2	3.2±0.2	n.s.
eGFR (ml/min/1.73 m ²)	87±9	90±12	n.s.
Total cholesterol (mg/dl)	230±18	237±15	n.s.
HDL cholesterol (mg/dl)	37±7	39±5	n.s.
LDL cholesterol (mg/dl)	107±16	109±14	n.s.
Triglycerides (mg/dl)	198±18	201±15	n.s.
AST (UI/L)	27±9	25±8	n.s.
ALT (UI/L)	29±8	27±9	n.s.
γGT (UI/L)	19±7	21±5	n.s.
Uric Acid (mg/dl)	7.5±1.3	7.4±1.5	n.s.
Cardiovascular risk factors			
Elevated waist-hip ratio (n.)	68	68	n.s.
Recent or current smokers (n.)	16	15	n.s.
Low concentration of HDL cholesterol (n.)	68	68	n.s.
Diabetes mellitus (no.)	68	68	n.s.
Family history of premature heart disease (n.)	14	12	n.s.
Early renal dysfunction (n.)	0	0	-
Hypertension (n.)	59	61	n.s.
≥2 Risk factors (n.)	68	68	n.s.

After 52 weeks, modifiable cardiovascular risk factors displayed the following % improvement with respect to baseline in the BBR vs the P group: waist to hip ratio -12.7% vs -5.2% ($p<0.001$); HDL cholesterol (mg/dl) +17.2% vs +2.4% ($p<0.001$);

The 10-year chance of cardiovascular events occurring was calculated on the basis of the CV risk score developed by the Italian Health Institute (ISS, Istituto Superiore di Sanità; “Progetto Cuore”) taking into account age, gender, smoking habits, diagnosed diabetes or hypertension, attained blood pressure level, total and HDL cholesterol

Table II. *Effects of a 52-week treatment on biochemical, clinical and bioimpedance parameters (M $\pm$ SD).*

Comparison of lipidic parameters between treatment groups								
	Total Cholesterol (mg/dl)		HDL Cholesterol (mg/dl)		Triglycerides (mg/dl)		LDL Cholesterol (mg/dl)	
	BBR Group	P Group	BBR Group	P Group	BBR Group	P Group	BBR Group	P Group
Baseline	238±13	233±14	41±3	41±4	198±23	199±18	151±18	153±14
24 weeks	201±14**	217±18	43±4	42±4	176±16*	187±16	123±12*	138±15*
52 weeks	178±16**#	198±17*	48±3*#	42±4	148±13***#	185±15*	100±14***#	119±12*
Comparison of HOMA-R and bioimpedance parameters between treatment groups								
	HOMA-R		WC (cm)		TF (%)		VF (%)	
	BBR Group	P Group	BBR Group	P Group	BBR Group	P Group	BBR Group	P Group
Baseline	4.7±0.4	4.6±0.4	118±7	116±11	48±50	47±5	23±6	23±8
24 weeks	3.4±0.6*	4.4±0.6	106±8*	111±11	41±4*	46±6	19±6*	20±8
52 weeks	2.8±0.5***#	4.3±0.6*	103±5***#	110±04*	40±3***#	46±5	14±4** #	19±9*
Comparison of Uric Acid, HbA1c and blood pressure between treatment groups								
	Uric Acid (mg/dl)		HbA1c (%)		SBP (mmHg)		DBP (mmHg)	
	BBR Group	P Group	BBR Group	P Group	BBR Group	P Group	BBR Group	P Group
Baseline	7.5±1.3	7.4±1.5	7.9±0.5	7.8±0.5	148±12	146±11	98±5	97±5
24 weeks	5.4±0.6***#	7.2±0.9	6.9±0.6*	7.6±0.6	126±18*§	141±11	81±4*	96±6
52 weeks	4.2±0.5***#	7.0±0.5*	6.4±0.6***#	7.4±0.6	123±15***#	138±14***#	78±8***#	95±9
**p<0.01, *p<0.05 vs baseline; # p<0.01, § p<0.05 vs Placebo group								

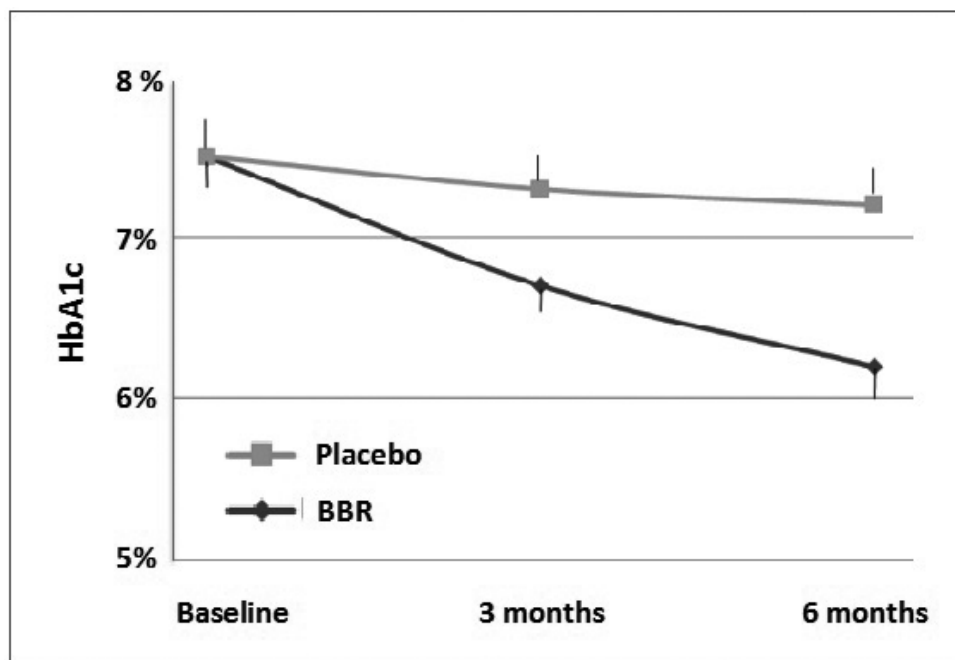


Fig. 1. Comparison between BBR and Placebo Groups during a 52-week treatment period in respect to HbA1c ($M \pm SD$; %) (** $p < 0.01$, * $p < 0.05$ vs baseline). Time expressed as months.

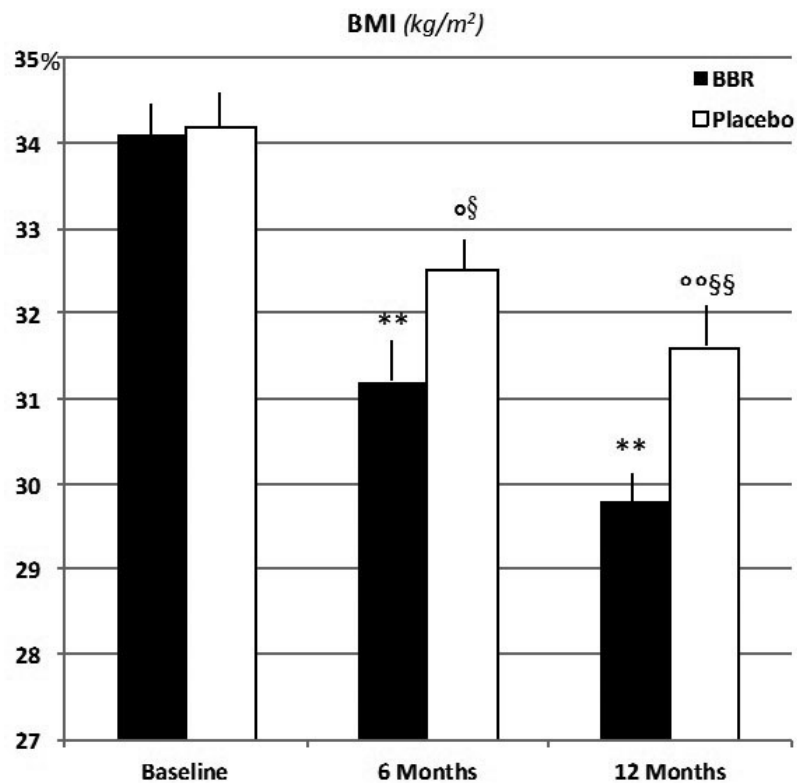


Fig. 2. Comparison between BBR and Placebo Groups in respect to BMI ($M \pm SD$; kg/m²) during a 52-week treatment period (** $p < 0.01$ vs baseline; °° $p < 0.01$; ° $p < 0.05$ vs baseline; §§ $p < 0.01$ and § $p < 0.05$ vs BBR Group). Time expressed as months.

(13). The two patient groups displayed statistically undistinguishable risk scores at baseline and were therefore combined and ranked by gender and smoking habits for further analysis: they were 13.3 ± 2.5 (range 9.2-17.7), with a marked difference between nonsmokers and smokers both in males (10.9 ± 1.5 , range 8.8-12.1 and 17.5 ± 2.7 , range 12.7-20.3, respectively) and in females (10.2 ± 1.3 , range 6.7-11.6 and 14.9 ± 2.9 , range 11.2-19.1, respectively). After the 52-week treatment period the risk score significantly decreased by a 25-32% range for men and a 8-11% range for women.

DISCUSSION

The BBR–silymarin association is a nutraceutical combination of highly standardized *Berberis aristata* and *Silybum marianum* herbal extracts, titrated as 85% BBR and 60–80% flavolignanes, respectively. In uncontrolled clinical trials BBR seems to have beneficial effects in patients with T2DM with suboptimal glycemic control when given alone (6, 8) or in addition to a conventional treatment regimen (i.e., metformin, dipeptidylpeptidase-4 inhibitors, glitazones, acarbose, or insulin alone or combined with one another), to improve the cholesterol-lowering properties of statins, and to have a positive effect on liver enzymes. Doses utilized in these preliminary studies caused virtually no relevant side effects, as witnessed by the fact that no patients discontinued treatment. The results of a recent pilot study we published on 50 obese hypercholesterolemic people with T2DM treated with BBR for 24 weeks also confirmed the ability of BBR to (i) significantly improve a series of metabolic parameters and body fat distribution, and (ii) affect energy balance at multiple sites by increasing insulin sensitivity, as witnessed by HOMA-R decrease (14).

These results were further supported by the present 52-week placebo-controlled study, showing a significant improvement of all investigated anthropometric, clinical and laboratory parameters in the BBR group.

Another interesting aspect of our study was the decrease of cardiovascular risk score in patients with a high pathological burden, represented by the metabolic

syndrome (MS) as defined by the ATP III criteria (15). In fact, due to its mechanism of action, BBR has been extensively used to date in people with high cardiovascular risk factors, such as T2DM, obesity, polycystic ovary syndrome, hyperlipidemia, hypertension, and gut dysbiosis, or with multiple chronic diseases including breast cancer.

The results obtained by others in patients with both subtle and overt glucose metabolism abnormalities and/or hyperinsulinemia and/or metabolic syndrome support the efficacy and safety of BBR in reducing cardiovascular risk factors, and our data provide statistical support to their field observations.

In fact, BBR fulfills various metabolic effects in subjects with MS by: i) exerting a protective role through its cholesterol-lowering activity; ii) protecting against atherosclerosis through its anti-inflammatory, anti-oxidant and smooth muscle cell anti-proliferative properties; iii) improving endothelial dysfunction; iv) lowering uric acid; v) increasing adipose and muscle cell glucose utilization, as well as, vi) decreasing gut glucose absorption and, consequently, body weight. The treatment seems to be safe and tolerated at the tested doses, with minimal unwanted effects, which in most cases tend to resolve spontaneously during treatment.

The originality of our findings lies in the following facts: (i) the study was conducted for 52 weeks on a homogeneous series of subjects with metabolic syndrome; (ii) a significantly lower abdominal adiposity was attained with BBR; and (iii) for the first time a significant decreasing effect was described with BBR on circulating uric acid.

Our results need further confirmation in larger trials, of course, but point to a new possible clinical use of BBR aimed at treating abdominal fat in overweight/obese patients with T2DM.

REFERENCES

1. Gentile S, Ceriello A, Strollo F on behalf of the AMD START DIAB Study Group. A multicenter Italian survey on diabetes care units reveals a somewhat slow attitude in treatment guideline implementation: Are we dealing with therapeutic inertia? Diabetes Res

- Open J 2016; 2:33-44.
2. Wang J, Xiong XJ. Current situation and perspectives of clinical study in integrative medicine in China. *Evid Based Complement Alternat Med* 2012; 2012:e268542, 1-11.
3. Vora CK, Mansoor GA. Herbs and alternative therapies: relevance to hypertension and cardiovascular diseases. *Curr Hypertens Rep* 2005; 7:275-80.
4. [No authors listed]. Berberine. *Altern Med Rev* 2000; 5:175-77.
5. Vuddanda PR, Chakraborty S, Singh S. Berberine: a potential phytochemical with multispectrum therapeutic activities. *Expert Opin Investig Drugs* 2010; 19:1297-307.
6. Cicero AF, Ferroni A, Ertek S. Tolerability and safety of commonly used dietary supplements and nutraceuticals with lipid-lowering effects. *Expert Opin Drug Saf* 2012; 11:753-66.
7. Di Pierro F, Villanova N, Agostini F, Marzocchi R, Soverini V, Marchesini G. Pilot study on the additive effects of berberine and oral type 2 diabetes agents for patients with suboptimal glycemic control. *Diabetes Metab Syndr Obes* 2012; 5:213-7.
8. Yin J, Xing H, Ye J. Efficacy of berberine in patients with type 2 diabetes mellitus. *Metabolism*. 2008; 57:712-7.
9. Xiong X, Xiaoke Li, Yuqing Zhang Y, Wang J. Chinese herbal medicine for resistant hypertension: a systematic review. *BMJ Open*. 2015; 5:e005355. Published online 2015 January 30. doi:10.1136/bmjopen-2014-005355.
10. Pan GY, Wang GJ, Liu XD, Fawcett JP, Xie YY. The involvement of P-glycoprotein in berberine absorption. *Pharmacol Toxicol* 2002; 91:193-97.
11. Kanbay M, Jensen T, Solak Y, Le M, et al. Uric acid in metabolic syndrome: From an innocent bystander to a central player. *Europ J Int Med* 2016; 29:3-8.
12. Vitturi N, Soattin M, De Stefano F, Vianello D, Zambon A, Plebani M, Busetto L. Ultrasound, anthropometry and bioimpedance: a comparison in predicting fat deposition in non-alcoholic fatty liver disease. *Eat Weight Disord* 2015; 20:241-47.
13. Rizzello V, Lucci D, Maggioni AP, et al.; IN-ACS Outcome Investigators. Clinical epidemiology, management and outcome of acute coronary syndromes in the Italian network on acute coronary syndromes (IN-ACS Outcome study). *Acute Card Care* 2012; 14:71-80.
14. Guarino G, Della Corte T, Sofia M, Carbone L, Marino G, Martedì E, Gentile S. Metabolic effects of the association *Berberis aristata*/*Silybum marianum*: a preliminary double-blind, placebo-controlled study in obese patients with type 2 diabetes. *Nutrafoods* 2015; 14:181.
15. Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). *Circulation* 2002; 106:3143-421.

LETTER TO THE EDITOR

BOTH 3,5-DIIODO-L-THYRONINE (T_2) AND T_3 MODULATE GLUCOSE-INDUCED INSULIN SECRETION

P. FALLAHI¹, S.M. FERRARI¹, E. SANTINI¹, S. CAMASTRA¹, G. FRENZILLI¹,
M. PUCCINI², F. GOGLIA³, A. LANNI⁴, P. MARCHETTI¹ and A. ANTONELLI¹

¹Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy; ²Department of Surgical, Medical, Molecular Pathology and Critical Area, University of Pisa, Pisa, Italy; ³Dipartimento di Scienze per la Biologia, la Geologia e l'ambiente, Università degli Studi del Sannio, Benevento, Italy; ⁴Department of General and Environmental Physiology, University of Naples, Naples, Italy

Received January 12, 2017 – Accepted March 8, 2017

3,5-diiodo-L-thyronine (T_2), a naturally existing iodothyronine, has biological effects on humans, but no information is available on its action on pancreatic β -cells. We evaluated its effect vs triiodothyronine (T_3), on glucose-induced insulin secretion in INS-1e cells, a rat insulinoma line, and on human islets. INS-1e were incubated in the presence/absence of T_2 or T_3 (0.1 nmol/L-10 μ mol/L), and glucose (3.3, 7.5, 11.0, and 20 mmol/L). Insulin release and content (at 11.0 and 20 mmol/L glucose) were significantly ($p < 0.01$) stimulated by 1-100 nmol/L T_2 and 0.1 nmol/L-1.0 μ mol/L T_3 , and inhibited with higher concentrations of both (1-10 μ mol/L T_2 and 10 μ mol/L T_3). Human islets were incubated with 3.3 mmol/L glucose in presence/absence of T_3 or T_2 (0.1 nmol/L, 0.1 μ mol/L, and 1 μ mol/L). T_2 (0.1 nmol/L-0.1 μ mol/L) significantly ($p < 0.01$) stimulated insulin secretion, while higher concentrations (1 μ mol/L) inhibited it. A modest increase in insulin secretion was evidenced with 1 μ mol/L T_3 . In conclusion, T_2 and T_3 have a direct regulatory role in insulin secretion, depending on their concentrations and the glucose level itself. At concentrations near the physiological range, T_2 enhances glucose-induced insulin secretion in both rat β -cells and human islets.

To the Editor,

Thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3), have various effects and are determinant for growth, differentiation and metabolism, depending on the positive or negative regulation of thyroid-responsive genes through thyroid hormone receptors (TRs), which bind to specific DNA elements, even if a mechanism apart from DNA binding has also been proposed (1). Also

various non-genomic effects of thyroid hormones have been shown, ascribing to iodothyronines other than T_4 and T_3 (2).

Until recently, it was supposed that T_3 was the only active iodothyronine, and that T_4 was its precursor. It has now become clear that other iodothyronines, especially 3,5-diiodo-L-thyronine (T_2), have biological effects (3, 4). Certainly, the resting metabolic rate (RMR) of hypothyroid rats is

Key words: diiodothyronine, triiodothyronine, islets, β -cells, INS-1e, insulin secretion

Mailing address:

Prof. Alessandro Antonelli, MD,
Immuno-Endocrine Section of Internal Medicine,
Laboratory of Primary Human Cells,
Department of Clinical and Experimental Medicine,
University of Pisa, School of Medicine,
Via Savi, 10, I-56126, Pisa, Italy
Tel.: +39 050 992318 - Fax: +39 050 993472
e-mail: alessandro.antonelli@med.unipi.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.

stimulated to the same extent (~40%) with a single dose of T_2 (25 $\mu\text{g}/100$ g body weight) or the same dose of T_3 (4).

In rats fed a high-fat diet (HFD), long-term treatment with T_2 results in reduced adiposity and less body weight gain without inducing a clinical syndrome reminiscent of the thyrotoxic state (5). More recently, it has been shown that T_2 , by activating mitochondrial processes, markedly reverses hepatic steatosis *in vivo* (6), and that T_2 (100-900 $\mu\text{g}/\text{day}$) increases RMR, decreases adiposity and body weight, without side effects in humans (7).

Literature data on the effect of thyroid hormones on insulin secretion are discordant. Most of the *in vivo* studies suggest that T_4 and T_3 can stimulate insulin secretion both in hypothyroidism and hyperthyroidism in humans (8, 9), while studies on rats have shown an inhibitory effect of thyroid hormones on insulin secretion by cultured islets (10). These contradictory results may be, at least in part, explained by the different biology of thyroid hormones in rats and humans, or the different concentrations of thyroid hormones used, mostly in the pharmacological range.

The present study evaluated the effect of T_2 , in comparison with T_3 , on glucose-induced insulin secretion in INS-1e cells, a rat insulinoma line, and on human islets.

MATERIALS AND METHODS

INS-1e cells

The INS-1e cell line was a gift from Professor Claes B. Wollheim (University of Geneva, Switzerland). Tissue culture reagents were acquired from Gibco Invitrogen (Basel, Switzerland), T_2 and T_3 were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA).

INS-1e cells were grown in monolayer culture in RPMI-1640 medium containing 11 mmol/L glucose. The culture medium was supplemented with 10% heat-inactivated fetal calf serum, 1 mmol/L sodium pyruvate, 10 mmol/L HEPES, 2 mmol/L glutamine, 50 $\mu\text{mol}/\text{L}$ β -mercaptoethanol, 100 IU/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin. Cells were cultured at 37°C in a humidified 95% air, 5% CO_2 atmosphere. Cells were seeded in wells at a density of 1.5×10^5 cells per culture well at least 96 h

before use in the insulin secretion experiments.

To measure insulin secretion, INS-1e cells were treated for 48 h in fresh RPMI-1640 medium with 11 mmol/L glucose in the presence/absence of T_2 or T_3 (0.1 nmol/L, 1 nmol/L, 0.1 $\mu\text{mol}/\text{L}$, 1 $\mu\text{mol}/\text{L}$ and 10 $\mu\text{mol}/\text{L}$). Cells were washed and then incubated for 60 min with Krebs-Hepes buffer with 3.3, 7.5, 11.0 or 20.0 mmol/L glucose. Insulin (measured by rat insulin radioimmunoassay - Linco Research, Inc., St. Charles, MO, USA) was dosed in the supernatants and cellular insulin content was determined from acid-ethanol extracts.

Human islets

Human islets were obtained from normal donors. Islet cell dissociation (11,12) was performed by resuspending cells in a calcium-free Krebs-Ringer-Hepes buffer with 1 mmol/L EGTA at 30°C. After 8 min, trypsin (16.5 $\mu\text{g}/\text{mL}$) and DNAase (2 $\mu\text{g}/\text{mL}$) were added, for 8-10 min at 30°C. Cell dissociation was evaluated observing aliquots of the suspension by an inverted light microscope. The protocol was stopped as most cells were single or in small groups of 2-3, adding Krebs-Ringer-Hepes buffer containing 1.67 mmol/L calcium and 1% calf serum. Then, cells were washed with PBS, resuspended in M199 culture medium and placed at 37°C in a CO_2 incubator.

Within 7-12 days of isolation, the insulin secretory function of the islets in response to 3.3 mmol/L glucose was evaluated by the batch incubation method, as previously reported (11). Groups of islets of comparable size (approximately 50/tube) were preincubated at 37°C for 45 min in Krebs Ringer Bicarbonate Buffer (KRB) solution, supplemented with 3.3 mmol/L glucose and 0.5% bovine serum albumin. The islets were then washed and incubated at 37°C for 45 min in KRB solution containing 3.3 mmol/L glucose, either with or without the addition of T_3 or T_2 (0.1 nmol/L, 0.1 $\mu\text{mol}/\text{L}$, 1 $\mu\text{mol}/\text{L}$). For each test, 2-4 groups of islets were incubated separately. Then, supernatants were collected to measure insulin concentration. At 3.3 mmol/L glucose, there was a positive relationship between insulin release expressed as insulin concentration in the medium and as percent of total islet content ($r=0.59$, $p<0.04$).

Data analysis

Data are given as mean $\pm$ SEM. Owing to their non-normal distribution, insulin concentrations were

expressed as median and interquartile range, and were log-transformed for use in parametric statistical analyses. Comparison of mean group values was made by ANOVA; simultaneous testing for group and treatment was carried out by two-way ANOVA, with Bonferroni-Dunn *post hoc* testing for individual group comparisons. The χ^2 test was used to evaluate association between categorical variables.

RESULTS

INS-1e

Insulin secretion was significantly ($p < 0.01$; ANOVA) stimulated (at 11.0 mmol/L and 20 mmol/L

glucose in the incubation medium) with T_2 in the concentration range 1-100 nmol/L, while inhibition of insulin secretion was evidenced with higher concentrations (1-10 μ mol/L) (Fig. 1a). Insulin content also was significantly ($p < 0.01$; ANOVA) stimulated (at 11.0 mmol/L and 20 mmol/L glucose) with 1-100 nmol/L T_2 , while it was inhibited with higher concentrations (1-10 μ mol/L) (data not shown). The insulin supernatant/content ratio was significantly ($p < 0.01$) increased (at 11.0 mmol/L and 20 mmol/L glucose) with 1-100 nmol/L T_2 , while inhibition was observed with higher concentrations (1-10 μ mol/L) (Fig. 1b).

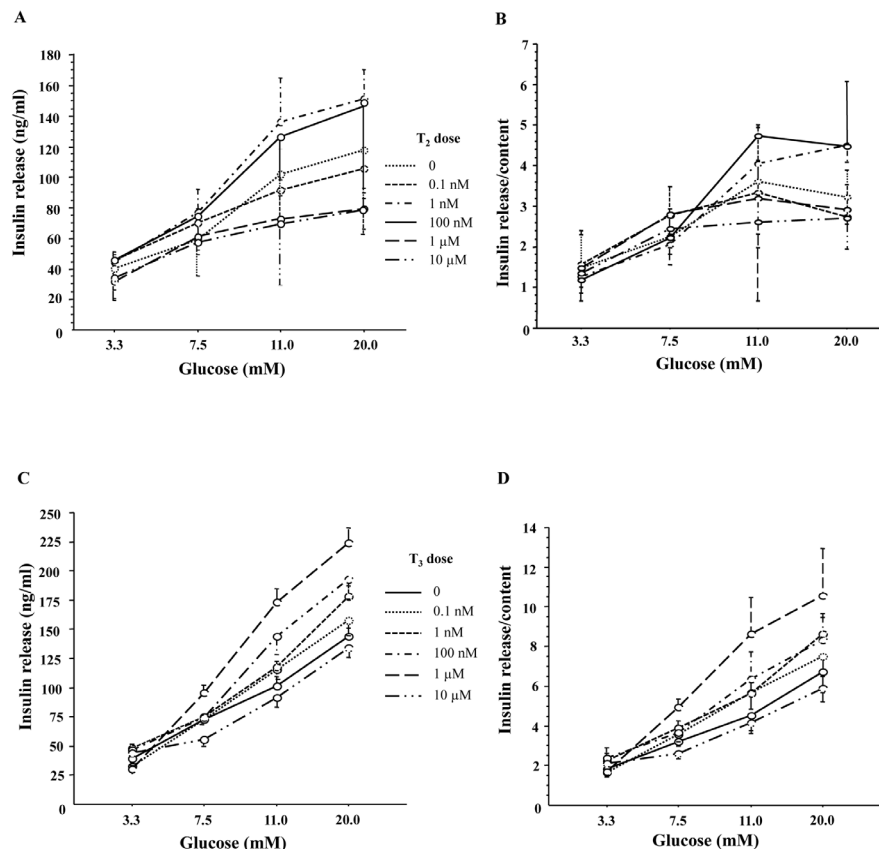


Fig. 1. Insulin secretion and/or insulin content after stimulation with T_2 or T_3 in INS-1e cells. **a)** T_2 (1-100 nmol/L) significantly ($p < 0.01$; ANOVA) stimulated (11.0 mmol/L and 20 mmol/L glucose) insulin secretion in INS-1e cells, while it was inhibited with higher concentrations of T_2 (1-10 μ mol/L). **b)** Insulin secretion/insulin content ratio was significantly ($p < 0.01$; ANOVA) increased (11.0 mmol/L and 20 mmol/L glucose) with T_2 1-100 nmol/L, while an inhibition was observed with higher concentrations (1-10 μ mol/L). **c)** T_3 had a similar pattern of stimulation than T_2 in INS-1e cells, in fact insulin secretion and insulin content were significantly ($p < 0.01$; ANOVA) stimulated (11.0 mmol/L and 20 mmol/L glucose) with T_3 0.1 nmol/L-1 μ mol/L, while an inhibition was observed at 10 μ mol/L. **d)** T_3 (0.1 nM-1 μ mol/L) increased significantly ($p < 0.01$; ANOVA) insulin secretion/insulin content ratio (11.0 mmol/L and 20 mmol/L glucose), while an inhibition was observed at 10 μ mol/L.

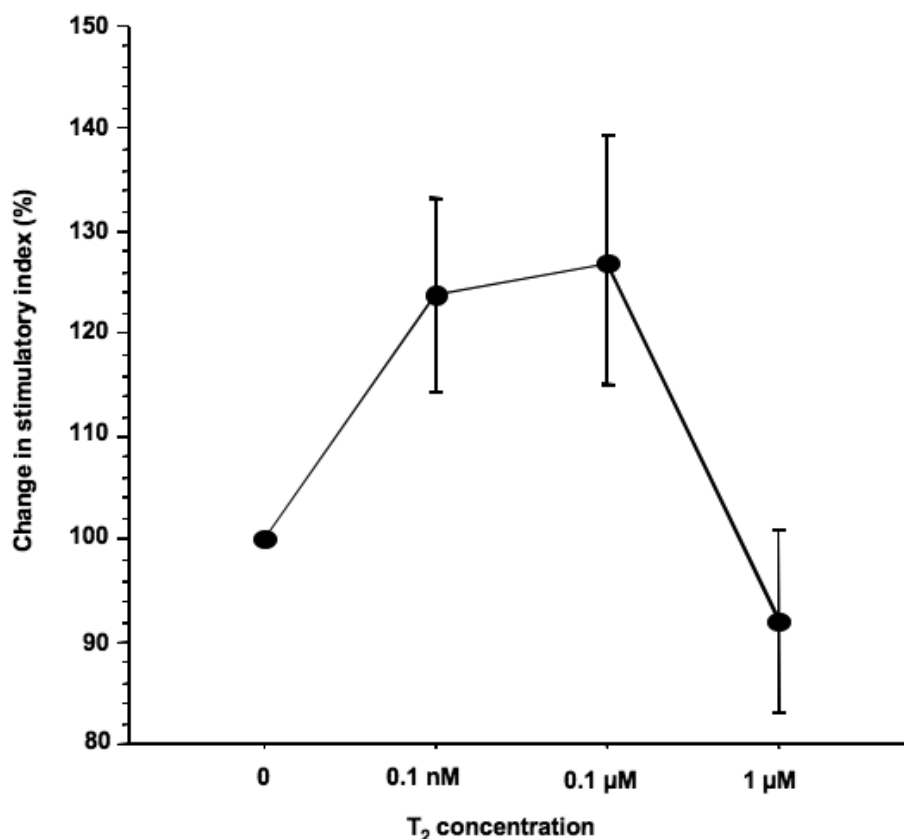


Fig. 2. Insulin secretion after treatment with T_2 in human islets. T_2 (0.1 nmol/L–0.1 μ mol/L) significantly ($p < 0.01$; ANOVA) stimulated insulin secretion in human islets, while higher concentrations (1 μ mol/L) inhibited it.

T_3 showed a similar pattern of stimulation to T_2 , as insulin secretion was significantly ($p < 0.01$; ANOVA) stimulated (at 11.0 mmol/L and 20 mmol/L glucose) with T_3 in the concentration range 0.1 nmol/L–1.0 μ mol/L, while inhibition was observed at 10 μ mol/L (Fig. 1c). Similarly, insulin content was significantly ($p < 0.01$) stimulated (at 11.0 mmol/L and 20 mmol/L glucose) with 0.1 nmol/L–1.0 μ mol/L T_3 , while it was inhibited with higher concentrations (10 μ mol/L) (data not shown). The insulin supernatant/content ratio showed the same pattern (Fig. 1d).

Human islets

Insulin secretion (at 3.3 mmol/L glucose) was significantly ($p < 0.01$; ANOVA) stimulated with 0.1–100 nmol/L T_2 , while it was inhibited with higher concentrations (1 μ mol/L) (Fig. 2). A slight increase of insulin secretion was evidenced with 1 μ mol/L T_3 ($p < 0.01$, ANOVA) (Fig. 3).

DISCUSSION

RMR is stimulated by a single dose of T_2 (25 μ g/100 g body weight) to the same extent (~40%) as the same dose of T_3 in hypothyroid rats, and T_2 induces metabolic inefficiency, perhaps stimulating energy loss through mechanisms comprising mitochondrial proton leakage/redox slippage (13), leading to reduced energy storage.

Serum concentrations of T_2 in humans have been reported to range from 10 to 190 pmol/L (average value of about 100 pmol/L). The production rate of T_2 is 22–55 nmol/day, cleared at a rate of 60–170 L/day. Tissue T_2 concentrations vary greatly in healthy subjects and in pathological conditions. To our knowledge, no study has measured T_2 levels in the human pancreas or isolated islets nor have the circulating concentrations of T_2 in animals been determined.

In our INS-1e cell cultures, we used T_2

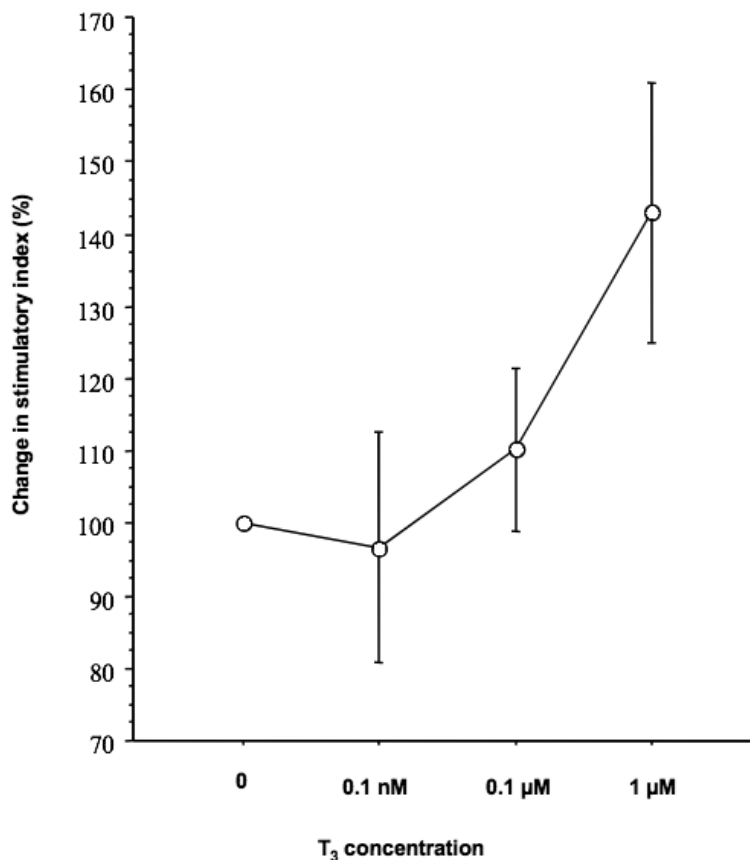


Fig. 3. Insulin secretion after treatment with T_3 on human islets. In human islets, a slight increase of insulin secretion was observed with T_3 1 $\mu\text{mol/L}$ ($p < 0.01$, ANOVA).

concentrations spanning the physiological and pharmacological range (0.1 nmol/L to 10 $\mu\text{mol/L}$). Within the probable rat physiological range (1-100 nmol/L), T_2 definitely stimulated glucose-induced insulin secretion. Higher concentrations (1-10 $\mu\text{mol/L}$) were inhibitory, probably by a toxic effect. T_3 also enhanced insulin secretion in a glucose-dependent manner, with higher concentrations becoming inhibitory. Therefore, physiological T_2 and T_3 concentrations sustain glucose-induced insulin secretion by a direct stimulatory action, such that their removal – as occurs in hypothyroidism – results in an impairment of insulin release, while their supraphysiological levels – such as in hyperthyroidism – depress insulin secretory function.

Interestingly, the effects of both T_2 and T_3 were only seen at high glucose concentrations (11.0 and 20 mmol/L glucose), suggesting that these hormones may become important regulators of insulin secretion

especially in hyperglycemic states.

A direct comparison of the interaction of glucose and thyroid hormones between isolated rat islets and INS-1e cells is inappropriate because the latter grow in a high-glucose (11 mmol/L) medium, but our findings are in agreement with others (18, 34).

T_2 effects on human islets are shown at a concentration (0.1 nmol/L – 0.1 $\mu\text{mol/L}$) about 100-1000 times lower than T_3 (1 $\mu\text{mol/L}$), and near the physiological circulating levels of T_2 (10 to 190 pmol/L), suggesting for the first time that T_2 may be involved in the physiological modulation of glucose-induced insulin secretion.

The mechanism by which thyroid hormones exert their effects on the β -cell has not been elucidated, and different hypotheses have been reported (5, 15).

In conclusion, our study indicates that both T_2 and T_3 play a direct regulatory role in insulin secretion, depending on their concentrations and the glucose

level itself. At concentrations near the physiological range, T_2 enhances glucose-induced insulin secretion in both rat β -cells and human islets. Further studies are necessary to elucidate the cellular and molecular pathways of T_2 action.

REFERENCES

- Shibusawa N, Hashimoto K, Nikrodhanond AA, et al. Thyroid hormone action in the absence of thyroid hormone receptor DNA-binding in vivo. *J Clin Invest* 2003; 112:588-97.
- Lanni A, Moreno M, Lombardi A, de Lange P, Goglia F. Control of energy metabolism by iodothyronines. *J Endocrinol Invest* 2001; 24:897-913.
- Goglia F. Biological effects of 3,5-diiodothyronine (T_2). *Biochemistry (Mosc)* 2005; 70:164-72.
- Moreno M, Lanni A, Lombardi A, Goglia F. How the thyroid controls metabolism in the rat: different roles for triiodothyronine and diiodothyronines. *J Physiol* 1997; 505:529-38.
- Lanni A, Moreno M, Lombardi A, et al. 3,5-diiodo-L-thyronine powerfully reduces adiposity in rats by increasing the burning of fats. *Faseb J* 2005; 19:1552-54.
- Mollica MP, Lionetti L, Moreno M, Lombardi A, De Lange P, Antonelli A, Lanni A, Cavaliere G, Barletta A, Goglia F. 3,5-diiodo-L-thyronine, by modulating mitochondrial functions, reverses hepatic fat accumulation in rats fed a high-fat diet. *J Hepatol* 2009; 51:363-70.
- Antonelli A, Fallahi P, Ferrari SM, Di Domenicantonio A, Moreno M, Lanni A, Goglia F. 3,5-diiodo-L-thyronine increases resting metabolic rate and reduces body weight without undesirable side effects. *J Biol Regul Homeost Agents* 2011; 25:655-60.
- Handisurya A, Pacini G, Tura A, Gessl A, Kautzky-Willer A. Effects of T_4 replacement therapy on glucose metabolism in subjects with subclinical (SH) and overt hypothyroidism (OH). *Clin Endocrinol (Oxf)* 2008; 69:963-9.
- O'Meara NM, Blackman JD, Sturis J, Polonsky KS. Alterations in the kinetics of C-peptide and insulin secretion in hyperthyroidism. *J Clin Endocrinol Metab* 1993; 76:79-84.
- Fragner P, Ladram A, Aratan de Leon S. Triiodothyronine down-regulates thyrotropin-releasing hormone (TRH) synthesis and decreases pTRH-(160-169) and insulin releases from fetal rat islets in culture. *Endocrinology* 1999; 140:4113-19.
- Antonelli A, Baj G, Marchetti P, et al. Human anti-CD38 autoantibodies raise intracellular calcium and stimulate insulin release in human pancreatic islets. *Diabetes* 2001; 50:985-91.
- Antonelli A, Tuomi T, Nannipieri M, et al. Autoimmunity to CD38 and GAD in Type I and Type II diabetes: CD38 and HLA genotypes and clinical phenotypes. *Diabetologia* 2002; 45:1298-306.
- Lombardi A, Lanni A, Moreno M, Brand MD, Goglia F. Effect of 3,5-diiodo-L-thyronine on the mitochondrial energy-transduction apparatus. *Biochem J* 1998; 330:521-26.
- Fukuchi M, Shimabukuro M, Shimajiri Y, et al. Evidence for a deficient pancreatic β -cell response in a rat model of hyperthyroidism. *Life Sci* 2002; 71:1059-70.
- Oberkofler H, Klein K, Felder TK, Krempler F, Patsch W. Role of peroxisome proliferator-activated receptor- γ coactivator-1 α in the transcriptional regulation of the human uncoupling protein 2 gene in INS-1E cells. *Endocrinology* 2006; 147:966-76.

LETTER TO THE EDITOR

SHORT TERM EFFICACY OF CAPACITIVE-RESISTIVE DIATHERMY THERAPY IN PATIENTS WITH LOW BACK PAIN: A PROSPECTIVE RANDOMIZED CONTROLLED TRIAL

A. NOTARNICOLA^{1,2}, G. MACCAGNANO², M.F. GALLONE³, I. COVELLI², S. TAFURI³
and B. MORETTI^{1,2}

¹*Course of Motor and Sports Sciences, Department of Medical Sciences of Basis, Neurosciences and Organs of Sense, Faculty of Medicine and Surgery, University of Study of Bari, Bari, Italy;*

²*Orthopedics Section, Department of Medical Sciences of Basis, Neurosciences and Organs of Sense, Faculty of Medicine and Surgery, University of Study of Bari, General Hospital, Bari, Italy;*

³*Department of Biomedical Sciences and Human Oncology, Faculty of Medicine and Surgery, University of Study of Bari, General Hospital, Bari, Italy*

Received December 24, 2016 – Accepted April 3, 2017

To compare the effectiveness of Doctor Tecar Therapy™ with that of laser therapy in the management of low back pain (LBP), a total of 60 patients with LBP were enrolled. The participants were randomly divided into two groups: a Tecar group (experimental group, 30 subjects), and a laser group (control group, 30 subjects). All the subjects received 10 sessions of therapy: one each day from Monday to Friday and the same again the following week. All the subjects were evaluated for pain (VAS) and disability (Roland and Morris score and Oswestry score) at baseline (T0), and 2 weeks (T1), 1 month (T2) and 2 months (T3) after the end of treatment. The pain and disability presented a trend to improvement over time in both groups. This improvement was statistically significant at all follow-ups (FUs) in the Tecar group but only at T1 for the Laser group ($p < 0.01$). Comparing the two methods, there emerged a significant difference in favour of the Tecar group at T2 and T3 ($p < 0.01$). The results show that Tecar therapy determined significant improvement already by the end of the treatment. Moreover, at the first and second month FUs, the Tecar therapy showed statistically better results than laser therapy.

To the Editor,

LBP is described as a pain in the lumbar region which may be associated with irradiation to the lower limbs, due to irritation of the nerve roots that originate at this level (1). The causes of pain are multifactorial. Conditions, such as degenerative disc disease or herniated disc, are causes of low back pain. A disc may herniate or bulge and compress a nearby spinal nerve root causing irritation and inflammation. The body attempts to immobilize the affected area to stop pain by tightening the surrounding musculature

and, as a result, painful muscle spasms occur. Radiculopathy, nerve root pain or leg pain is defined as pain radiating to the leg, above the knee or below the knee and into the foot with variable neurological findings (2). The association between degeneration of the intervertebral disc and the nociceptive mechanism of back pain has been demonstrated (3). When there is no indication for surgery, LBP is commonly treated with drugs and rehabilitation, which include analgesic, nonsteroidal anti-inflammatory drugs, steroid injections, exercise therapy, transcutaneous

Key words: Low back pain, diathermy, laser therapy

Mailing address:

Angela Notarnicola, MD, Ph.D., Associate Professor
Orthopedics Section, Department of Medical Sciences of Basis,
Neurosciences and Organs of Sense, Faculty of Medicine and Surgery,
University of Study of Bari, General Hospital,
Piazza Giulio Cesare 11, 70124 Bari, Italy
Tel./fax: +39 080 5592938
e-mail: angelanotarnicola@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.

electrical nerve stimulation (TENS), superficial heat or cold, infrared, ultrasound, body cast, laser therapy, diathermy, individual patient education, massage, behavioral treatment, lumbar supports, traction, and multidisciplinary rehabilitation (4-8). The fact that there are so many types of treatment for chronic LBP, testifies that no single approach has yet been able to demonstrate its definitive superiority (9).

TECAR (Energy Transfer CAPacitive and Resistive) therapy represents the technological evolution of diathermy (9-12). Despite the popularity of this treatment, unfortunately, there are no randomized controlled studies that have evaluated its therapeutic benefits. The initial hypothesis in the use of Tecar therapy is that diathermy warms up the tissues by contact. It is accepted that the passage of current which induces an electromagnetic field increases tissue temperature, causes vasodilatation, increases pain threshold, reduces muscle spasm, quickens cell activity and increases the elasticity of the connective tissues (10-12).

Clinical trials are needed to verify the clinical effects of Tecar therapy. This is the aim of our study.

MATERIALS AND METHODS

We conducted a prospective randomized case control study in order to verify the efficacy of Tecar therapy in the treatment of LBP. The control group consisted of patients treated with laser therapy. This trial was conducted at the Orthopaedics Unit of our General Hospital. The study was approved by the local research Ethics Committee. All subjects were informed and signed consent to participate in the study.

Patients and recruitment

Sixty patients with symptoms of LBP and documented discopathy or disc herniation were enrolled. The patients were selected according to the following criteria: age >18 years, a diagnosis of chronic low back pain lasting at least 12 weeks, made on the basis of clinical symptoms and instrumental tests, and a functional VAS score ≥ 4 as well as computed tomography (CT) or nuclear magnetic resonance (MRI) documented discopathy or disc hernia. Patients were excluded from the study if they had contraindications to Tecar therapy or laser therapy:

neoplasia or current or previous infections of the affected area, a cardiac pacemaker, or pregnancy. They were also excluded if they had the presence of active vertebral fractures, a history of previous spine surgery, rheumatic diseases such as rheumatoid arthritis, infections and systemic diseases that could affect pain perception (diabetes, etc) or local injections (local anesthetic and/or corticosteroids; O2O3, etc), drugs or physiotherapy administered within the previous 4 weeks.

All baseline epidemiological and clinical data were obtained before treatment. Musculoskeletal physical examination included range of motion of the lumbar spine and full neurological examination which was composed of evaluation of gait, ability to walk on the heel/toe, muscle strength, sensory and reflex assessments in the lower extremity, and straight leg raise test.

Clinical results were evaluated using visual analogic scale (VAS), Roland Morris Disability Questionnaire and Oswestry Low Back Pain Disability Questionnaire at the time of recruitment (T0) at first follow-up (FU) two weeks after the end of treatment (T1), and respectively 1 and 2 months later (T2 and T3).

The intensity of pain in the leg and lower back was measured using visual analogue scale set from 0 (no pain) to 10 (worst possible pain) (12). Disability was measured using the Roland Morris Disability Questionnaire and the Oswestry Disability Index, where respectively 24 and 100 indicate worst possible disability. We recommended taking acetaminophen if the pain was unbearable.

Treatment

The patients of the study group received 10 min of capacitive phase and 10 min of resistive phase of Tecar therapy (Doctor Tecar Therapy™, Mectronic Medical SRL, Bergamo, Italy) at each session. Tecar therapy is an endogenous thermotherapy (diathermy) that uses electrical currents in the frequency range between 0.45 and 0.60 MHz and generates thermal effects (heating) in the deep layers. The tool consists of a generator of short wave currents and electrodes with multifrequency sequential systems of emission. The thermic effects generated in the tissues were monitored and controlled by Biotransfer. This device regulates the intensity and frequency of the impulses and maintains the tissue temperature within a naturally thermic range of 37 to 42°C. The device consists of a generator of short wave currents and electrodes with

multifrequency sequential systems of emission. The treatment has two phases. The first is called 'capacitive' and is characterized by a frequency of 600 kHz which determines an increase in superficial temperature and thus vasodilatation and catabolic liquid reabsorption. The second is called 'resistive' and is characterized by a frequency of 450 kHz and generates a deep warming of the tissues in order to increase oxygenation of the deep muscular structures. The power of energy in both phases is the same: 10–12 VA, equivalent to 0.42 J/cm², applied to an area of 28.2 cm².

The patients of the control group were treated with laser therapy (Ixyon, Mectronic Medicale, Italy). The protocol requires continuous mode emission and a wavelength of 980 nm. The power was set constantly at 500 mW. The spot size was 1 cm². In a single session, each patient was treated with laser therapy using a dosage of 10 J per each cm² of this painful area at the level of the column.

In both groups, each patient was treated five times a week, for a total of 10 sessions. The patient was placed in a prone position on the treatment couch with hip and knee extended and the ankle in maximal extension. The treatment surface area was the lumbar paravertebral zone in correspondence with the vertebra affected by discal disease. For both therapies, the treatment was carried out using manual scanning following the direction of muscle fibers.

Statistical analysis

For each enrolled patient a standardized form was filled in that included information regarding sex, age, weight, height, body mass index (BMI), initiation of treatment, smoking habit, flexion and extension range of motion (ROM), Lasegue test and femoral stretch test positivity, sensitivity, strength, tone and trophic alteration, rotation degrees, type of treatment and scores from each functional scale. Each patient underwent a functional evaluation by means of three different scales: Visual Analogue Scale (VAS), Roland-Morris Disability Questionnaire (RMDQ), Oswestry Disability Index (ODI). The functional evaluation was performed before the treatment.

All information was collected in a database made by FileMaker Pro software and data were analyzed using Stata MP12 statistical software. For categorical variables, absolute frequency and proportion were evaluated. The proportions in the two treatment groups were compared using *chi*-squared test. Continuous variables and all the

scores, measured at different times for each group, were described as mean with the indication of the standard deviation and as median with the indication of the range. In order to evaluate the differences regarding the functional scores, in each group, measured at different times of follow-up, the median scores were compared using Wilcoxon sign-rank test, and two follow-ups were compared at a time (T0 vs T1; T1 vs T2; T2 vs T3; T0 vs T3). In order to evaluate the differences between the two groups of treatment as regards the functional scores measured at each follow-up, the median scores were compared using Kruskal-Wallis test for independent samples. For each test we considered as statistically significant a value of $p < 0.05$.

RESULTS

The study population was made up of 17 males (28.3%) and 43 females (71.7%) (Table I). The average age was 59.7 (DS: 13.9; range: 20-89), weight 71.9 Kg (DS: 10.7; range: 50-90), height 164.8 cm (DS: 8.9; range: 147-182), BMI 26.5 kg/m² (DS: 19; range: 19-36.2). The average time of LBP onset was 6 months (DS: 4; range: 3 – 24); 15% were smokers. The clinical evaluation showed the following results: 12% were characterized by sensibility alteration, 10% by decreased muscle strength, 5% by decreased muscle mass, 40% were positive to Lasegue test and 25% to Wassermann test.

Each treatment group was made up of 30 patients. Between the two groups we did not reveal any statistically significant differences regarding epidemiological and clinical features apart from alteration of sensitivity (Table I). There were no drop-outs. The patients did not refer any side effects. No patient reported to have taken acetaminophen.

VAS

In each of the two treatment groups, a statistically significant difference emerged between individuals regarding the mean of the measured scores before treatment and the last follow-up (Tecar group: $z=4.8$, $p<0.01$; laser group: $z=4.3$, $p<0.01$). However, the patients treated with laser therapy showed differences only at the first follow-up ($z=4.7$, $p<0.01$), whereas in the patients treated with Tecar

Table I. Comparison of epidemiological features and clinical features of the enrolled population, expressed as average and range or as percentage, in the two groups by univariate analysis. Significant value for $p < 0.05$.

Epidemiological features	Total sample (n=60)	Laser-therapy (n=30)	Tecar-therapy (n=30)	χ^2	p
	Average $\pm$ DS (range)	Average $\pm$ DS (range)	Average $\pm$ DS (range)		
Age (years)	59.7 $\pm$ 13.9 (20 – 89)	61 $\pm$ 11.8 (21 - 76)	59.4 $\pm$ 16.0 (28 - 89)	0.08	0.8
Weight (kg)	71.9 $\pm$ 10.7 (50.0 – 90.0)	73.5 $\pm$ 11.0 (54 - 90)	70.2 $\pm$ 10.3 (50 - 88)	1.4	0.4
Height (cm)	164.8 $\pm$ 8.9 (147 – 182)	165.3 $\pm$ 7.7 (154 - 182)	164.3 $\pm$ 10.1 (147- 182)	0.3	0.6
BMI (kg/m ²)	26.5 $\pm$ 19.0 (19.0 - 36.2)	26.8 $\pm$ 2.7 (19.0 – 30.0)	26.1 $\pm$ 4.0 (19.5 – 36.2)	1.4	0.2
Beggining of pain(months)	6.6 $\pm$ 4.0 (3.0 – 24.0)	6.2 $\pm$ 3.7 (4.0 – 24.0)	7.1 $\pm$ 4.2 (1.0 – 18.0)	1.1	0.3
Active maximum estension (degree)	12.3 $\pm$ 8.7 (19.0 - 36.2)	14.7 $\pm$ 6.3 (10.0 – 30.0)	10.0 $\pm$ 10.2 (0.0 – 30.0)	3.7	0.05
Active maximum flexion (degree)	39.5 $\pm$ 13.4 (20.0 – 65.0)	39.3 $\pm$ 12.8 (20.0 – 60.0)	39.7 $\pm$ 14.2 (20.0 – 65.0)	0.04	0.8
Features	Total sample (n=60)	Laser (n=30)	Tecar (n=30)	χ^2	p
	N (%)	N (%)	N (%)		
Male gender	17 (28.3)	7 (23.3)	10 (33.3)	0.7	0.4
Smoking	9 (15.0)	6 (20.0)	3 (10.0)	1.2	0.3
Lasegue test +	24 (40.0)	15 (50.0)	9 (30.0)	2.5	0.1
Wassermann test +	15 (25.0)	8 (26.7)	7 (23.3)	0.09	0.8
Altered sensibility	12 (20.0)	10 (33.3)	2 (6.7)	6.7	0.01
Alteration of tendin reflexes	24 (40.0)	17 (56.7)	7 (23.3)	6.9	<0.01
Alteration of muscle stenght	6 (10.0)	4 (11.3)	2 (6.7)	0.7	0.4
Alteration of muscle tone and trophism	3 (5.0)	1 (3.3)	2 (6.7)	0.4	0.6

Significant value for $p < 0.05$

Table II. Comparison of the average scores of the different scales (VAS score for pain; Roland and Oswestry scores for disability) in the two treatment groups at the different FUs (Wilcoxon sign-rank test and Kruskal-wallis test for independent sample).

Scale	Group	T0	T1	T2	T3
		Average $\pm$ DS (range)	Average $\pm$ DS (range)	Average $\pm$ DS (range)	Average $\pm$ DS (range)
VAS	Laser	7.4 $\pm$ 1.6 (4 - 10)	3.6 $\pm$ 2.4 (0 - 10)	3.5 $\pm$ 2.5 (0 - 10)	4.2 $\pm$ 2.4 (0 - 9)
	Tecar	8.0 $\pm$ 1.5 (5 - 10)	3.7 $\pm$ 1.8 (0 - 8)	1.6 $\pm$ 0.9 (0 - 4)	0.8 $\pm$ 0.7 (0 - 2)
Scale	Group	T0	T1	T2	T3
		media $\pm$ DS (range)	media $\pm$ DS (range)	media $\pm$ DS (range)	media $\pm$ DS (range)
ROLAND 24S	Laser	14.4 $\pm$ 5.1 (3 - 24)	8.8 $\pm$ 6.3 (0 - 21)	9.1 $\pm$ 7.0 (0 - 24)	8.6 $\pm$ 6.5 (0 - 22)
	Tecar	14.2 $\pm$ 4.4 (3 - 21)	4.7 $\pm$ 2.5 (1 - 9)	2.0 $\pm$ 1.9 (0 - 7)	1.5 $\pm$ 1.4 (0 - 4)
Scale	Group	T0	T1	T2	T3
		media $\pm$ DS (range)	media $\pm$ DS (range)	media $\pm$ DS (range)	media $\pm$ DS (range)
OSWESTRY	Laser	43.0 $\pm$ 16.5 (16 - 88)	28.7 $\pm$ 18 (0 - 82)	26.8 $\pm$ 19.8 (0 - 88)	26.0 $\pm$ 18.6 (0 - 78)
	Tecar	46.0 $\pm$ 15.4 (18 - 74)	22.3 $\pm$ 11.0 (8 - 42)	9.4 $\pm$ 14.6 (4 - 22)	6.0 $\pm$ 2.7 (4 - 12)

(T0: recruitment, T1: at the end of treatment, T2 and T3: after 1 and 2 months).

The data are expressed as average, standard deviation and range. The significant value was for $p < 0.05$.

therapy an improvement emerged at each follow-up. Indeed, in this group the scores decreased from T0 to T1 ($z=4.8$, $p<0.01$), from T1 to T2 ($z=4.5$, $p<0.01$) and from T2 to T3 ($z=2.8$, $p<0.01$).

No statistically significant differences emerged between the two treatment groups with regard to VAS scores at T0 ($\chi^2=2.1$, $p=0.2$) and T1 ($\chi^2=0.4$, $p=0.6$). However, significant differences were revealed at T2 ($\chi^2=15.7$, $p<0.01$) and T3 ($\chi^2=30.7$, $p<0.01$), with the lowest scores being reached in patients treated with Tecar therapy. The scores measured at T0 and T3 showed differences within the two groups ($\chi^2=24.9$, $p<0.01$).

Roland Morris Disability Questionnaire

In each of the treated groups, patients showed statistical differences regarding mean measured scores at T0 and T3 (Tecar therapy: $z=4.8$, $p<0.01$; laser therapy: $z=4.3$, $p<0.01$) (Table II). The patients treated with laser therapy showed improvement only at T1 ($z=4.7$, $p<0.01$) and remained stable at the following follow-ups. The patients treated with Tecar therapy showed improvement at all follow-ups (T0-T1: $z=4.8$, $p<0.01$; T1-T2: $z=4$, $p<0.01$; T2-T3: $z=4.8$, $p<0.01$).

No statistically significant differences emerged between the two groups at T0 ($X^2=0.2$, $p=0.6$).

However, significant differences between the two groups were revealed at all follow-ups, with the lowest scores being achieved by patients treated with Tecar therapy (T1: $\chi^2=6.6$, $p=0.01$; T2: $\chi^2=18.1$; T3: $\chi^2=24.8$, $p<0.01$). The variation in the scores measured at T0 and T3 were different between the two groups ($\chi^2=20$, $p<0.01$).

Oswestry Disability Index

In each of the treated groups, patients showed statistical differences regarding mean measured scores at T0 and T3 (Tecar therapy: $z=4.8$, $p<0.01$; Laser therapy: $z=4.3$, $p<0.01$) (Table II). The patients treated with Laser therapy showed improvement only at T1 ($z=4.7$, $p<0.01$) and remained stable at the following follow-ups. The patients treated with Tecar therapy showed improvement at all follow-ups with greater variation between T0 and T1 ($z=4.7$, $p<0.01$) and T1 and T2 ($z=4.1$, $p<0.01$).

No statistically significant differences emerged between the two groups regarding the Oswestry Disability Index at T0 ($\chi^2=0.8$, $p=0.4$) and T1 ($\chi^2=1.8$, $p=0.2$). However, significant differences between the two groups were revealed at T2 ($\chi^2=16.5$, $p<0.01$) and T3 ($\chi^2=21.9$, $p<0.01$), with the lowest scores being achieved by patients treated with Tecar therapy. The variation in the scores measured at T0 and T3 were different between the two groups ($\chi^2=22.7$, $p<0.01$).

DISCUSSION

The present study is the first to compare the efficacy of Tecar therapy with another treatment in patients with LBP. Our results show that both methods, Tecar therapy and laser therapy, administered separately, determined significant improvement as regards pain and disability. The reduction of pain (VAS) and the improvement of function (Roland Morris Disability Questionnaire and the Oswestry Disability Index) within both the groups began already at the end of the 2 weeks of treatments. However, no significant difference was found between the groups at this time. The trend of improvements was continued throughout the whole period of 2 months of the study. At the FUs at 1st and 2nd months the patients treated with Tecar therapy showed results statistically better than

patients treated with laser therapy.

The clinic and functional improvement that we found in patients treated with Tecar therapy can be interpreted by considering the biological effects of the treatment applied to the specific pathological picture: anti-oedema and anti-inflammation action due to wash-out of inflammatory cytokines involved in the degeneration of the disc and responsible for nerve damage; muscle relaxant action on paravertebral contracted muscle due to inflammatory mediators present; antalgic action due to the release of endorphins that control the nociceptive and neuropathic pain; bio-stimulative action due to the increase in cellular metabolism which contrasts degenerative processes (13).

There were three limitations to this study. Firstly, we did not perform a long-term follow-up. Secondly, the treating physician who collected the data was not blinded to the group allocation. Thirdly, the absence of a placebo group.

The results of the present study are encouraging in supporting that Tecar therapy could be an effective modality in the treatment of patients with low back pain. Further studies could clarify the potential differences in application between Tecar and Laser therapy, related to physical and biological effects. It would be useful to compare different protocols which differ in energy parameters, number of sessions, combination with other treatments, etc, to define a standard treatment.

ACKNOWLEDGEMENTS

The authors thank Brian John Molloy B.A. for language revision.

REFERENCES

1. Macfarlane GJ, Jones GT, Hannaford PC. Managing low back pain presenting to primary care: where do we go from here? *Pain* 2006; 122(3):219-22.
2. Konstantinovic LM, Cutovic MR, Milovanovic AN, Jovic SJ, Dragin AS, Letic MDj, Miler VM. Low level laser therapy for acute neck pain with radiculopathy. *Pain Med* 2010; 11:1169-78.
3. Vincent AM, Edwards JL, Sadidi M, Feldman EL.

- The antioxidant response as a drug target in diabetic neuropathy. *Curr Drug Targets* 2008; 9(1):94-100.
4. Notarnicola A, Fischetti F, Maccagnano G, Comes R, Tafuri S, Moretti B. Daily pilates exercise or inactivity for patients with low back pain: a clinical prospective observational study. *Eur J Phys Rehabil Med* 2014; 50(1):59-66.
 5. Osti R, Pari C, Salvatori G, Massari L. Tri-length laser therapy associated to tecar therapy in the treatment of low-back pain in adults: a preliminary report of a prospective case series. *Lasers Med Sci* 2015; 30(1):407-12.
 6. Notarnicola A, Maccagnano G, Tafuri S, Gallone MF, Moretti L, Moretti B. High level laser therapy for the treatment of lower back pain: clinical efficacy and comparison of different wavelengths. *J Biol Regul Homeost Agents* 2016; 30(4):1157-64.
 7. Fiore P, Panza F, Cassatella G, Russo A, Frisardi V, Solfrizzi V, Ranieri M, Di Teo L, Santamato A. Short-term effects of high-intensity laser therapy versus ultrasound therapy in the treatment of low back pain: a randomized controlled trial. *Eur J Phys Rehabil Med* 2011; 47(3):367-73.
 8. Manchikanti L, Hirsch JA. An update on the management of chronic lumbar discogenic pain. *Pain Manag* 2015; 10:1-14.
 9. Haldeman S, Dagenais S. What have we learned about the evidence informed management of chronic low back pain? *Spine J* 2008; 8:266-77.
 10. Shields N, Gormley J, O'Hare N. Short-wave diathermy: current clinical and safety practices. *Physiother Res Int* 2002; 7:191e202
 11. Cameron MH. *Physical Agents in Rehabilitation: from Research to Practice*. Philadelphia: W.B. Saunders; 1999.
 12. Low J. Dosage of some pulsed shortwave clinical trials. *Physiotherapy* 1995; 81:611e6
 13. Zati A, Valent A. *Terapia Fisica: Nuove Tecnologie In Medicina Riabilitativa*. Edizioni Minerva medica, 2006.

LETTER TO THE EDITOR

**BIOMECHANICAL CONSIDERATIONS ON MACROMORPHOLOGY
OF ENDOSSEOUS DENTAL IMPLANTS**

S. FANALI and R. CELLETTI

*Department of Medical Oral and Biotechnological Sciences, University G. d'Annunzio
Chieti-Pescara, Chieti, Italy**Received January 25, 2017 – Accepted April 4, 2017*

The aim of the present study is to define the optimal thread form and why the macroscopic shape of the dental intra-osseous implant interacts with the biological environment thus conditioning its lifespan and long-term success.

To the Editor,

One of the major topics of research in implant dentistry is to improve dental implant shapes to accelerate the osseointegration process, to preserve marginal bone and to prevent peri-implantitis (1-5). The thread design can also influence the alterations of marginal bone level and the progression of peri-implantitis. Thread configuration is also an important objective in biomechanical optimization of dental implants and is strongly related to long-term maintenance of the interface between implant and bone (6-9). The interface can be easily compromised by compressive forces that cannot dissipate through the implant configuration (9-18).

The aim of the present study is to define the optimal thread form and why the macroscopic shape of the dental intra-osseous implant interacts with the biological environment thus conditioning its lifespan and long-term success.

Implant design

The implant design should be considered as totally independent from the replaced tooth: since it is useless to duplicate the form, the materials and

the bone connection, the implant-prosthetic system duplicates only the function. It is evident that by maintaining a circular section like the tooth, the macroscopic shape of the endosseous implant can be either cylindrical or conical. A conical implant shows some advantages: easier to insert, because during insertion the screw passively enters the socket before engaging the bone, by almost its total length. This makes the surgical insertion easier which, automatically, will coincide with the surgical socket.

As the fixture is already in the socket middle point, the time necessary to complete the insertion and tighten the implant to the surrounding bone is less, speeding up the work.

The disadvantages are by far more important:

- The limited bone contact area that a conical implant offers in apical zone, due to the apex tapered shape, could jeopardize its primary stability and, at the same time, increase the stress on the coronal portion with lateral movements.

- If a conical implant is not tightened properly with the right amount of torque, consequences would be quite different. In the case of a cylindrical

Key words: thread, biomechanic, loading, bone resorption, stress

Mailing address:

Prof. Stefano Fanali,
Chairman Implantology Course 1,
Via A Sartorio 2,
00147 Rome, Italy
Tel.: +39 06 5140908
Fax: +39 06 5140908
e-mail: fanali@odontoiatra.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.**

implant there would be no effects, taking into account the minimal position change, so that the stability would remain unaltered

- Vice versa, the conical implant, would immediately lose osseous contact and initial stability leading to failure

- That which considerably makes the difference between a cylindrical and a conical implant is the reaction to vertical loads.

Usually the implant diameter is smaller than the prosthetic crown; if a vertical load acts on the external part of the occlusal surface of the crown or, worse, has an oblique direction, the entire crown-implant complex system is stressed not only with a simple vertical force but with a torque following a bending moment. Since the fulcrum is placed more or less in the middle of the fixture body (depending on the bone quality) this moment will tend to spin the implant-prosthetic complex (11-17).

Another disadvantage for the conical-shaped implant consists in the apex distance from the wall cortical, always wider in respect to a cylindrical implant, so that the fixture functional instability will worsen.

Strong et al. (18) analyzed how vector forces quantitatively behave on an implant in function. To make things easier, they didactically formed

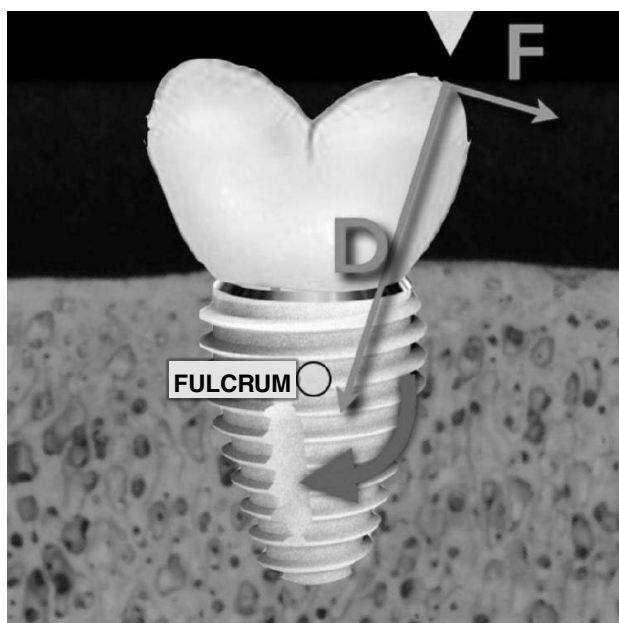


Fig. 1. *Cylindrical Implant.*

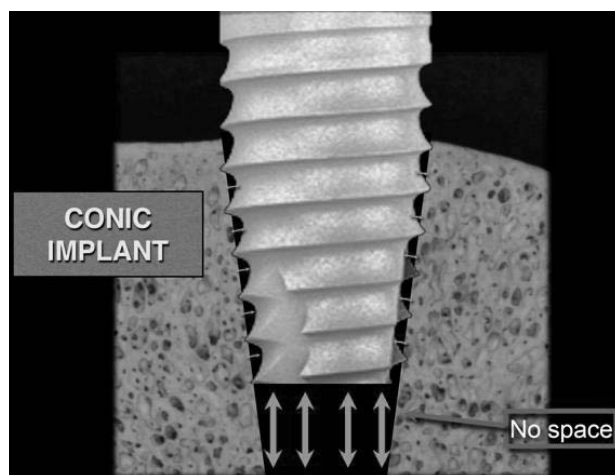


Fig. 2. *Conical Implant.*

three non-homogeneous groups composed of an infinite number of vectors that varied in direction and intensity. For example, rarely a compressive stress will be pure and perfectly directed on the implant axis, but in this group the authors also included all the “para-axials” vectors which have a preponderant compressive component.

i) The first group includes the compression vectors, namely the co-axial vectors of the implant axis, the direction of which goes towards the socket: the implant opposes them the highest resistance and for this reason a value of 100 has been assigned.

ii) The second group is formed by the tension vectors, co-axial to the implant axis, which direction goes toward the oral cavity and against which the implant opposes a minor force; to them a value of 65 has been assigned.

iii) The third group is composed of transversal vectors, namely those which are cross-sectional to the implant axis, to which the implant makes a much weaker opposition; their value is 35.

All this emphasizes the need to limit all the lateral occlusal forces, especially in the osseous low density areas in which the stability is already precarious (13-16).

Implant core

Thread geometry includes thread pitch, depth and configuration. Although thread pitch and depth could affect the stress distribution, traditionally, the manufacturers have provided implant system with a

constant pitch and depth. The first screw implants were composed of a thin core and implant stability was achieved by means of the width of the threads. On the contrary, with the introduction of the bi-phasic implant, the core needs width for the screw connecting the prosthetic abutment and therefore threads and pitches become smaller.

Functional surface area

In implantology, we define as 'functional surface area' the implant surface intended to eliminate compressive and tensile loads, therefore excluding the oblique ones. It determines, together with the osseous density, the load bio-mechanical distribution. Strong (18) compared three different implant threads: "V"-shaped, "spur"-shaped and "square"-shaped.

The larger surface is given by the square surface, therefore it should be considered as the most advantageous. The "spur"-shaped ones do not show many advantages in respect to the "V"- and "square"-shaped. On the contrary, they also lead to a number of construction problems with cost increases without giving any consistent solution. It has been demonstrated (14, 16) that square threads distribute the axial loads better compared to the side loads; in fact, the majority of them are supported by the implant core (Figs. 1-4).

In the "V"-shaped thread, due to the 30° thread angulation the axial loads vector is almost equal to the side loads vector thus obtaining a more balanced

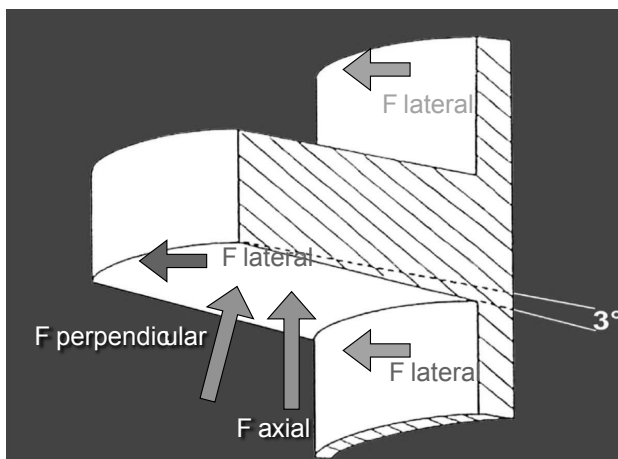


Fig. 3. Square threads strongly support axial forces. Lateral forces are lower because they are supported by the implant core.

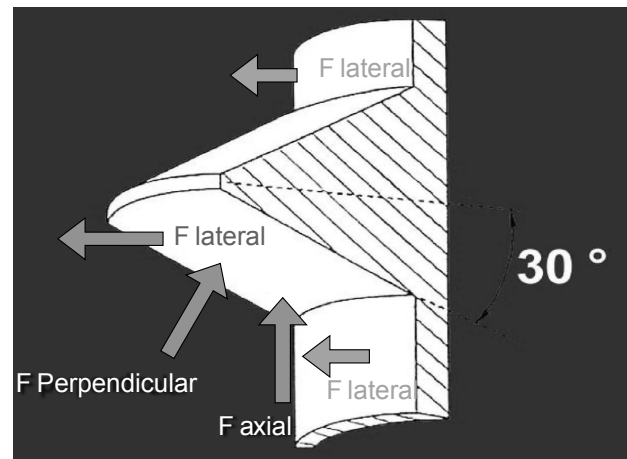


Fig. 4. With 30° threads, lateral forces and perpendicular forces are similar, enhancing the system biomechanically.

biomechanical system.

However functional loads are not always axial, because we also have to consider the protrusion and laterality. But if we consider only the centric movements, the functional surface area will be important if the implant axis corresponds to the direction of this force vector: however this is not frequent since an implant is rarely perfectly perpendicular to the Spee's and Wilson's curves.

Another limit to the use of the square thread is related to its difficulty to penetrate in the osseous substance: the square shape is not a self-tapping thread and does not combine properly with the monophasic implants. The insertion of these implants is achieved by under-sizing the socket with a cilindric bur by 2 mm diameter, and then relying only on the self-tapping capacities of their threads. Moreover, we have to consider the lateral and vertical properties of the osseous condensation, according to Summers's principle. The "V" shape of the threads of the implant allows bone condensation during the insertion: in this way the bone is not removed but "condensed" laterally and vertically around threads becoming a sort of osseous "self-graft".

To summarize, the advantages of a "V" thread are the following:

- i) A correct distribution of the axial and para-axial loads;
- ii) Excellent self-tapping capacity;
- iii) Lateral and vertical osteo-condensation;
- iv) Low cost.

DISCUSSION

In the early days of dental implantology, immediate loading was positively perceived as stimulating bone apposition around implant and avoiding crestal bone loss. At that time however fibrous tissue interposition was considered to be optimal response to implants because it was mimicking the natural periodontal ligament (1). Realizing that fibrous tissue interposition was not the optimal bone response to implants was the results of many studies (1,2). Branemark et al. guided implant dentistry to high clinical success rates through a strict surgical and prosthetic protocol (1, 2). One of the prerequisites for success was a long stress-free healing period. However this time frame was recognized by Branemark et al. as empirical. Therefore the relevance of such extended healing periods prior to loading has been questioned. Several studies compared 4 group of implants: blades, screws, cylinders and conical implant design (1-3). All prosthetically early loaded blades were associated to fibrous tissue interposition, in contrast to blades early loaded screws were not found to exhibit a systematic fibrous encapsulation. The obvious difference between those groups is the design of the implant, screw type implants allows a significant primary stability. This primary stability seems to be able to withstand early loading without inducing deleterious micromotions and is suggested to have an important clinical relevance (4, 5).

Bone resorption has been reported around both cylinder and screw implants (4, 5). In the resorption models, the bone defects were lined by a cortical shell, to simulate the increase in bone density (lamina dura) radiographically observed in clinically stable implants with bone resorption (6). The tendency of the most coronal cortical bone to remodel adjacent to implants and form a new cortical bone layer at a lower level was also found in a dog study (7).

Obviously, one major goal in implant dentistry is to avoid implant failure. Although the failure rate of implants used in two-stage procedures is rather low, it is likely that higher failure rates are associated with immediate loaded or grafted implants.

As implant failures are often related to biomechanical factors, an assessment of implant stability may significantly lower the risk of failure.

Studies have shown that high resonance frequency analysis values are indicative of a successful implant treatment with a small risk for future failure (8).

From the occlusal loads point of view, implants must be compared to the periodontally damaged teeth: loads have to be distributed along the root of the tooth and occlusal surfaces should be reduced to prevent lateral occlusal forces; a rigid stabilization must be provided immediately after implant application.

REFERENCES

1. Adell R, Leckholm U, Rocker B, Brånemark PI. A 15 years study of osseointegrated implants in the treatment of the edentulous jaw. *Int J Oral Surg* 1981; 10:387-416.
1. Branemark PI Adell R, Breine U, Hansson BO, Lindström J, Ohlsson A. Intraosseous anchorage of dental prosthesis. Experimental studies. *Scand J Plastic Recon Surg* 1969; 3:81-100.
2. Brunski JB, Trachtenberg DI. The influence of functional use of endosseous dental implants on the tissue implant interface. *Histol aspects. J Dent Res* 1979; 58:1953-69.
3. Spiekermann H, Jansen VK, Richter EJ. A 10-year follow-up study of IMZ and TPS implants in the edentulous mandible using bar-retained overdentures. *Int J of Oral Maxfac Implants* 1995; 10:231-43.
4. Mericske-Stern RD, Buser D, Geering AH. Long-term evaluation of non-submerged hollow cylinder implants. Clinical and radiographic results. *Clin Oral Implants Res* 2001; 12:252-59.
5. Jung Y-C, Han C-H, Lee K-W. A 1-year radiographic evaluation of marginal bone around dental implants. *Int J Oral MaxFac Impl* 1996; 11:811.
6. Cochran DL, Hermann JS, Schenk RK, Higginbottom FL, Buser D. Biologic width around titanium implants. A histometric analysis of the implanto-gingival junction around unloaded and loaded nonsubmerged implants in the canine mandible. *J Period* 1997; 68:186-98.
7. Sennerby L, Meredith N. Implant stability measurements using resonance frequency analysis: biological and biomechanical aspects and clinical implications. *Periodon* 2000 2008; 47:51-66.
8. Akin-Nergiz N, Nergiz I, Schulz A, Arpak N,

- Niedermeier W. Reactions of periimplant tissues to continuous loading of implants. *Am J Orth DentFac Orthop* 1998; (3):292-98.
9. Bergkvist G, Simonsson K, Rydberg K, Johansson F, Dérand T. A finite element analysis of stress distribution in bone tissue surrounding uncoupled or splinted dental implants. *Clin Impl Dent Rel Res* 2008; 10:40-46.
 10. Bidez MW, Misch CE. Force transfer in implant dentistry. Basic concepts and principles. *Oral implant* 1992; 18:264-74.
 11. Fanali S, Iezzi G, Mangano C, Traini T, Petrone G, Orsini G, Scarano A. Fluid penetration in conical and screwed implant-abutment connections: an in vitro study at stereomicroscopy and SEM. *J Dent Res* 2001; 80(4):1246.
 12. 13.Heckmann SM, Karl M, Wichmann MG, Winter W, Graef F, Taylor TD. Cement fixation and screw retention: parameters of passive fit. In vitro study of 3-unit implant-supported fixed partial dentures. *Clin Or Impl Res* 2004; 15(4):466-73.
 13. Meyer U, Vollmer D, Runte C, Bourauel C, Joos U. Bone loading pattern around implants in average and atrophic edentulous maxillae: a finite-element analysis. *J Cranio MaxFac Surg* 2001; 29(2):100-105.
 14. Rieger MR, Mayberry M, Brose MO. Finite element analysis of six endosseous implants. *J Prosthet Dent* 1990; 63:671-76.
 15. Roberts WE, Turley PK, Brezniak N, Fielder PJ. Implants: bone physiology and metabolism. *CDA J* 1987; 15:54-61.
 16. Skalak R. Biomechanical considerations in osseointegrated prostheses. *J Prosthet Dent* 1983; 49:843-48.
 17. Strong JT, Misch CE, Bidez MW Nalluri P. Functional surface area. Thread form parameter optimization for implant body design. *Compend Contin Educ Dent* 1998; 19:4-9.

LETTER TO THE EDITOR

STATIC AND FATIGUE RESISTANCE OF TWO TYPES
OF IMPLANT/ABUTMENT CONNECTORSS. FANALI¹, T. VILLA², S. LICO³ and R. CELLETTI¹

¹University G. d'Annunzio Chieti-Pescara, Department of Medical Oral and Biotechnological Sciences, Chieti, Italy; ²Politecnico di Milano, Department of Chemistry, Material and Chemical Engineering "Giulio Natta" Laboratory of Biological Structure Mechanics, Milano, Italy;

³Private Practice, Rome, Italy

Received January 25, 2017 – Accepted April 3, 2017

The aim of this study is to determine in an experimental way through mechanical tests the static, fatigue and torque resistance of two types of implant/abutment connectors with diameters of 3.4–5.2 mm.

To the Editor,

Modern implantology is based on the use of endosseous dental implants and on the study of osseointegration processes. The loss of marginal bone around a dental implant can be caused by many factors; the proper distribution of the masticatory loads is important and is closely dependent on the quality and quantity of bone tissue surrounding the implant. In fact, bone has the ability to adapt its microstructure through processes of resorption and neoformation of new bone matrix, as a result of the mechanical stimuli generated during the chewing cycles. The purpose of this study is to determine, through mechanical tests, the static, fatigue and the torque resistance of two types of implant/abutment connectors with diameters of 3.4–5.2 mm (i-Fix UniQo F.M.D. srl - Rome, Italy).

MATERIALS AND METHODS

Specimens

Tests were performed using the implant typologies above reported. The static and fatigue compression test

specimens were composed of an endosseous screw-type component of two different diameters (i-Fix UniQo F.M.D. srl - Rome, Italy - 3.4 and 5.2 mm) and a 25° angulated abutment: implants were mounted on cylindrical aluminum supports to mount the specimen on the testing machine as illustrated in Fig. I.

For static bending test, specimens were provided with the endosseous part already inserted into a custom-made aluminum cylinder for the testing machine grips.

Testing machine

Tests were performed on an MTS 858 Bionix servohydraulic testing machine (S/N 1014952, MTS, Minneapolis, MN) equipped by an axial - bending hydraulic actuator, with 25 kN axial capacity and 250 Nm bending capacity, a ± 100 mm range LVDT displacement transducer and a $\pm 140^\circ$ range ADT angular transducer mounted on the actuator. The load applied to the test sample was measured by an MTS axial/bending load cell (model 662.20D-05, S/N 1007099, ± 25 kN maximum axial load, ± 250 Nm maximum bending load). The machine was driven by Test Star 790.01 digital controller.

Key words: torque resistance, implants bending, loading forces

Mailing address:

Prof. Stefano Fanali,
Chairman Implantology Course I,
Via A Sartorio 2,
00147 Rome, Italy
Tel.: +39 06 5140908
Fax: +39 06 5140908
e-mail: fanali@odontoiatra.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.**

Test procedure

Static tests criteria. The bending that arises when masticatory loads act with an angle to the implant axis, is mostly responsible for implant failure; in this situation the stress state of the implant due to the load and the way of applying it is particularly severe and determines the implant specimen failure due to bending.

Test setup and configuration. All tests were carried out in monotonic compression under displacement control mode at a rate of 2 mm/min. Using the control software of test machine, the values of the displacement of the actuator and the force measured by the load cell were acquired. For the bending test, the implant is held between the lower jaws of the testing machine while the screwdriver is placed between the upper ones: a picture of the test is shown in Figure II.

Test procedure. Tests were performed by increasing the load until specimen failure. All tests were carried out in monotonic compression under displacement control mode at a rate of 2 mm/min. Using the control software of the test machine the values of the displacement of the actuator and the force measured by the load cell were acquired.

The bending test is run by rotating the upper head of the testing machine at the speed of 20°/min. The bending is stopped after specimen failure. Using the control software of the test machine the values of the angle of the actuator and the torque measured by the load cell were acquired (Fig. III).

Fatigue tests criteria. Tests were performed by applying the load in the same way as described for compressive static tests.

Test set-up and configuration. For the fatigue test the same configuration used for the static tests was used. Tests were carried out at a temperature of $25 \pm 2^\circ\text{C}$ and a humidity of $60 \pm 5\%$, and were conducted under force control, applying a sinusoidal waveform at 10Hz frequency.

Test procedure was conducted at the maximum frequency compatible with the control capabilities of the testing machine and, eventually not higher than 10Hz. A zero value for the displacement was set, when the actuator came in contact with the spherical head placed on the top of the abutment; a 0.3 mm displacement limit was then set so that the machine automatically stopped if such value was reached, thus indicating a failure of the implant.

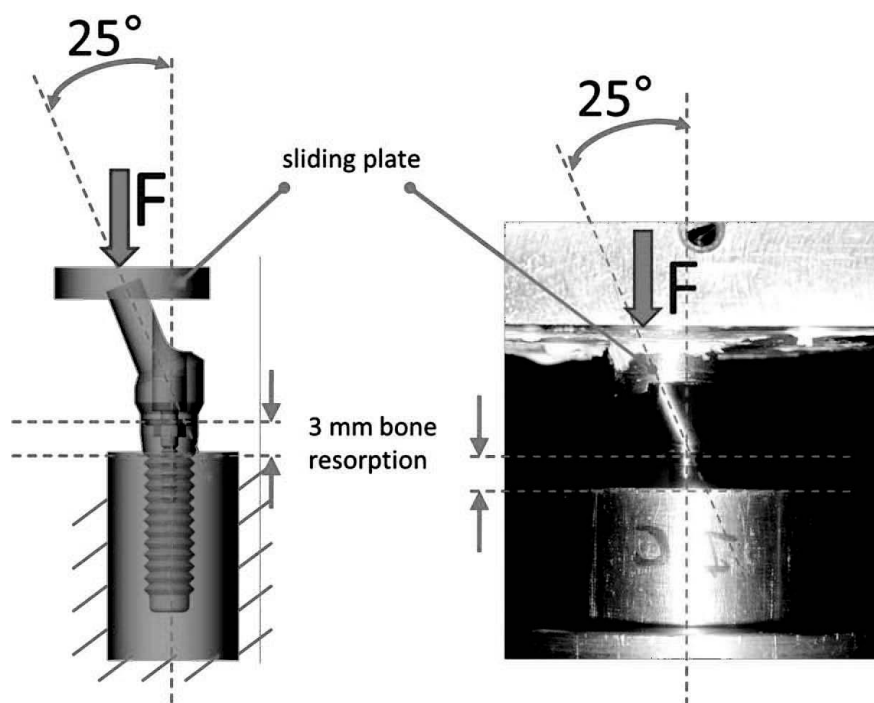


Fig. 1. Testing machine.

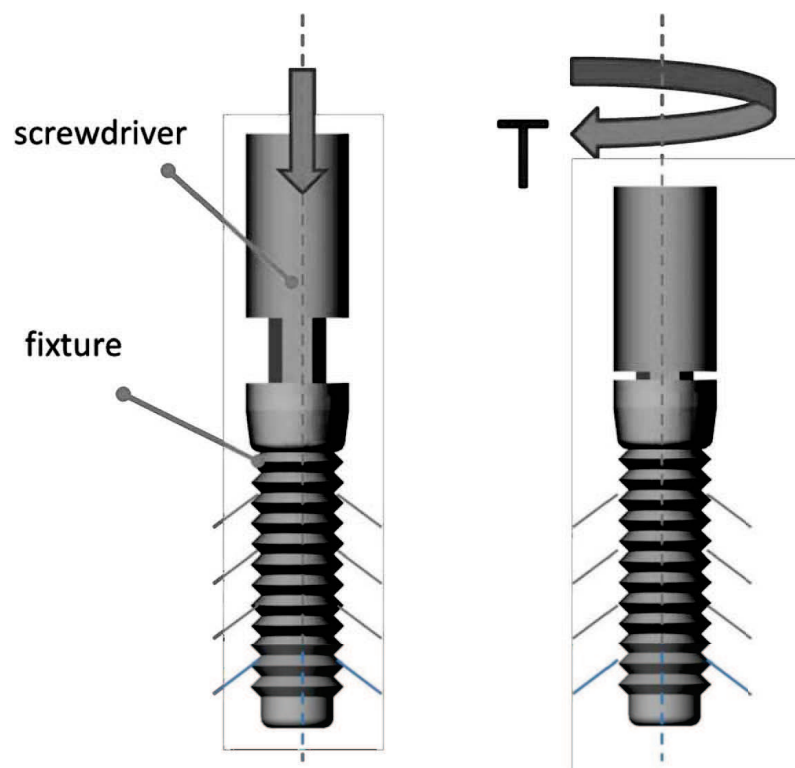


Fig. 2. *Picture of the test.*



Fig. 3. *The torque measuring.*

Table I. *Results of the Static Compression Test.*

ID	Yield load [N]	Ultimate load [N]
3.4-1	1025	2265
3.4-2	970	2380
3.4-3	905	2359
3.4-4	950	2346
mean	962.5	2337.50
std. dev.	49.75	50.32
5.2-1	1170	1316
5.2-2	1133	1140
5.2-3	1214	1360
5.2-4	1198	1273
mean	1178.75	1272.25
std. dev.	35.50	95.05

Table II. *Results of fatigue Compression Test.*

ID	Applied load [N]	Cycles at failure
3.4-5	108-1080	>5.000.000
3.4-6	125-1250	395.935
3.4-7	125-1250	79.085
3.4-8	125-1250	78.257
mean		1449297.67
std. dev.		971537.20
5.2-5	108-1080	>5.000.000
5.2-6	125-1250	467.704
5.2-7	125-1250	1.469.733
5.2-8	125-1250	2.410.456
mean		184425.67
std. dev.	1831	72.92

RESULTS

The results of the static compression tests are reported in Table I. The two groups compared by Student's *t*-test resulted statistically and significantly different for both yield load ($p < 0.001$) and ultimate load ($p < 0.001$). The results of the fatigue compression tests are shown in Table II. The two groups compared by Student's *t*-test resulted statistically and significantly different regarding the number of cycles at rupture.

Results of the static bending tests compared by Student's *t*-test were not statistically different with regard to the ultimate torque ($p > 0.05$).

DISCUSSION

For implant-supported fixed prostheses, the factors that affect bone-implant stress distribution and ultimately the success of the prostheses include implant inclination, implant number and position, the prostheses splinting scheme, the occlusal surface, framework material properties (1-8). Canay (9) compared vertically orientated implants with angled implants and found that the inclination of implants greatly influenced stress concentrations around the implant-supported fixed prosthesis. Many clinicians believe that a correct implant positions and the appropriate scheme of prostheses are critical for the long term success and stability of an implant-supported fixed prostheses. Kregzde (10) reported that induced stresses in bone are sensitive to the scheme of prostheses splinting and implant positions. Stress distribution in implant-supported fixed prostheses has been shown by FEA (11) to be influenced in various ways by implant inclination, implant number and position, the prosthetic framework, material properties. The bending that arises when masticatory loads act with an angle to the implant axis is mostly responsible for implant failure; in this situation, the stress state of the implant due to the load and the way of applying it is particularly severe and determines the implant specimen failure due to bending. It has been shown (12) that stress distribution in cortical bone does not seem to be greatly influenced by thread form configuration and that stress difference

is also not affected greatly by the load inclinations and cortical bone properties. However, in the trabecular bone-implant interface, stress distribution is influenced greatly by thread form configurations. Rieger et al. (13) stated that v-thread (V) threads are better than threads in cylindrical or tapered implant. Other studies (14, 15) showed that square threads are better for compressive load transmission than a standard v-thread or a cylindrical implant. Both v-thread and large square thread configurations appear to be suitable for use in a screw implant design but thin thread form should be strongly avoided and small square thread form is not satisfactory. Stress distribution in cortical bone does not greatly affect thread form configurations. Stress difference in cortical bone does not seem to be greatly influenced by support type constraint positions among various thread models. However, minimal support constraints allow clearer differentiation of the stress picture between the different screw types at the trabecular bone-implant interface. The fatigue strength of implants is influenced by occlusal masticatory forces. These residual stresses are redistributed by cyclic loading, and the bending momentum that arises when masticatory loads act with an angle to the implant axis is mostly responsible of implant failure.

REFERENCES

1. Basten CH, Nicholls JJ, Daly CH, Taggart R. Load fatigue performance of two implant-abutment combinations. *Int J Oral MaxFac Impl* 1996; 11(4):522-28.
2. Rangert B, Krogh PH, Langer B, Van Roekel N. Bending overload and implant fracture: a retrospective clinical analysis. *Int J Oral Maxillofac Impl* 1995; 10(3):326-34.
3. Mollersten L, Lockowandt P, Linden LA. Comparison of strength and failure mode of seven implant systems: an in vitro test. *J Prosthet Dent* 1997 78(6):582-91.
4. Khraisat A, Stegaroiu R, Nomura S, Miyakawa O. Fatigue resistance of two implant/abutment join designs. *J Prosthet Dent* 2002; 88(6):604-10.
5. Piattelli A, Scarano A, Paolantonio M, et al. Fluids

- and microbial penetration in the int part of cement-retained versus screw ret impl-abut connections. *J Period* 2001 72(9):1146-50.
6. Huang HM, Tsai CM, Chang CC, Lin CT, Lee SY. Evaluation of loading conditions on fatigue-failed implants by fracture surface analysis. *Int J Oral Maxillofac Impl* 2005; 20(6):854-9.
 7. Steinebrunner L, Wolfart S, Ludwig K, Kern M. Implant-abutment interface design affects fatigue and fracture strength of implants. *Clin Oral Impl Res* 2008; 19(12):1276-84.
 8. Quaresma SE, Cury PR, Sendyk WR, Sendyk C. A finite element analysis of two different dental implants: stress distribution in the prosthesis, abutment, implant and supporting bone. *J Oral Impl* 2008; 34(1):1-6.
 9. Canay S, Hersek N, Asik Z. Comparison of stress distribution around vertical and angled implants with finite element analysis. *Quintessence Int* 1996; 27:591-8.
 10. Kregzde M. A method of selecting the best implant prosthesis design option using three-dimensional finite element analysis. *Int J Oral MaxFac Impl* 1993; 8:662-73.
 11. Geng JP, Tan KBC, Liu GR. Applications of finite element analysis in implant dentistry, a review of literatures. *J Prosthet Dent* 2001; 85:585.
 12. Geng JP, Ma QS, Tan KBC, Liu GR. Finite element analysis of four thread-form configurations in a stepped screw implant. *J Oral Rehab* 2004; 31:233-239.
 13. Rieger MR, Fareed K, Tanquist RA. Bone stress distribution for three endosseous implants. *J Prosthet Dent* 1989; 61:223.
 14. Strong JT, Misch CE, Bidez MW, Nalluri P. Functional surface area: thread-form parameter optimization for implant body design. *Compend Contin Educ Dent* 1998; 19:4.
 15. Patra AK, DePaolo JM, Meenaghan MA. Guidelines for analysis and redesign of dental implants. *Implant Dent* 1998; 7:355.

LETTER TO THE EDITOR

ALLERGIC CONTACT DERMATITIS TO ACRYLATES

P. ROMITA^{1*}, C. FOTI^{1*}, L. MASCIOPINTO^{1,2}, E. NETTIS², E. DI LEO^{2,3},
G. CALOGIURI⁴, D. BONAMONTE¹, G. ANGELINI¹, G. DIPALMA⁵, A. BALLINI^{6*}
and F. INCHINGOLO^{5*}

¹Department of Biomedical Science and Human Oncology, Dermatological Clinic, University of Bari "Aldo Moro", Bari, Italy; ²Department of Internal Medicine and Infectious Diseases, Section of Allergy and Clinical Immunology, University of Bari "Aldo Moro", Bari, Italy; ³Sezione di Allergologia e Immunologia Clinica, Unità di Medicina Interna, Ospedale "F. Miulli", Acquaviva delle Fonti, Bari, Italy; ⁴IV Dipartimento di Pneumologia, Ospedale A. Galateo, San Cesario di Lecce, Italy; ⁵Interdisciplinary Department of Medicine, University of Bari "Aldo Moro", Bari, Italy; ⁶Department of Basic Medical Science, Neurosciences and Sense Organs, University of Bari "Aldo Moro", Bari, Italy

Received December 21, 2016 - Accepted April 11, 2017

*These authors contributed equally to the work

Acrylates and methacrylates are a large group of chemically reactive monomers that are polymerized into acrylic plastics. These have very broad applications in glues, coatings, and various plastic materials. Allergic contact dermatitis (ACD) caused by acrylates can be occupational, mainly in dentistry workers during the manufacturing and implantation of dental prosthesis, and in nail technicians during the sculpturing and application of artificial nails. The clinical manifestations vary according to the location of the contact. In non-occupational ACD, hand eczema, pulpitis and stomatitis are more frequent. We conducted a study to investigate the frequency of sensitization to acrylates, determining the most frequently sensitizing acrylates and assessing the possible role of 2-hydroxyethyl methacrylate (2-HEMA) as a screening allergen. From January 2013 to December 2014, 217 patients with a personal history and symptoms suggestive of contact dermatitis were patch tested with an extended series of acrylates at the Dermatology and Allergology Units of the University Hospital of Bari. Seven patients (3.2%) had positive reactions. The reactions were related to artificial nails in 2 patients (28.6%), both beauticians, and dental material in 5 patients (71.4%) with dental prosthesis. 2-HEMA detected 100% of sensitized patients to acrylates.

To the Editor,

Acrylates and methacrylates are a large group of chemically reactive monomers that are polymerized into acrylic plastics and are commonly used in glues, coatings, and various plastic materials.

A typical formula of a modified acrylic structural adhesive is: (i) resin, which is composed of a monomer (70-85%) and a polymer (15-20%); (ii) a catalyst; and (iii) an accelerator lacquer (1).

The mixing of all these compounds together, at

Key words; acrylates, allergic contact dermatitis, artificial nails, dental prosthesis, 2-hydroxyethyl methacrylates

Mailing address:

Dr. Paolo Romita,
Department of Biomedical Science and Human Oncology,
Dermatological Clinic,
University of Bari,
70124, Bari, Italy
Tel/Fax: +39 0805478107
e-mail: romitapaolo@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.

room temperature, with heat or UV radiation, results in a polymerized (cured) compound, which has little or no sensitizing potential. In the unpolymerized state, the monomers of these resins, as well as the additives used in their manufacture, are potential allergens and can sensitize workers in contact with them.

Allergic contact dermatitis (ACD) to acrylates was first reported with methyl methacrylate in 1941 (2). Since then, acrylate dermatitis has been reported in the occupational, printing and cosmetic industry (3).

In the hospital setting, acrylate based adhesives are used to attach orthopedic prostheses and splints, dental prostheses, tooth fillings, and histological preparations (4-6). They are also used in the adhesives of some surgical tapes, sanitary towels, ECG dots and all disposable diathermy pads. In these situations, acrylates are commonly used as they allow easy removal of the adhesive without trauma to the skin (4).

In the present study, the prevalence of ACD to acrylates was investigated and the most frequently sensitizing acrylic monomers in a population of subjects with a history of exposition to acrylates and symptoms suggestive of contact dermatitis.

MATERIALS AND METHODS

From January 2013 to December 2014, a total of 217 patients with a history of a suspected contact reaction to acrylates underwent patch testing with "series acrylates" (Euromedical. Calolziocorte, Lecco, Italy) (Table I) at the Dermatology and Allergology Units of the University Hospital of Bari.

All the subjects were given oral information about the products and the purpose of the study and gave an informed consent, in accordance with the ethical principles originating in the Declaration of Helsinki and consistent with good clinical practice. Subsequently, each patient filled in a questionnaire that included clinical demographic and job-related information. Patch tests were applied on their back and left in occlusion for 2 days with Al Test® (Euromedical) on Scanpor® Tape (Norgesplaster, Vennesla, Norway). Test readings were performed on day (D) 2 and D4 according to the European Society of Contact Dermatitis guidelines (7).

RESULTS

Patch tests resulted positive to acrylates in 7 patients (3.2%) (Table II), all females, aged between 26 and 65 years (mean 45.5 years). Four patients (57.14%) were atopic. Positive reactions were related to artificial nails in 2 patients, both beauticians (28.6%), and to dental materials in 5 patients, users of dental prostheses (71.4%).

Both beauticians (mean age 26.5 years) came into contact with acrylates at work during a gel application for nail art; eczematous lesions were localized on fingertips, periungual region, wrists and the ulnar side of the forearms. One patient also showed involvement of the interdigital spaces, palm and back of the hands.

The 5 users of dental prostheses (mean age 61.2 years) complained of burning and itching sensations to oral mucosa close to dental prostheses.

One patient (1/7, 14.29%) was positive only to one hapten [2-hydroxyethyl methacrylate (2-HEMA)], while 6 of them (6/7, 85.71%) were positive to multiple haptens.

Positive reactions were observed to: 2-HEMA in all cases (100%); ethylene glycol dimethacrylate (EGDMA) in 6 cases (85.71%); 2-hydroxypropyl methacrylate (2-HPMA) in 3 cases (42.9%); triethyleneglycol dimethacrylate (TREGDMA) in 3 cases (42.9%); methyl methacrylate (MMA) in 4 cases (57.14%); ethyl cyanoacrylate (ECA) in 2 cases (28.57%).

DISCUSSION

The number of dermatology consultations for contact dermatitis caused by acrylate sensitization is increasing and this has important repercussions for both treatment and work. As polymerized acrylates are relatively hypoallergenic, they are widely used in the industrial, cosmetic and hospital settings. Dentists and dental technicians are the professionals most affected by ACD, followed by workers in the paints and coatings, printing, and fiberglass industries, and then by professional beauticians (8, 9).

In a recent study by Ramos et al. (4), of 2,263 patch tested patients, 122 underwent aimed testing,

Table I. *Acrylates series (acrylic materials and activator substances and inhibiting polymerization).*

Allergens	Allergens and Vehicle
Methyl Methacrylate	2% Vaseline
Ethylene Glycol Dimethacrylate	2% Vaseline
Bisphenol A Dimethacrylate	1% Vaseline
2-Hydroxyethyl Methacrylate	2% Vaseline
Tetrahydrofurfuryl Methacrylate	2% Vaseline
Urethane Dimethacrylate	2% Vaseline
N-N Dimethyl-P-Toluidine	5% Vaseline
Hydroquinone	1% Vaseline
2-Hydroxy-4-Methoxybenzophenone	2% Vaseline
N-Methyl-4-Toluenesulfonamide	1% Vaseline
Epoxy Acrylate	0.5% Vaseline
4-Methyldiethanolamine	2% Vaseline
Morpholine Mercaptobenzothiazole	2% Vaseline
Benzoyl Peroxide	1% Vaseline

with an extended (meth)acrylate series, and 37 showed positive and relevant reactions. Twenty-five cases (67.6%) were occupational. Hand eczema with pulpitis was observed in 32 patients. Twenty-eight cases were related to artificial nails, 3 were related to dental materials, and 2 were industrial workers. Oral lesions associated with dental prostheses were observed in 4 patients. Thirty-one patients reacted to more than one (meth) acrylate. In a non-professional contest, ACD to acrylates may arise in dental patients and users of artificial nails. The conservative dentistry interventions predispose the oral mucosa to contact with potentially sensitizing acrylic monomers (10). The symptoms in this case may include stomatitis, lip angioedema, peri-oral dermatitis (11).

Allergic reactions to artificial (sculpted) nails may appear in months or years, after their use. In this case, the ACD to acrylates seems to have a lower incidence than in the professional form, probably thanks to the protection performed by the nail plate

against exogenous agents. In this form, the clinical signs are at the base of the nail with pain, paronychia, and occasionally paraesthesia. In some cases, the nail bed becomes dry and thick accompanied by onycholysis (12).

When there is a suspect of ACD to acrylates, it is necessary to proceed with a careful anamnesis to assess the possible contacts, occupational or not, with allergens and the execution of patch tests with baseline standard series associated with a supplementary acrylates series.

In some cases it may be necessary to test the products used by a patient provided that the concentration of the single constituents is well known in order to proceed with appropriate dilutions (13). In fact, it is recommended that the concentration of acrylate and methacrylate substances in the preparations of acrylic products must never be greater than 0.1% and 2%, respectively (14).

We observed professional ACD to acrylates in

Table II. Positive reaction to “patch testing with “series acrylates” in the seven exposed subjects.

Year	Age	Sex	Atopy	Occupazionale	Source	Symptoms	Dental series	Other series
2013	26	F	Y	Y	Nails Gel	Eczema (hands, wrists)	2-HEMA ++ EGDMA++	Benzocaine++
2013	58	F	Y	N	Prosthesis	Stomatitis	2-HEMA+++ 2-HPMA++ EGDMA++ TREGDMA+ MMA++	
2013	62	F	N	N	Prosthesis	Gingival stomatitis	2-HEMA+++ 2-HPMA+++ EGDMA++ TREGDMA++ MMA++ ECA+	Fragrance mix++
2014	65	F	N	N	Prosthesis	Gingival stomatitis	2-HEMA+++ 2-HPMA++ EGDMA++ TREGDMA++ MMA++ ECA++	Eugenol++
2014	61	F	N	N	Prosthesis	Stomatitis	2-HEMA++ EGDMA++ MMA++	
2014	60	F	Y	N	Prosthesis	Stomatitis	2-HEMA++	
2014	27	F	Y	Y	Nails Gel	Eczema (hands, wrists)	2-HEMA++ EGDMA	

2-HEMA: 2-hydroxyethyl methacrylate; EGDMA: ethylene glycol dimethacrylate; 2-HPMA: 2-hydroxypropyl methacrylate; TREGDMA: triethyleneglycol dimethacrylate; MMA: methyl methacrylate; ECA: ethyl cyanoacrylate.

Table III. Allergens marker of ACD to acrylates.

Category	Supplementary series
Workers in acrylates industries	TREGDA + 2-HPMA + BUDA/HDDA
Beauticians or artificial nails sellers	2-HEMA + EGDMA + TREGDA
Dental equipe	2-HEMA
Patients with dental diseases	2-HEMA + bis-GMA

Bis-GMA: 2,2-bis [4- (2-hydroxy-3-methacryloxy propoxy) phenyl] propane; BUDA: 1,4- butanediol diacrylate; HDDA: 1,6-hexanediol diacrylate.

29% of patients (2/7) who were positive to one or more haptens of “acrylates series”. Both of these patients were beauticians who came into contact with these substances during the application of sculptured nails.

Our “acrylates series” included 14 allergens (Table I). Seven patients showed one or more positive reactions to: 2-HEMA (100%), EGDMA (85.71%), ECA (67%); 2-HPMA (42.9%), TREGDMA (42.9%) and MMA (51.14%). Concomitant reactions to different acrylates can be explained by concomitant sensitization to acrylates or different cross-reactions. Cross-reactivity among acrylates and their strong sensitizing effect were studied in 2007 by Teik Jin-Goon et al. (15). The authors also proposed different supplementary acrylate series (Table III) to be used to reduce the exposure of patients to these substances, in order to avoid possible cases of active sensitization.

Although some authors (4-6) have shown the higher prevalence of acrylate ACD for dentists and dental technicians, our study highlighted the preponderance of patients sensitized in the extra-professional environment: 71% of the patients (5/7) were users of dental prostheses. The application of dentures and other dental materials often relies on the use of acrylates exploiting their curing properties. The acrylic monomers released from prosthetic dentures for the permanence in the oral cavity are capable of acting as allergens, developing stomatitis and gingival-stomatitis. The ACD localized to the oral cavity, linked to the presence of dental materials and prostheses, can be prevented by the removal of

excess monomers at the end of the dental procedure.

Cases of professional ACD can be avoided by taking some precautions. The replacement of nitrile gloves immediately after their contamination with acrylate monomers is critical. Acrylates, in fact, easily penetrate the gloves. Therefore, the use of double vinyl or natural rubber gloves is recommended for a 15-minute activity, or thicker nitrile gloves for a 30-minute activity in contact with acrylates (16).

The airborne pattern related to ACD, rhinitis, asthma and rhino-conjunctivitis in hand-beauticians and dental hygienists can be avoided by installing air extraction in the workplace and immediately closing the monomer containers (17, 18).

Our study confirms the role of acrylates as culprit of allergic contact dermatitis in both occupational and non-occupational cases. A careful anamnesis and patch tests play a key role in the diagnosis, and the results of our study highlight the role of 2-HEMA as a good screening marker in standard series for ACD to acrylates.

REFERENCES

1. Romaguera C, Grimalt F, Vilaplana J. Methyl methacrylate prostheses dermatitis. *Contact Dermatitis* 1985; 12:172-83.
2. Stevenson WJ. Methyl methacrylate dermatitis. *Contact Point* 1941; 18:171.
3. Freeman S, Lee MS, Gudmundsen K. Adverse contact reactions to sculptured acrylic nails: 4 case

- reports and a literature review. *Contact Dermatitis* 1995; 33:381-85.
4. Ramos L, Cabral R, Gonalo M. Allergic contact dermatitis caused by acrylates and methacrylates-a 7-year study. *Contact Dermatitis* 2014; 71:102-7.
 5. Anderson SE, Meade BJ. Potential health effects associated with dermal exposure to occupational chemicals. *Environ Health Insights* 2014; 8:51-62.
 6. Guerra L, Vincenzi C, Peluso AM, Tosti A. Prevalence and sources of occupational contact sensitization to acrylates in Italy. *Contact Dermatitis* 1993; 28:101-3.
 7. http://www.escd.org/education/guidelines/Guidelineversion6_afterIRW_150211final.pdf
 8. Bjorkner B. Acrylic resins. In: Frosch PJ, Menne T, Lepoittevin JP, eds. *Contact Dermatitis*. 4th ed. Berlin, Germany: Springer; 2006; 584.
 9. Geukens S, Goossens A. Occupational contact allergy to (meth)acrylates. *Contact Dermatitis* 2001; 44:153-59.
 10. Michelsen VB, Moe G, Str m MB, Jensen E, Lygre H. Quantitative analysis of TEGDMA and HEMA eluted into saliva from two dental composites by use of GC/MS and tailor-made internal standards. *Dent Mater* 2008; 24:724-31.
 11. Conde-Salazar L, Romero L, Guimaraens D, et al. Dermatitis al rgica de contacto por acrilatos en odont logo y prot sico dental. *Actas Dermosifiliogr* 1988; 79:13.
 12. Fitzgerald DA, English JSC. Widespread contact dermatitis from sculptured nails. *Contact Dermatitis* 1994; 30:118.
 13. Kanerva L, Estlander T, Jolanki R. Sensitization to patch test acrylates. *Contact Dermatitis* 1988; 18:10-15.
 14. Bruze M, Bjorkner B, Lepoittevin JP. Occupational allergic contact dermatitis from ethyl cyanoacrylate. *Contact Dermatitis* 1995; 32:156-59.
 15. Teik-Jin Goon A, Bruze M, Zimerson E, Goh CL, Isaksson M. Contact allergy to acrylates/methacrylates in the acrylate and nail acrylics series in southern Sweden: simultaneous positive patch test reaction patterns and possible screening allergens. *Contact Dermatitis* 2007; 57:21-7.
 16. Makela EA, Jolanki R. Chemical permeation through disposable gloves. In: Boman E, Estlander T, Wahlberg JE, et al., eds. *Protective Gloves for Occupational Use*. 2nd ed. Boca Raton, FL: CRC Press; 2005; 279.
 17. Torres MC, Linares T, Hernandez MD. Acrylates induced rhinitis and contact dermatitis. *Contact Dermatitis* 2005; 53:114.
 18. Tosti A, Rapacchiale B, Piraccini BM, Perluso AM. Occupational airborne contact dermatitis due to ethylene glycol dimethacrylate. *Contact Dermatitis* 1991; 24:152-3.

