

Editorials

W. Kranc, J. Budna, R. Kahan, A. Chachula, A. Bryja, S. Ciesiolka, S. Borys, M.P. Antosik, D. Bukowska, K.P. Brussow, M. Bruska, M. Nowicki, M. Zabel and B. Kempisty. Molecular basis of growth, proliferation, and differentiation of mammalian follicular granulosa cells	1
G. Tomasello, F. Giordano, M. Mazzola, R. Jurjus, A. Jurjus, P. Damiani, S. Nobile, F. Carini and A. Leone. <i>Helicobacter pylori</i> and Barrett's esophagus: a protective factor or a real cause?	9
MC. Di Tommaso Morrison, F. Carinci, G. Lessiani, E. Spinaz, S.K. Kritas, G. Ronconi, Al. Caraffa and P. Conti. Fibromyalgia and bipolar disorder: extent of comorbidity and therapeutic implications	17
C. Conti, Al. Caraffa, S.K. Kritas, G. Ronconi and M. Fulcheri. Alexithymia and its relationships with inflammatory response mediated by IL-1 family members	21

Original articles

L.J. Hu, W.Y. Ren, Q.J. Shen, H.Y. Ji and L. Zhu. Inflammation in lung after acute myocardial infarction is induced by dendritic cell-mediated immune response	29
C. Pan, Y. Wang, M-K. Qiu, S-Q. Wang, Y-B. Liu, Z-W. Quan and J-M. Ou. Knockdown of HMGB1 inhibits cell proliferation and induces apoptosis in hemangioma via downregulation of AKT pathway	41
L-X. Fu, Q-W. Lian, J-D. Pan, Z-L. Xu, T-M. Zhou and B. Ye. JAK2 tyrosine kinase inhibitor AG490 suppresses cell growth and invasion of gallbladder cancer cells via inhibition of JAK2/STAT3 signaling	51
S. Marinari, V. De Iulius, V. Dadorante, S. Colella, A. Marino, A. Nunziata, V. Flati, M. Caruso, A. Pennelli, F. De Benedetto, S. Matera, S. Capodifoglio, S. Martinotti, S. Caputi and E. Toniato. Cytokine modulation in patients with idiopathic pulmonary fibrosis undergoing treatment with steroids, immunosuppressants, and IFN- γ 1b	59

Letters to the Editor

Q. Liu, J.J. Wang, H.Y. Yan, Y.Q. Wang, C.W. Qin and M.L. Li. Safety and pharmacodynamic mechanism of ropivacaine lumbar anesthesia in cesarean section	71
S.R. Rao, R. Subbarayan and D.M. Girija. Cyclosporine-A augments endoplasmic reticulum stress markers and expression of matrix protein mRNA	77
A. Bryja, M. Dyszkiewicz-Konwińska, J. Budna, W. Kranc, A. Chachula, S. Borys, S. Ciesiolka, J. Sokalski, M. Prylinski, D. Bukowska, P. Antosik, M. Bruska, M. Nowicki, M. Zabel and B. Kempisty. The biomedical aspects of oral mucosal epithelial cell culture in mammals	81
A. Socha-Banasiak, S. Pierzynowski, J. Woliński, D. Grujic, M. Boryczka, P. Grzesiak, P. Szczurek, E. Czkwianianc, B. Westrom and K. Goncharova. The pig as a model for premature infants - the importance of immunoglobulin supplementation for growth and development	87
M. Li, R. Li, Q. Jin, J. Pang and Z. Xu. The efficacy of proanthocyanidins and secnidazole in the treatment of chronic periodontitis after scaling and root planing therapy	93
C. Qian, G.M. Wan, PS. Yan, WZ. Wang, SZ. Liang and Y. Dong. Correlation between Cystatin C and retinopathy of type-two diabetes mellitus patients	99
A. Bruzda-Zwiech, M. Konieczka, A. Hilt, M. Daszkowska, J. Grzegorezyk and J. Szczepańska. Salivary cortisol, alpha-amylase and immunoglobulin A responses to a morning session of basketball or volleyball training in boys aged 14–18 years	105
S.Q. Wu, S.Z. Ma, C. Zhang, D.Q. Li and C.Z. Gao. Effects of human parathyroid hormone on bone morphogenetic protein signal pathway following spinal fusion in diabetic rats	111
Y. Wang, J. Zhang and S.J. Zhang. Effects of anesthesia using propofol and etomidate on T lymphocyte subpopulation of infectious shock patients in perioperative period	119
K. Sznurkowska, A. Żawrocki, J. Sznurkowski, E. Iżycka-Świeszeńska, P. Landowski, A. Szlagaty-Sidorkiewicz, K. Plata-Nazar and B. Kamińska. Indoleamine 2,3-dioxygenase and regulatory T cells in intestinal mucosa in children with inflammatory bowel disease	125
D. Wang, L.H. Lai, P. Yan and PS. Dong. Correlation between blood asymmetric dimethylarginine level and the complications of patients with cardiovascular diseases	133
L.W. Qiao, Q.S. Qu and X. Jiang. Evaluation of tolerance and safety of conversion from mycophenolate mofetil to enteric-coated mycophenolic acid in renal transplant recipients ...	141
D.O. Martins, F.M. Santos, L.R.G. Britto, J.B.D. Lemos and M. Chacur. Neurochemical effects of photobiostimulation in the trigeminal ganglion after inferior alveolar nerve injury	147
F. Javed, S.V. Kellesarian and G.E. Romanos. Role of diode lasers in oro-facial pain management	153
L. Cheng, X. He, Y. Che, H. Che and M. Qian. Osteogenesis-promoting activity of composites SBA-15 mesoporous particles carrying oxytocin <i>in vitro</i> and <i>in vivo</i>	157
E. Hao, J. Yu, S. Xie, W. Zhang and G. Wang. Up-regulation of miR-888-5p in hepatocellular carcinoma cell lines and its effect on malignant characteristics of cells	163
X-L. Liu, X. Zhao, C. Wang, S-J. Gao and Y-H. Tan. Decitabine treatment for acute myeloid leukemia relapse after allogeneic hematopoietic stem cell transplantation	171
YL. Li, LC. Zhai, JH. Ji and LY. Liu. Detection of combined procalcitonin and C-reactive protein applied in the diagnosis of bacterial infections	177
J. Yuan, B. Jiang, K. Li, W. Shen and J-L. Tang. Beneficial effects of protein hydrolysates in exercise and sports nutrition	183
XP. Wang, B. Wang, P. Hou, R. Li and JB. Gao. Screening and comparison of polychromatic and monochromatic image reconstruction of abdominal arterial energy spectrum CT ..	189
M. Barbagallo, G. Vitaliti, F. Greco P. Pavone, N. Matin, G. Panta, R. Lubrano and R. Falsaperla. Idiopathic intracranial hypertension in a paediatric population: a retrospective observational study on epidemiology, symptoms and treatment	195
M. Gelardi, C. De Luca, S. Taliente, M.L. Fiorella, N. Quaranta, C. Russo, A. Ciofalo, A. Macchi, M. Mancini, P. Rosso, V. Seccia, F. Guagnini and G. Ciprandi. Adjuvant treatment with a symbiotic in patients with inflammatory non-allergic rhinitis	201
V. Galeandro, A. Notarnicola, A. Bianco, S. Tafuri, L. Russo, V. Pesce, B. Moretti and V. Petruzzella. <i>ACTN3/ACE</i> genotypes and mitochondrial genome in professional soccer players' performance	207
F. Bambini, A. Santarelli, A. Putignano, M. Procaccini, G. Orsini, L. Memè, D. Sartini, M. Emanuelli and L. Lo Muzio. Use of supercharged cover screw as static magnetic field generator for bone healing. 1 st part: <i>in vitro</i> enhancement of osteoblast-like cell differentiation	215
S. Negro, T. Boutsikou, D.D. Briana, M.L. Tataranno, M. Longini, F. Proietti, F. Bazzini, C. Dani, A. Malamitsi-Puchner, G. Buonocore and S. Perrone. Maternal obesity and perinatal oxidative stress: the strength of the association	221
G.E. Carpagnano, D. Lacedonia, M. Malerba, D. Martinelli, G. Cotugno and M.P. Foschino-Barbaro. Exhaled breath temperature measurement: influence of circadian rhythm	229
A. Goren, M. Kovacevic and T. Lotti. Melanin of the nipple areola complex	237
G.M. Abbate, A. Mangano, P. Sacerdote, G. Amodio, G. Moschetti and L. Levrini. Substance P expression in the gingival tissue after upper third molar extraction: effect of ketoprofen, a preliminary study	239
G. Tomasello, M. Mazzola, A. Jurjus F. Cappello, F. Carini, P. Damiani, A. Gerges Geagea, M.N. Zeenny and A. Leone. The fingerprint of the human gastrointestinal tract microbiota: a hypothesis of molecular mapping	245
A. Notarnicola, G. Maccagnano, L. Moretti, S. Tafuri and B. Moretti. Cardiopathy and osteoporosis: the epidemiology in a region of Italy	251
L. Azzi, F. Carinci, S. Gabaglio, F. Cura, F. Croveri, L. Tettamanti, A. Tagliabue and S. Segato. <i>Helicobacter pylori</i> in periodontal pockets and saliva: a possible role in gastric infection relapses	257

EDITORIAL

MOLECULAR BASIS OF GROWTH, PROLIFERATION, AND DIFFERENTIATION OF MAMMALIAN FOLLICULAR GRANULOSA CELLS

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For normal folliculogenesis and oogenesis to occur many intrinsic and extrinsic factors are needed, i.e. positive feedback of hormone secretion and local ovarian-follicular growth factors distribution. During follicle formation, granulosa cells (GCs) change their morphology and physiological properties. The factors needed for GCs to differentiate within each layer are transforming growth factor beta (TGFB) and insulin-like growth factor (IGF), as well as the activation and modification of biochemical pathways involved in folliculogenesis. Physiological alterations occur when GC genes are characterized by several differences in their gene expression profile. Studies in recent years indicate a variety of processes involved in follicle morphology and biochemical remodeling during growth and development. It was demonstrated that IGFs play a central role in the differentiation of GCs both *in vivo* and *in vitro*. Moreover, the primary role of FSH and LH in the formation of the ovarian follicle, was also described. Our review article characterizes the most important pathways involved in the differentiation of GCs and the effect of various factors on gene expression in GCs during folliculogenesis.

GENE CHARACTERISTICS RESPONSIBLE FOR *IN VITRO* AND *IN VIVO* PROLIFERATION

Mammalian fertility depends on normal ovarian follicle growth. Normal follicular development is regulated by many factors such as the presence of specific hormones, the interactions between the oocyte and the cumulus cells (CCs) or GCs, and certain growth factors (1).

The role of growth factors in the regulation of mammalian oocyte growth and development is well recognized, but an association between proliferation

capability of ovarian somatic cells and oocytes requires further investigation. Studies suggest the oocyte controls differentiation, maturation, and proliferation of GCs. Additionally, gonadotropins have a significant influence on these processes (2). Moreover, it is suggested that the differentiation and transformation of oocytes and ovarian somatic cells during antrum formation is accompanied by activation of the whole transcriptome, proteome, and/or metabolome (3). Recent studies indicate that separated CCs or cumulus – enclosed oocytes reflect a different proliferation ability during short-term,

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primary *in vitro* culture. Additionally, it was shown that separated GCs may be cultured *in vitro* under short or long-term conditions. Analysis of the results showed that the expression of genes involved in the dialogue between GC-CC-oocyte are genes encoding hormone receptors such as luteinizing hormone receptor (LHR), follicle-stimulating hormone (FSHR), progesterone receptor (PGR), and progesterone receptor membrane component 1 (PGRMC1). The role of these growth factors during mammalian oocyte development and maturation *in vitro* is well understood in several species, however, the impact of insulin growth factor (IGF) and/or gonadotropin administration on GC-CC growth capability, differentiation, and proliferation *in vitro*, still requires further investigation (4).

During the transformation of the primary follicle into the secondary follicle, changes occur in the primary oocyte and connective stroma of the ovary. The most spectacular changes occur in the GCs surrounding the oocyte. As a result of mitotic divisions, approximately four layers of GCs arise surrounding the oocyte. These cells increase in size and change their shape. Within the GCs, the number of mitochondria, endoplasmic reticulum, ribosomes, and spare Golgi apparatus increases. During antral follicle formation, there is increased gene expression of ZP-1, ZP-2, and ZP-3 proteins, which also comprise the zona pellucida (ZP) (5). The protein products of these genes play an important role in GC differentiation. Transcription of these genes is under the control of FIG- α factor (factor in the germline α), which is intensely expressed in the primary oocyte follicle (6). The GC layer undergoes many mitotic divisions during formation of the pre-ovulatory antral follicle, and as a result GCs form 10 - 18 layers. Additionally, the cumulus oophorus also increases its size. GCs at this point have a large nucleus and little cytoplasm. During the creation of the antral follicle, grainy Call-Exner bodies form between cells, however, the function of these cells is not fully understood (7).

The formation of the antrum causes GCs to divide into two populations: CCs that are directly adjacent to the oocyte and mural granulosa cells that line the follicle wall. The GCs surrounding the antrum are called mural granulosa cells. The preovulatory release of gonadotropins induces changes in the oocyte and in

GCs. After expansion of the cumulus oophorus (CO), the remaining GCs (mural granulosa cells) are prone to further differentiation and luteinization. Mural granulosa cells and theca cells, which form the corpus luteum, are necessary to maintain pregnancy (2). The population of GCs can be tracked successfully by distinguishing cell morphology and utilizing molecular techniques. For instance, LHR receptor characterizes mural granulosa from CCs, which do not have LHR receptors. It was also observed that the highest expression of LHR occurs in cells adjacent to the basal lamina. LHR is a protein product of the gene *Lhcgr*. *In vitro* oocytes, isolated from small antral follicles, inhibit *Lhcgr* mRNA production by GCs. The oocytes suppress *Lhcgr* mRNA expression in GCs from large antral follicles that were derived from females stimulated by gonadotropins. It appears that the oocyte suppresses gonadotropin-dependent differentiation of GCs and promotes the GCs phenotype typical for CCs (2).

Luteinization, the last stage of GC differentiation, occurs in the periovulatory period. Luteinization is characterized by progesterone production (8). It was observed that isolated oocytes secrete anti-luteinizing factors, preventing the differentiation of the final GCs.

THE ROLE OF SELECTED GROWTH FACTORS

One growth factor involved in GC differentiation is IGF. IGF was initially isolated from human serum and has two forms: IGF-1 and IGF-2. IGF-1 is a protein composed of 70 amino acids while IGF-2 is composed of 67 amino acids. These factors are structurally similar to insulin so they work with insulin-like receptors and have insulin-like activity (9). In humans, the gene encoding IGF-1 is located on the long arm of chromosome 12. It is composed of six exons and five introns, and it contains two promoters and two leader exons (10).

IGF circulates in the plasma in complexes associated with specific binding proteins of IGFBP (insulin-like growth factor-binding protein). The six IGFBP binding proteins have been identified as: IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5, and IGFBP-6 (11). IGFBP expression is regulated by various hormones, including estrogens,

glucocorticoids, follicular stimulating hormone (FSH), insulin, and thyroid hormones (12, 13).

In the cell membrane there are two types of IGF receptors: type 1 for IGF-1 and type 2 for IGF-2. IGF-1 induces cell growth, prevents apoptosis, and promotes cell proliferation (11).

The literature discusses the importance of growth factors for normal ovarian cycle. In the ovary, IGF-1 acts as a paracrine modulator that influences gonadotropin hormones. In theca cells, it increases the aromatization of androstenedione and testosterone to estradiol. IGF-1 works with follicle stimulating hormone (FSH) and luteinizing hormone (LH) to induce GC proliferation, both in the early stages of follicular growth (with FSH) as well as in the preovulatory phase (with LH). IGF-1 interacts with LH to increase progesterone production in the GC layer and also acts as a regulator of estradiol synthesis in luteal cells. Similar to IGF-1, IGF-2 is produced by thecal and luteal GCs. It works to enhance the effect of gonadotropins and to induce aromatase activity and progesterone synthesis in GCs. However, in women, overexpression of the *IGF-1* gene can cause polycystic ovary syndrome (PCO) and/or endometriosis (14).

The stimulating action of IGF-1 on GCs is well documented, and is believed to enhance the effect of gonadotropins on ovarian steroidogenesis (14).

In *in vitro* cultures of GCs, the binding protein IGFBP-3 is stimulated by growth hormone (GH) and is inhibited by FSH. Prostaglandin F₂ α (PGF₂ α) stimulates production of IGFBP3 in luteal GCs and inhibits IGF-1-induced progesterone production. IGF-1 is involved in the stimulation of follicular development and steroidogenesis in dominant follicles. Other IGF-1 binding proteins, IGFBP-4 and IGFBP-5, are produced by GCs and lead to follicular atresia of non-dominant follicles (15).

Spicer et al. demonstrated that adult *IGF-1* knockout mice have reduced levels of FSHR mRNA in their GCs, and renal ovarian steroidogenesis and infertility were observed. They studied the role of IGF-2 and IGF-2R in the ovary, and found that IGF-2 increased GC proliferation during folliculogenesis. Moreover, they discovered IGF-2 increased progesterone production in the GCs of large follicles, competing with IGF-1 for IGF-1R. This led to the hypothesis that increased

IGF-1 in developing follicles enhanced synthesis of IGF-2R (16).

In other another study, Gao et al. showed the *Wilms' tumors 1 (WT1)* gene was involved in GCs differentiation. They demonstrated that mice with WT1 (+/R394W) had defects in their reproductive system, and females had abnormal folliculogenesis. *In vitro* studies showed that FSHR, 3 β -hydroxysteroid dehydrogenase, and aromatase expression were reduced by WT1. Mutations in WT1 caused defects in GC polarity, which contributed to abnormal folliculogenesis. Furthermore, the data showed that WT1 may participate in GC development and differentiation via regulation of E-cadherin and Par6b expression (17).

Until recently, it was suggested that LH is responsible for changes in preovulatory follicles, which leads to ovulation and corpus luteum formation, while FSH is responsible for follicle formation. Eppig et al. suggested that FSH stimulated processes inside the follicle. Highly purified FSH, but not LH, induced cumulus expansion by oocyte-cumulus cell complexes. It stimulated cumulus oophorus GCs to produce hyaluronic acid, which was dispersed in *in vitro* culture (18).

Davis et al. reported that female mice lacking the *Cox-2* (cyclooxygenase-2), which controls the synthesis of prostaglandin gene or PGE2 receptor gene, had a malformed cumulus oophorus (19). Results of this study showed that growth differentiation factor-9 (GDF-9) is involved in GC differentiation and proliferation. GDF-9 is a protein belonging to transforming growth factor beta (TGF β) family and is secreted by the oocyte. Previously, it was suggested that GDF-9 acts as an autocrine regulator of oocyte development and maturation. However, in this case, it was involved in regulating proliferation and differentiation of GCs through a paracrine effect (20). Expression of GDF-9 was observed in oocytes of several species: rodent, bovine, ovine, and human (21-24). Studies by Dong et al. showed that, in knockout mice, GDF-9-mediated development of follicles was inhibited (20).

It has been suggested that TGF β belongs to the main mediators of cell survival, and it was also postulated that it may play a significant role in the regulation of GC proliferation (25).

REGULATION OF FOLLICULAR GRANULOSA CELL PROLIFERATION *IN VITRO*

During the transformation of a primary follicle to a secondary follicle, extensive changes occurred in the oocyte (such as the creation of the zona pellucida) and GC morphology. GCs changed shape from flat to cuboidal and began to proliferate extensively. The secondary follicle had the oocyte surrounded by a double layer of GCs. Simultaneously, the stromal cells formed the theca layer of the follicle (2). Then, during antrum formation, GCs were separated into two subpopulations: mural cells, which covered the inner wall of the follicle, and corona radiata cells. The latter mentioned cells surround the oocyte directly and combine with the cumulus oophorus. GCs of the corona radiata have characteristics that distinguish them from the remainder GCs. Cumulus oophorus granulosa cells are characterized by intense proliferation, synthesis of hyaluronic acid, and a weak intensity of steroidogenesis (2). The LH signal is distributed in the preovulatory follicle through peptide synthesis by mural granulosa cells. These peptides belong to the epidermal growth factor (EGF) family and include amphiregulin, epiregulin, and beta-celulin. These factors bind to receptors on the GC surface of the corona radiata and ultimately influence the production of the matrix and oocyte maturation (26).

For proper GC function, there is communication between the oocyte and the secreted hormones and growth factors, such as TGF β , GDF-9, and bone morphogenetic proteins (BMPs). GDF-9 and bone morphogenetic protein-15 (BMP-15) are growth factors related to the mechanism of GH action, which affects the growth and maturation of oocytes. It was observed that the presence of these factors is important for GC proliferation (27). The expression of GDF-9, its regulation, and/or activation of signaling pathways were associated with expression of other target genes, involved in GC-CC differentiation, growth, and transformation *in vitro*. GDF-9 is a member of the TGF β superfamily and affects the proper formation of the cumulus oophorus matrix by stimulating expression of various genes such as *has-2*, *EPSPS* (Hyaluronan 2), *PTX 3* (pentraxin 3), *Tsg6* (tumor necrosis factor stimulated gene 6), and *Cox-2/Ptgs2*

(cyclooxygenase 2). Furthermore, GDF-9 affects the inhibition of LH receptor expression, thus countering the luteinization GCs organized around the oocyte. Another role of GDF-9 is the inhibition of urokinase plasminogen activator (uPA) expression, which is produced by GCs located in the follicle wall. Other factors derived from the oocyte are BMP-15, BMP-6, and activin, reinforcing the impact of GDF-9 on the structure of the cumulus (28).

As previously mentioned, IGF-1 plays an important role in mammalian reproduction and fertility by affecting the hypothalamic-pituitary-gonadal axis. Hypothalamic and pituitary IGF-1 regulates the secretion of GnRH, LH, and prolactin (29). IGF-1 enhances the mechanism of action of FSH on GCs proliferation and differentiation. It was observed that, in the case of GH administration during ovarian stimulation, IGF-1 concentrations in the serum were increased daily. IGFBP1 inhibits GH secretion by a negative feedback loop.

Additionally, Mason et al. showed in *in vitro* culture that the absence of IGFBP1 and presence of FSH and IGF-1 increased estradiol production and secretion by GCs. In turn, the presence of FSH, IGF-1, and IGFBP3 in culture diminished the estradiol production and secretion (29). In other experiments, Mani et al. studied the mechanism of IGF-1 action in bovine GCs in order to determine its role on apoptosis- and steroidogenesis-regulated gene expression. GCs cultured with IGF-1 showed increased production of 17 β -estradiol (E2), increased cell number, and mRNA expression of *CYP11A1*, *HSD3B1*, *CYP19A1*, *BAX*, *IGF-1R*, and *FSHR* genes. The combined use of IGF-1 and FSH caused increased GC number and expression of *CYP19A1* and *IGF1R* genes, however, the production of E2 was unchanged. IGF-1 regulated phosphoinositide 3'-OH kinase (PI3K) levels but not in the mitogen-activated protein kinases (MAPK) pathway in the GCs. The test results showed that IGF-1 increased the activation of steroidogenic and apoptotic regulatory genes by activating the PI3K/AKT pathway in bovine GCs (27).

It was suggested that FSH changes gene expression in GCs. Research conducted by Castanon et al. proved that the GCs treated with FSH had a greater abundance of beta catenin (CTNNB1) protein and

increased expression of *WNT2* gene mRNA. These results showed that FSH regulates synthesis of WNT2 and CTNNB1 proteins in the GCs. These studies suggest that the canonical WNT signaling pathway plays a role in steroidogenesis and folliculogenesis in cattle (30). Moreover, FSH together with IGF-I affect the production of gonadal steroids. Mutations of *FSH* and *IGF-I* genes lead to fertility disorders. Zhou et al. demonstrated that inhibition of activity and expression of IGF-IR leads to inhibition of cAMP and FSH-induced expression of steroidogenic genes as well as estradiol production. These studies showed that IGF-I enhances the action of FSH and expression of cAMP, as well as indicating that FSH reduces IGH-I production by GCs (31). The hormonal regulation of oocyte maturation both *in vivo* and *in vitro* is well described, however, the possible association between selected hormones i.e. gonadotropins (LH, FSH) and their receptors in conjunction with the proliferation and/or differentiation ability of GC-CC, are still to be explored. It was partially investigated by Wu et al. who reported that FSH is the primary inducer of GC maturation, activating their proliferation and differentiation. During these processes increased aromatase activity, estradiol and inhibin production, as well as LHR expression in GCs have been observed (32). It is thought that thyroid hormones are involved in the regulation of ovarian follicle function. One hypothesis involves participation of thyroid hormones in the regulation of ovarian follicle function by acting on GCs. GCs present receptors for these hormones (33). Maruo et al. demonstrated *in vitro* that thyroid hormones, together with FSH, affect the function of GCs by stimulating the formation of LH/CH (luteinizing hormone/chorionic gonadotropin) receptors (32). Additionally, El-Fouly et al. suggested that oocytes produce anti-luteinizing factors, preventing final differentiation (8).

Also, it was found that GC function was affected by BMP, GDF-9, and TGF α synthesized by the oocyte (33). Vitt et al. showed that, *in vitro*, GDF-9 causes GC proliferation from both small antral and preovulatory follicles. Furthermore, they demonstrated that GDF-9 reduced cell differentiation induced by FSH (35). FSH is produced by the anterior pituitary and influences follicular GCs maturation. FSH receptors are located

in the plasma membrane. Tilly et al. examined FSHR expression in *in vitro* cultured GCs. GCs derived from rats were stimulated by estrogen, and cells were cultured for 48 h in medium supplemented with FSH. They concluded that FSH prevents a decrease in FSHR expression and has a similar effect on GCs as the adenylyl cyclase activator, forskolin. GCs were cultured in medium with the addition of EGF, basic fibroblast growth factor (bFGF), and IGF-1. The effect of these factors on FSHR expression depended on the concentration of the agent. The highest concentrations of bFGF and EGF completely inhibited FSHR expression (12).

INDUCTION OF BIOCHEMICAL PATHWAYS REGULATING GRANULOSA CELL DIFFERENTIATION

It was recently reported that human and animal GCs successfully proliferate and differentiate *in vitro* during primary culture. The morphological changes involved formation of spindle-shaped cell architecture, which may differentiate into fibroblast-like cells during long-term, primary culture. On the other hand, the biochemical modifications included induction of signaling pathways and activation of signaling mediators, necessary for signal transduction and cellular response. It is well known that Gonadotropin administration and the induction of cellular response causes the maturation of oocytes and/or ovarian somatic cells in several species of mammals. However, the mechanism responsible for activation of gonadotropin-related signaling pathways, both *in vivo* and *in vitro*, requires further research. The mechanism of hypothalamus-related gonadotropin-releasing hormone (GnRH) action on LH and FSH secretion, as a result of primary or secondary transmitter activation, is unknown.

GnRH is a decapeptide and plays a major role in mammalian reproduction. GnRH is produced and released by hypothalamic cells. This neuropeptide stimulates the synthesis and secretion of FSH and LH from the pituitary gland. The latter is responsible for the proper gonadal function in both females and males (36). Recent studies showed that in addition to GnRH produced by the hypothalamus, there

are two other forms of this hormone, GnRH-I and GnRH-II, in different compartments of the ovary, including GCs (12). In recent years, it was shown that both forms of GnRH and its receptors are present in the human ovary. It was suggested that they have paracrine function and are autocrine factors in gonads. Genes encoding GnRH-I and GnRH-II proteins are expressed in different compartments of the ovary, i.e.: ovarian surface epithelial cells (OSE) and granulosa-luteal cells. Expression of these genes is controlled by gonadal steroids, gonadotropins, and melatonin (36).

It was observed that 17 β -estradiol may regulate oocyte maturation and GC development and differentiation *in vitro*. Leung et al. demonstrated that the level of the GnRH-I mRNA was down-regulated by 17 β -estradiol in GCs (36). Similarly, Chiang et al. showed that GnRH-I suppresses estrogen receptor expression (ER α and ER β) through a PKC-dependent pathway in GCs. As a result, GnRH-I inhibits the action of estrogens on its own expression (37).

It was well recognized that GnRH modulates signal transduction pathways via activation of LHR and FSHR as well as inducing the secretion of sex hormone from the pituitary. Expression of GnRH depends on FSH and LH binding to their respective receptors, which then stimulates intracellular cAMP production. It was found that FSH increases the level of GnRH-II and decreases GnRH-I mRNA in GCs (37). Furthermore, Tamura et al. suggested that melatonin plays a role in reproduction. The main function of melatonin is the regulation of the physiological adaptation to day length changes in mammals. The role of melatonin in the ovary is unclear, but it is believed that melatonin is a modifier of ovarian function. In humans, two types of receptors are present in GCs: mt₁-R and MT₂-R. In GCs, GnRH-I is a luteolytic factor. Furthermore, melatonin downregulates expression of GnRH-I and its receptors in GCs and thus may interfere with the atrophy of the corpus luteum in the middle and final stages of the luteal phase (38). Choi et al. found that in the preovulatory follicles GnRH-I and GnRH-II are located in the GC layer. Significant levels of GnRH-I, GnRH-II, and GnRHR in granulosa luteal cells were also demonstrated in the corpus luteum (39). Additionally, Tilly et al. reported that GnRH receptors reduce FSH expression, LH production,

and cAMP production of steroidogenic enzymes (12). Kang et al. showed that treatment with a GnRH agonist-I stimulates phosphorylation of extracellular signal-regulated kinase (ERK). The use of GnRH agonist-I caused an increased phosphorylation of Elk-1 and c-fos mRNA in human GCs. Activation of the ERK pathway affected protein kinase C (PKC) and the G α protein. The results of these studies indicate that GnRH-I reduces steroidogenesis in GCs via PKC and ERK signaling pathways. It was also noted that GnRH-II had an inhibitory effect on the expression of gonadotropin receptors and progesterone secretion in GCs (40). Research demonstrated that expression of GnRH-I, GnRH-II, and GnRHR proteins in GCs is specific for follicular development phase. These therefore influenced the differentiation of GCs and formation of the corpus luteum (39).

In conclusion, growth and differentiation of the individual layers of GCs in follicles depends on many factors and biochemical pathways. In the past decade an increased interest in the factors affecting changes in GCs has been observed. It is known that the GCs affect the proper conduct of the ovarian cycle. In recent years, scientists have noticed that GCs have stem cell potential, but whether GCs may be used in regenerative medicine and *in vitro* biomedical engineering requires further investigation.

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EDITORIAL

**HELICOBACTER PYLORI AND BARRETT'S ESOPHAGUS:
A PROTECTIVE FACTOR OR A REAL CAUSE?**

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Notwithstanding the definite aetiopathogenetic path of certain diseases, the relationship between *Helicobacter pylori* (*H. pylori*) and Barrett's esophagus (BE), a condition that increases the risk for dysplasia and consequently adenocarcinoma of the distal esophagus and esophagogastric junction, remains uncertain. This paper reviews the current scientific literature with emphasis on the protective correlation between *H. pylori* infection and BE and demonstrates that a causal relationship has not been disproved with certainty. Furthermore, *H. pylori* infection could pose a risk for the onset of gastroesophageal reflux disease (GERD), which could in turn trigger BE, a precancerous lesion, and subsequently cause cancer. By analyzing the current available data, this article tries to verify that *H. pylori* infection is the underlying cause of esophageal cancer.

Approximately 10¹⁴ microorganisms colonize the human gastrointestinal tract. Though the part with the highest microbial concentration is the colon, the oral cavity, esophagus and stomach are also colonized. This large variety of microorganisms constitutes the microbiota, a complex ecosystem of over 800-1000 different microbial species (bacteria, viruses, fungi) with more than 3 million genes. Evolutionary processes have established a symbiotic

relationship between the microbiota and the host, however, sudden changes could result in dysbiosis with strong imbalances in the autonomic nervous and immune systems. Each individual possesses his/her own characteristic microbiota, a "bacterial fingerprint" composed of at least sixty common microbial species. Microbial changes are the result of infant delivery method (vaginal birth or cesarean) and/or infant feeding option (breast milk or formula

Key words: esophageal cancer, Barrett's esophagus, Helicobacter pylori, Barrett's adenocarcinoma

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feeds). Early exposure to maternal IgA determines a bowel cell gene pattern expression unlike that expressed by formula-fed infants who exhibit genes associated with chronic inflammatory diseases. Microbiota composition changes are also related to age, diet, stress and antibiotic use, among others (1).

Many studies have examined the function of microbiota and the relationship between healthy intestinal flora maintenance and the host's overall health status. The microbiota is predominantly composed of two major phyla, Bacteroidetes and Firmicutes. Important functions of these phyla include the production of enzymes that foster digestion, pH adjustment, intestinal motility, the production of important vitamins, such as K and B12 and the synthesis of conjugated linoleic acid (CLA), an essential short-chain fatty acid (SCFA) known for its anti-diabetic and anti-atherogenic properties, all of which seem to implicate the prevention of colon cancer. Among the more important functions of butyric acid, are its trophic, beneficial, and protective roles of the colonic mucosa. Butyric acid may be viewed as a valuable complementary therapeutic agent if used together with traditional drugs (corticosteroids and mesalazine) for the treatment of apoptosis and atrophy of colocytes. Moreover, the flora modulates the intestinal immune system (gut associated lymphoid tissues, GALT) in terms of development and operation, resistance to the invasion of and colonization by territory competing drug metabolizing pathogenic bacteria (immunomodulation) and xenobiotici (2).

It is therefore easy to understand how changes in microbiota conditions (dysbiosis) could be associated with different clinical conditions, for example: metabolic diseases; autoimmune diseases; allergies; inflammatory bowel disease (IBD); irritable bowel syndrome (IBS); ulcerative colitis; and colorectal cancer. Furthermore, the data suggest how these diseases are associated with altered expression of Heat Shock Protein (HSP) to the extent that they were called chaperonopathies (3).

Numerous studies have focused on the detection of high concentrations of HSP 10 and HSP 90 in adenocarcinoma samples. The physiological function of HSPs is to help other proteins folding to guide the

degradation of defective proteins. However, during evolution, HSPs have acquired additional roles and are involved in immunomodulation and tumor progression (4).

Based on this evidence, it was inferred that microbiota modulation with probiotics, which the World Health Organization (WHO) defined as beneficial live microorganisms to human health, and prebiotics, or food ingredients that contain non-digestible oligosaccharides, such as galactooligosaccharides and inulin, could be useful for the treatment or prevention of numerous disorders.

However, the microbiota affects also other body functions. A connection between dysbiosis and obesity, based on the microbiota compositions of lean and overweight subjects, indicates a prevalence of Bacteroidetes in the former and Firmicutes in the latter (5). In addition, this connection shows that alterations in the intestinal microbial composition are associated with large variations in enterochromaffin cells, via the effect of the brain-gut axis. Such results could also be linked to psychiatric and behavioral disorders, thus indicating the crucial importance of the two-way communication path between the microbiota and the brain, in health and disease (6).

Helicobacter pylori infection

Earlier studies focused on the microbiota of the lower segments of the digestive tract, neglecting the upper tract such as the stomach long considered sterile due to its acidic pH. However, Barry Marshall and Robin Warren, recipients of the Nobel Prize in Physiology and Medicine in 2005 for their isolation of *Helicobacter pylori* (*H. pylori*) in the acidic environment of the stomach, found the Gram negative bacterium (named for its spiral shape and peculiar pyloric location) scourging the stomach. Furthermore, they found an acidophilus natural habitat in the stomach. Consequently, *H. pylori* has become accustomed to living in the gastric mucus of about 50% of the population. It is actually localized in the superficial mucin and the lower epithelium as a result of adhesions. Occasionally, *H. pylori* associates with the accessing epithelial cells, however, in general, it remains immersed in the mucus gel coating

the gastric mucosa where it can move faster due to its flagella. On the other hand, *H. pylori* is incapable of crossing the epithelium to infiltrate the lamina propria of the mucosa. Nonetheless, it is able to consistently induce an inflammatory reaction that affects the mucosa thickness, though not substantial enough to push the patient to seek medical care. However, left undiagnosed, *H. pylori* is acknowledged to cause more serious diseases of the stomach, such as peptic ulcer, atrophic gastritis, and consequently stomach cancer. In addition, *H. pylori* has been associated with other diseases of the digestive tract such as duodenal ulcer which is linked to antral gastritis caused by acid hypersecretion: a consequence of an increased release of gastrin and to infection by *H. pylori* (7). Furthermore, emerging evidence suggests that *H. pylori* infection may increase the risk of acute coronary syndrome, contribute to insulin resistance, and be associated with neurodegenerative respiratory diseases, among others (8). Moreover, accumulating evidence supports the fact that the onset of chronic gastroesophageal reflux disease (GERD) mediates the connection between *H. pylori* and Barrett's esophagus (BE), and that in the absence of drug treatment, this link may be the basis for the onset of esophageal cancer (7, 8).

Esophagitis and Barrett's esophagus

The stratified squamous epithelium of the esophagus is known to be abrasion resistant, but sensitive to acid. It is now well documented that the content of gastric reflux into the esophagus is the most common cause of esophagitis. All conditions

that reduce the lower esophageal sphincter quality or that increase abdominal pressure, such as alcohol abuse, smoking, obesity, delayed gastric emptying and hypotonia of the LES musculature, contribute to GERD, and consequently to the emergence of esophagitis reflux. Esophageal lesions are endoscopically visible; their extent and severity are described in the Los Angeles Classification of Gastroesophageal Reflux Diseases (9). This classification system is based on endoscopic data and provides a breakdown of the four possible clinical grades:

- i) GRADE A: one or more erosions of the mucosal length, 5 mm or less in extent, with no overlap at the edges of two mucosal folds;
- ii) GRADE B: one or more erosions of the mucosal length, greater than 5 mm in extent, with no overlap at the edges and continuity of two mucosal folds;
- iii) GRADE C: mucous discharge with overlap between the ends of one or more folds, but involving less than 75% of the esophageal circumference;
- iv) GRADE D: mucous discharge affecting at least 75% of esophageal circumference.

An interesting correlation between *H. pylori* infection and BE, a complication that affects about 10% of patients with chronic GERD, is that 1% of patients with Barrett's will develop adenocarcinoma. Barrett's esophagus (BE) was first described in 1950 by Norman Barrett who characterized this relatively rare condition as epithelial metaplasia of the columnar type which develops through the replacement of stratified squamous esophageal

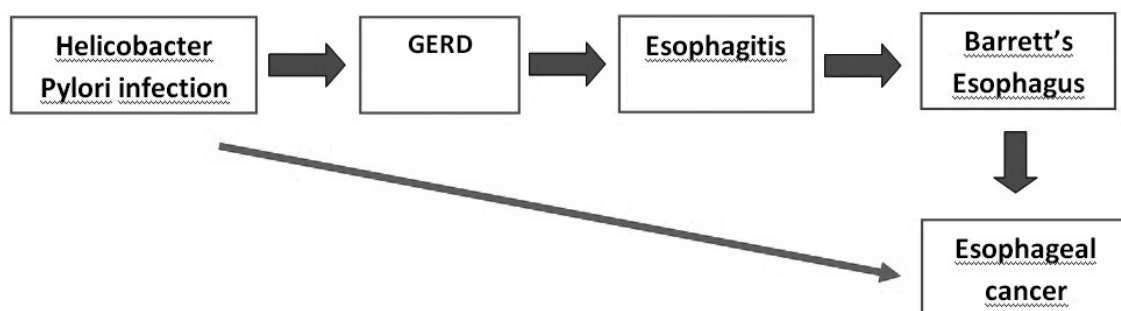


Fig. 1. Sequence of cellular changes related to infection by *Helicobacter pylori*.

epithelium with columnar epithelium (2). The lesion, with digits of variable lengths, originates from the gastroesophageal junction and extends in a proximal direction appearing as a ring of velvety erythematous mucosa. It is considered as an important pre-cancerous lesion for esophageal adenocarcinoma. It is also associated with microbiota variations and genetic alterations, most commonly observed in the pathological Barrett-carcinogenesis path. This path includes disorders in important cell cycle regulators, such as: the inactivation of P53; the deregulation of the TGF- β /Smad and receptor tyrosine kinases; the presence of a mitogen activated protein kinase (MAPK); and increased expression of COX-2. Each of these factors is related to increased cell proliferation. In addition, chronic exposure to the components of gastro-esophageal reflux triggers the release of inflammatory cytokines, chemokines, reactive oxygen species (ROS), and reactive nitrogen species (RNS). Such factors promote active migration of inflammatory cells, and damage in DNA, and play a crucial role in the initiation of the carcinogenic path (10). There are three variants of Barrett's epithelium: columnar epithelium, cardia columnar epithelium gastric-fundic, and intestinal columnar epithelium.

Knowledge of the three variants is essential when evaluating the Barrett mosaic. However, the evaluation can be achieved by endoscopic examination as long as there is a histological confirmation. With BE, there is a risk of developing dysplasia and adenocarcinoma as a result of specialized intestinal metaplasia. As such, some researchers advocate for a limited definition of BE and to follow-up endoscopically only in patients with intestinal metaplasia (7, 9, 11).

Metaplasia is a major factor in the development of GERD, which can be caused by *H. pylori* infection. The build-up of material in the esophagus along with bile acid induces such transformation, however, a mucosal resistance to insult the refluxate takes place. Moreover, cancer risk increases in direct proportion to the length and severity of the reflux and increases even more if the GERD is associated with hiatal hernia, esophagitis, or ulcer (11).

Our investigation is based on reading and

evaluating most studies that examine the relationship between *H. pylori* and BE. Our analysis depicts discrepancies among the studies. For example, some studies emphasize that both the protection from Barrett in subjects Cag A positive (11-14) and the possible mechanisms through which *H. pylori* may decrease the risk of BE are due to their association with atrophic gastritis and the resultant damaging effects of acid produced by parietal cells. Because of the loss of parietal cells, esophageal exposure to the harmful stomach acid effects would be less likely, thus reducing the likelihood of acid reflux, erosive esophagitis, and BE (15). However, other studies support the cause and effect relationship between *H. pylori* and BE, or indicate that the primary cause of *H. pylori* infection is chronic GERD (16-20).

DISCUSSION

Though the mechanisms underlying the pathogenic action of *H. pylori* are largely unknown, one could still distinguish bacterial virulence factors from those related to the inflammatory reaction of the host. Key virulence factors contributing to the onset of symptoms include first, the microbe's gastric motility, which allows bacterial penetration of the mucus layer covering the mucous membrane. Second, the production of high amounts of urease, an enzyme necessary for the colonization of the area, hydrolyzes the urea present in the gastric lumen, and ultimately produces ammonia and bicarbonates that thoroughly neutralize the immediate vicinity. And third, the secretion of hydrochloric acid by gastric parietal cells creates an acidic pH around the bacterium, which promotes its ability to adhere to the mucosa and makes the bacterium capable of transmitting its cytopathic effect to the cells. Once in contact with the epithelial cell, *H. pylori* is able to induce a lesion in the form of numerous cytoplasmic vacuoles. This secondary injury leads to the production of a cytotoxin called Vacuolating cytotoxin A (VacA) which can be incorporated into the epithelial cells and lead to the induction of fusion between endosomes and lysosomes (20).

The structure of this cytotoxin, which requires an acidic environment for its synthesis, appears

Table I. Comparison between studies on *Helicobacter pylori* and gastrointestinal diseases.

STUDIES	POSITIVE RELATIONSHIP	NEGATIVE RELATIONSHIP
<u>Vicari J et al</u> ¹⁸ <i>Helicobacter pylori</i> and acid peptic disorders of the esophagus: is it conceivable?	X	
<u>Ghoshal UC et al</u> ¹⁹ Gastroesophageal Reflux disease and <i>Helicobacter pylori</i> : What may be the relationship?	X	
<u>Lai CH et al</u> ²⁰ Lower prevalence of <i>Helicobacter pylori</i> infection with vacAs1a, cagA-positive, and babA2-positive genotype in erosive reflux esophagitis disease.	X	
<u>Kountouras J et al</u> ¹⁶ <i>Helicobacter pylori</i> infection might contribute to esophageal adenocarcinoma progress in subpopulations with gastroesophageal reflux disease and Barrett's esophagus.	X	
<u>Rossi G, Gambi R. et al</u> ¹⁷ Severe gastritis with double <i>Helicobacter</i> spp. infection associated with Barrett's esophagus in a cheetah.	X	
<u>Rubenstein JH et al</u> ¹¹ Association between <i>Helicobacter pylori</i> and Barrett's esophagus, erosive esophagitis, and gastroesophageal reflux symptoms.		X
<u>Loffeld RJ et al</u> ¹² Colonization with cagA-positiven <i>Helicobacter pylori</i> strains inversely associated with reflux esophagitis and Barrett's esophagus.		X
<u>Lord RV et al</u> ¹³ Prevalence of <i>Helicobacter pylori</i> infection in 160 patients with Barrett's oesophagus or Barrett's adenocarcinoma.		X
<u>Fischbach LA et al</u> ¹⁴ The association between Barrett's esophagus and <i>Helicobacter pylori</i> infection: a meta-analysis.		X
<u>Corley DA et al</u> ¹⁵ <i>Helicobacter pylori</i> infection and the risk of Barrett's oesophagus: a community-based study.		X

to be subject to variations between the numerous bacterial strains. Some forms are associated with the presence of duodenal ulcer, while others with the absence of significant inflammatory lesions or symptoms. All strains of *H. pylori* are capable of hosting the VacA gene. However, for unknown reasons, the gene is active only in a percentage of the strains of *H. pylori*. VacA interacts with numerous receptor molecules of the host surface and can trigger a number of responses including insertion in the cell membrane, endosomal function changes, and apoptosis. A second factor is the pathogenetic CagA (*cytotoxin associated gene*) pathogenicity island which consists of approximately thirty genes that appear to be injected into the skin cell to induce the production of IL-8. Together, IL-8 and other cytokines produced during the inflammatory reaction induce the massive destruction of cell junctions and the loss of cell polarity. Hence, the neutrophils experience a chemotactic action emanating from the lamina propria capillaries which eventually infiltrate the epithelium. The release of protease and the derivative, ROS, coupled with vacuolation, may be the mechanism that *H. pylori* uses to cause peptic ulcers (20).

Gastric and duodenal *H. pylori* mucosal infections are consistently associated with an inflammatory reaction. This reaction is characterized by an immune response, both humoral and cellular, which is directed towards bacterial antigens and extracellular surfaces. The interaction between the bacteria and the mucosal surface gives rise to the release of pro-inflammatory factors, particularly interleukin-8, which regulates polymorphonuclear leukocyte accumulation. Epithelial cells are induced to express class II histocompatibility antigens which are self-induced to introduce bacterial antigens to the immune system, thus amplifying inflammation. The numerous inflammatory mediators present in the gastric mucosa, in particular TNF- α and several cytokines (IL-6, IL-12, CCL2-5), may exert a pathological effect on the mucosa. Furthermore, the activated leukocytes release, into the interstitial spaces, oxygen free radicals which tend to produce genetic damage to the DNA of skin cells and B lymphocytes. These mutations, which accumulate

with persistent infection, encourage the progression to neoplastic transformation (12-14).

In addition to gastritis and peptic ulcer, *H. pylori* infection has been linked to GERD and its countless manifestations in chronic BE. Roughly 40% of patients with GERD experience infection. This infection seems to increase with age regardless of sex. Some other studies consider *H. pylori* as a risk factor and that its presence might aggravate GERD (17-19). Others saw it as a protective factor, confirming that the infection signals the onset of GERD in patients in whom *H. pylori* was eradicated. Therefore, several explanations have been proposed regarding the aetiopathogenetic mechanism. In brief, *H. pylori* can influence gastric acid secretion in two ways:

i) Inflammation limited to the stomach is associated with the destruction of the D cells, which secrete somatostatin, therefore, there is no negative feedback on gastric acid secretion which would result in hyperchlorhydria, leading to an increase in the severity of reflux disease gastroesophageo (20);

ii) Helicobacter-induced gastritis leads to the destruction of the parietal cells which secrete acid and cause gastric atrophy, resulting in hypo- or achlorhydria which can reduce the severity of GERD and its complications.

This dual, hypothetical, yet opposing vision is the foundation of our question: Is *H. pylori* a protective or a causal factor? Hereby, some studies are presented in tabular form in support of each of the two hypotheses.

CONCLUSION

This review confirms the protective theory, with an *H. pylori*-Barrett association visibly negative. However, there are many studies that say the exact opposite. The evaluated case-control studies, meta-analysis, and cohort studies were significantly more in support of this theory. Though multiple confounding factors appear to have been thoroughly evaluated, several issues remain inexplicable, thus pointing the need for a large cohort, therefore, requiring further investigation.

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EDITORIAL

FIBROMYALGIA AND BIPOLAR DISORDER: EXTENT OF COMORBIDITY AND THERAPEUTIC IMPLICATIONS

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Fibromyalgia (FM) is a syndrome that affects muscles and soft tissues. Presenting symptoms include chronic muscle pain, fatigue, sleep problems and psychological symptoms, including depression and anxiety. There exists strong evidence of a comorbidity between FM and Bipolar Disorder (BD). In this study, papers from 2006 to February 2016 that examined the comorbidity and etiological similarities of FM and BD were reviewed, as well as the therapeutic implications of these findings. The reviewed articles showed that an adequate psychiatric screening for BD is recommended in FM patients with depressive symptoms, in order to decrease administration of antidepressants for BD, due to the lack of proven efficacy, and to limit antidepressant-induced mania. Alternative therapies, such as agomelatine, memantine and psychotherapeutic treatment should be considered.

In order to further investigate the comorbidity of fibromyalgia (FM) and bipolar disorder (BD), papers were reviewed from 2006 to February 2016 using the NIH PubMed Literature Search System to search for relevant articles. Inclusion criteria were that manuscripts i) be primary peer-review research articles; ii) concisely explained or investigated etiological similarities and therapeutic implications of FM and BP; iii) be published in English. For selected manuscripts these criteria were relaxed to allow the inclusion of studies deemed particularly useful.

A chronic disease, FM presents without a

fully-known etiology and pathophysiology (1). Characterized by chronic widespread pain, fatigue and psychological symptoms such as depression and anxiety, FM can be a severely debilitating disorder (2). In addition, markers, such as cytokines and chemokines, which play a role in inflammatory disorders (3-5), are also involved in FM as shown in biopsies of muscle.

Over the years, there have been countless papers reporting on the association of FM and BD (6-8). The recognition of the poorly understood pathophysiological processes and symptoms between

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BD and FM has relevant clinical and therapeutic implications (9). The crossover, likewise, deserves future research consideration. In a meta-analysis study, Kudlow et al. (10) aimed to investigate the association between BD and FM, and nine studies were included. The rate of BD co-morbidity in samples of FM patients was 21% ($n=678$); the rate of BD in the general population is 2.6%.

Further unequivocal evidence of FM-BD association was shown by Arnold et al. (11) in a study conducted from September 1999 to April 2002. The study included 78 FM patients and 146 of their relatives. The odds ratio for BD in individuals with versus those without FM was 153 (95% CI = 26 to 902, $p < .001$) and the highest among other comorbid disorders (major depression, schizophrenia, anxiety, substance use disorder, etc).

Moreover, according to Gota et al. (12), increasing levels of depression severity, as well as a positive screen for BD, were significantly associated with increasing prevalence and severity of FM symptoms.

In 2012, Alciati et al. (13) supposed that increase in activity of patients with FM is linked to the manic/hypomanic states associated with bipolar spectrum disorders. Using the DSM-IV and Zurich criteria, elevated incidence of bipolar spectrum disorders were found in patients with FM: 70% and 86.3%, respectively; 79% of subjects with FM scored 14 (threshold for hypomania) or above. These results suggested that FM is strongly related to the hypomania/overactivity component of BD.

Some recent papers further investigated the well-known association of FM with BD at a genetic level (14, 15). In a 2012 retrospective case control, Schmechel and Edwards (15) demonstrated an association of fibromyalgia syndrome (FMS) and A1AT in a consecutive series of patients. Furthermore, patients with anxiety disorders, BD I, BD II, or PTSD, showed increased proportion of A1AT polymorphisms and significant overlap with FMS phenotype and “ICE- intense creative energy”, a complex behavioral trait.

In order to screen patients with FM for BD, and to determine whether there are clinical methods other than the Mood Disorders Questionnaire (MDQ), Wilke et al. (9) screened 128 FM patients using

MDQ for BD, Fibromyalgia Impact Questionnaire Disability Index, Epworth Sleepiness Scale for daytime sleepiness and Beck Depression Inventory for depression. From this, 25.19%, of the FM subjects had a positive result for BD; 78.12% were clinically depressed; 52.13% reported daytime sleepiness. Depressive symptoms were more severe in FM when BD was comorbid.

Proven effective treatments of patients with FM depressive symptoms are serotonin and norepinephrine reuptake inhibitors, especially duloxetine and milnacipran (16-21), however, these may cause destabilization of mood (9, 22, 23); thus, Wilke et al. concluded that patients with FM should be systematically screened for BD prior to treatment.

In a study with the return to clinic time of one year, Carta et al. (24) advanced the hypothesis that SNRI antidepressants are not effective in long-term treatment of FM: at the end of the one-year follow-up, quality of life and bipolar symptoms were worse in the group treated with antidepressants than in the controls. These results should be read in the light of the high FM-BD comorbidity and of the well-known poor response of bipolar depression to SNRI antidepressants.

Several studies suggested that some psychotherapeutic treatments and SNRI antidepressants showed virtually identical results in FM (25, 26). Thus, Carta et al. (27) suggested that psychotherapeutic treatments of proven efficacy in FM should be considered first choice when compared to antidepressants.

In 2014, Calandre et al. (28) and Bruno et al. (29) reported that agomelatine, a melatonin agonist, slightly improved depression, anxiety, and pain but did not significantly improve sleep quality and executive/cognitive symptoms associated with FM.

In a 2014 case-report, Serra et al. (30) reported the case of a young woman, suffering from BD II with associated FM, in whom memantine, an NMDA antagonist, showed positive effects on both BD as well as the associated FM syndrome.

CONCLUSION

Comorbidity of FM and BD is clearly evident,

therefore, adequate psychiatric screening for BD is recommended in FM patients, prior to administration of antidepressants or sleep aids, in the light of the well-known poor response of the mood symptoms to antidepressants in BD. This approach would also limit antidepressant-induced mania, a well known complication of patients with depressive BD symptoms. Alternative therapies, such as agomelatine, memantine and psychotherapeutic treatments of proven efficacy, should be considered.

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EDITORIAL

ALEXITHYMIA AND ITS RELATIONSHIPS WITH INFLAMMATORY RESPONSE MEDIATED BY IL-1 FAMILY MEMBERS

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The major aim of this study is to provide a review of the research studies regarding the clinical link between alexithymia and interleukins (IL). We performed a search for the relevant literature by using search terms as “alexithymia” combined with “interleukin or IL”. A total of 9 original research studies were identified and included. Alexithymia was found to be prevalent in inflammatory response and associated with inflammatory cytokines. Our review emphasized for the first time the relationships of alexithymia with inflammatory response mediated by IL-1 family members. Therefore, the clinical psychological assessment of alexithymic traits and the administration of appropriate psychological and psychotherapeutical interventions should be integral parts of inflammatory disease management programs.

Alexithymia, literally translated as “no words for mood”, was introduced by Sifneod in 1973 to refer to a specific emotional disturbance characterized by an inability to identify internal affective states and describe them verbally (1). The alexithymia construct comprises four distinct features: difficulty in identifying (DIF) and describing feelings (DDF); difficulty in distinguishing feelings from the bodily sensations; diminution of fantasy, and concrete and poorly introspective thinking (1). Alexithymics may have affective dysregulation, the inability to self-appease and deal with emotions because of a lack of awareness of emotions (1). These psychological characteristics have been ascribed to an impaired capability to elevate emotions from a sensorimotor

level of experience to a representational level, where they can be used as signaling responses to internal or external events and modulated by psychological mechanisms (1). Because alexithymic subjects are psychologically poorly equipped, they generally demonstrate significantly higher levels of anxiety, depression and general psychological distress and are inclined to suffer both “functional” somatic symptoms and symptoms of emotional turmoil (1-3).

Several recent findings indicate that various interactions between the nervous and immune systems are important in the pathophysiology of alexithymia (4-10). These findings strongly suggest that pro-inflammatory cytokines such as interleukin (IL)-1, IL-2, IL-6, IL-8 and TNF-alpha may play

Key words: Interleukin, alexithymia, inflammation, cytokine, psychological stress

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a significant role in developing alexithymia and can mediate its psychological and neurobiological manifestations (7-9, 11). According to recent evidence, cytokine production may be also directly stimulated by negative emotions (12) and, conversely, cytokines may be responsible for influencing the HPA and SAM systems (13-15).

Guilbaud et al. (8) suggested that alexithymia may skew the T helper (Th)1/Th2 balance toward a pronounced Th2 immune response and lowered Th1 immune response. In this context, recent study found significantly lower IL-6 concentrations in alexithymic than in non-alexithymic upper endoscopy patients (16). However, among patients with major depression, those who were alexithymic had higher C-reactive protein (CRP) levels than the non-alexithymics (17).

Despite emerging evidence suggesting a link between alexithymia and immune function, previous studies yielded contrasting results. The proposed link between alexithymia and immune function remains controversial as does the role.

On this background, the specific aim of this manuscript is to provide new insights, performing a focused review by only reporting the relevant studies analyzing the relationship (i.e., both association, mediating role, and prediction) between alexithymia and interleukin-1 family members.

MATERIALS AND METHODS

Published original research reports comprising the study of alexithymia linked to interleukins were identified and then selected by searching in PubMed, Scopus, ScienceDirect, and Google Scholar from 1995 to March 2017. In addition, a manual search was performed by analyzing the bibliographical reference lists from all selected articles in order to identify other potentially relevant papers. The search terms on which we have focused on were “alexithymia” combined with “interleukin or IL”. With regard to the inclusion and exclusion criteria, we selected only original research studies in English language examining the relationship (i.e., both association, mediating role, and prediction) between alexithymia and interleukins. By contrast, we excluded all other different papers, namely reviews,

systematic reviews, meta-analyses, and commentaries. In order to evaluate the validity of the research studies according to the above-mentioned eligibility criteria, two investigators independently rated each study by examining the titles and abstracts, as well as relevant full reports, on the basis of the main topic of our focused review paper.

RESULTS

When focusing both on the search of PubMed, Scopus, ScienceDirect, Google Scholar databases, and on full bibliographical lists from all selected articles, after adjusting for duplicates and reviewing the titles and abstracts in order to exclude those which clearly did not meet the eligibility criteria, a total of 9 research studies were identified and selected for inclusion in the review.-

The link between alexithymia and inflammatory cytokines

One of the most recent studies on alexithymia-related physiological changes showed that an increased level of IL-8 in Cerebral Spinal Fluid (CSF) may be related to symptoms of alexithymia in patients with non-inflammatory neurological disorders, even after controlling for depression (11).

In another recent study, Honkalampi et al. (18) found that the levels of IL-6 (in picograms per milliliter) and hs-CRP (in milligrams per liter) were significantly higher in alexithymic than in non-alexithymic subjects (IL-6 effect size, ES: 0.50; hs-CRP ES: 0.27). In addition, although alexithymia was related to depressive symptoms, the association observed was independent of depressive symptoms (18).

In subjects undergoing upper endoscopy, Mandarelli et al. (16) detected significantly lower IL-4 and IL-6 concentrations in alexithymic than in non-alexithymic patients. According to multiple linear regression analysis, alexithymia predicted low IL-4 and IL-6 levels in the sample overall, independently of stress, anxiety, depression and other possible confounders.

Studying stroke patients, Bussù et al. (19) showed that serum levels of IL-18 were significantly related to stroke severity. Furthermore, a strong

positive correlation was observed between IL-18 levels and severity of alexithymia, particularly among patients with right-hemisphere lesions. Specifically, circulating concentrations of IL-18 were significantly increased in patients with alexithymia score at the Toronto Alexithymia Scale (TAS-20), as compared with both non-alexithymic patients and control subjects. In addition, stroke was more severe in alexithymic patients, as compared to non-alexithymic patients. Following multivariate regression, serum IL-18 levels appeared to be specifically associated with alexithymia rather than with stroke severity in patients with right-hemisphere lesions only.

In a previous study, Guilbaud et al. (8) found that women with alexithymia exhibited decreased interleukin-1beta, IL-2 and IL-4 production with reduced ratios of Th1/Th2 (IL-2/IL-10) and of CD4/CD8, as well as reduced CD4 percentages. IL-2 and IL-4 production remained significantly diminished in the alexithymic group, even after

adjusting for between-group differences in anxiety and depression levels.

A clinical study by Vadacca et al. (20) found increased prevalence of alexithymia in patients with rheumatoid arthritis (54%) and also in patients with systemic lupus erythematosus (42%). Both groups of patients had increased values of IL-6 and TNF-alpha.

Temoshok et al. (21), examined relationships between psychological, cardiovascular, and immune variables in a baseline sample of 200 HIV-infected, predominantly African American outpatients attending an HIV primary care, and alexithymia was correlated with significantly lower stimulated production of HIV-inhibiting MIP-1 alpha, but they did not find a relationship between alexithymia and IL-6.

Among patients with somatoform disorders, alexithymia has been found to be associated with a reduction in Th1-mediated immune function and an increase in the activation of Th2 immune

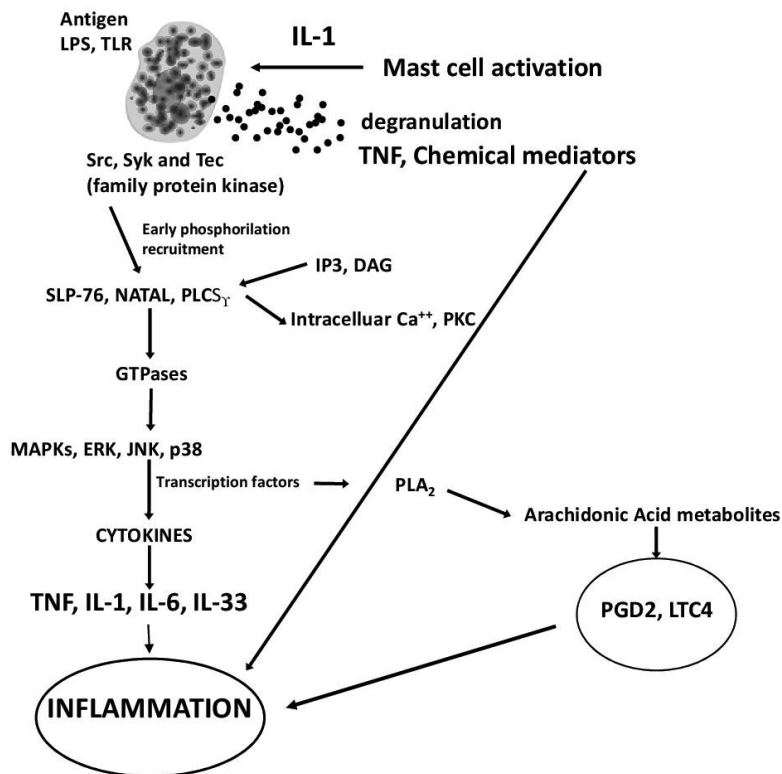


Fig. 1. Mast cell activation by IL-1 leads to a network cascade, releasing inflammatory cytokines, mediating inflammation.

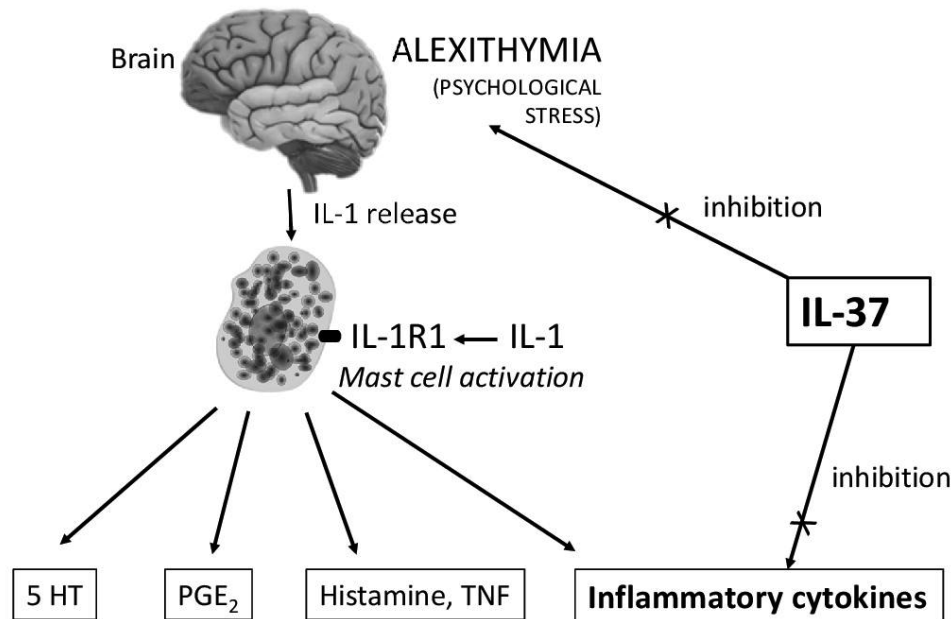


Fig. 2. Generation of IL-1 by psychological stress, activating mast cell and releasing inflammatory cytokines inhibited by IL-37.

function, indicated by the augmented serum levels of IL-6 and IL-10 and elevated immunoglobulin E. Thus, in patients with SFD, serum levels of IL-6 ($p < 0.001$), IL-10 ($p = 0.047$) and immunoglobulin E ($p = 0.045$) were significantly increased in comparison with healthy controls (10).

A remarkable study was conducted by Corcos et al. (9), who evaluated the relationship between alexithymia and circulating levels of IL-1, IL-2 and IL-4 in healthy women and found a significant association between alexithymia (particularly with the DIF dimension of TAS-20) and serum levels of IL-4.

DISCUSSION

To the very best of our knowledge, this is the first review paper aimed at investigating the published original research reports analyzing the highly relevant relationship between alexithymia and interleukins in order to better understand the main consequences deriving from this association.

Cytokines have been suggested to be potential

mediators of the interaction between immune and neuroendocrine systems. However, only few studies have investigated the association between alexithymia and interleukins.

One of the earliest was the research by Corcos et al. (9), in which a significant association was found between alexithymia and serum levels of IL-4 which is an inhibitor of IL-1. Other studies demonstrated a significant association between alexithymia and IL-18 (19), IL-6 (10, 18, 20) and IL-10 (10). Only one study did not report a significant association between IL-6 and alexithymia. In that study, alexithymia was correlated with significantly lower stimulated production of HIV-inhibiting the chemokine MIP-1 alpha (21).

Among healthy women, Guilbaud et al. (8) found that the production of anti-inflammatory cytokine IL-4 remained diminished in an alexithymic group even after adjusting for anxiety and depressive symptoms. IL-6 is a proinflammatory cytokine produced by several immune cells, and is considered to be a marker for depression. Thus, these findings suggest that

both depression and alexithymia are conditions characterized by a pronounced inflammatory state, and could be considered state reactions to stressful situations (16).

An interesting study demonstrated an increased level of IL-8 (in CSF) related to symptoms of alexithymia in patients with non-inflammatory neurological disorders.

These findings broaden our view on the stress-alexithymia studies, demonstrating its association with inflammatory cytokines.

There is now increasing recognition that psychological stress leads to a risk of development of several medical conditions, including infections, obesity, diabetes, heart disease, pain inflammation and psychiatric disorders (2-5, 22). Several lines of evidence support that early stress exposure may lead to chronic inflammation and is also a risk factor for psychiatric diseases including alexithymia (2, 3). Dysregulation of the neuroimmune system is one of the most important biological mechanisms relevant in psychological and psychiatric disorders, an effect that affects Hypothalamus-Pituitary-Adrenal (HPA) axis hyperactivity (23). The inflammatory response may involve the generation of inflammatory cytokines which may mediate the activation of immune/inflammatory cells such as macrophages, lymphocytes, neutrophils and mast cells.

Cytokines are proteins that mediate inflammation and immune reaction and are generated by immune and non-immune cells. IL-1 is a cytokine produced mainly by activated mononuclear cells which mediate immunity and inflammation.

Mast cells

Mast cells (MCs) derive from a committed precursor in the bone marrow, involved in immune response inflammation and psychiatric disorders (24). Mast cell maturation and growth from human cord blood CD34⁺ cells requires the presence of certain cytokines such as stem cell factor (SCF) and IL-6.

MCs express *c-kit* receptor, and the high affinity receptor for immunoglobulin E (IgE) FcεRI. They participate in human innate and adaptive immune reactions and are activated by cross-linking of bound IgE by antigen activating protein tyrosine kinases.

These reactions lead to a mitogen-activated protein (MAP) kinase cascade and a phosphatidylinositol-specific phospholipase C (PI-PLC). MAP kinase acts on the MC nucleus and activates cytokine gene expression with the generation of inflammatory cytokines (25) (Fig. 1).

After activation, MCs release many different cytokines and chemokines (MCP-1, Eotaxin, and others) which are mediators of the immune responses and the growth and development of neurons. An abnormal production of cytokines leads to a potent inflammatory response and is associated with major depression (MDD) and stress disorders. Interleukin (IL)-1 and its receptor IL-1 type I receptors (IL-1RI) are involved in the regulation of HPA axis and in mast cells affecting the generation of corticotrophin-releasing hormone (CRH) from hypothalamus, ACTH from the pituitary gland, and glucocorticoids from the adrenal cortex. IL-1 receptor is expressed in many tissues, including the anterior pituitary and the adrenal cortex (26-27).

IL-1β acts through IL-1 receptor type 1 (IL-1RI) in the brain, activates the hypothalamic-pituitary-adrenal axis, thereby stimulating inflammatory cells, and glucocorticoid release. Activated inflammatory cells may induce the generation of arachidonic acid cascade with a release of inflammatory compounds, including prostaglandins, leukotrienes and lipoxines. In fact, repeated stress leads to the release of IL-1 and PGE₂. In addition, Nitric oxide synthase (NOS) is constitutively expressed in neurons under psychological conditions, and NO generated by iNOS in the brain is involved in the elevation of CRH released by MCs (28). In these cases, there is a release of inflammatory IL-1, IL-33, IL-18, and IL-6 which may reduce neurogenesis and cell survival.

IL-1 may play a crucial role in the brain function, activating the HPA axis, and is necessary for stress to impair neurogenesis (Fig. 2). IL-1 concentration is implicated in the pathophysiology of MDD, and rodents exposed to stress show an increase in IL-1. An increase of IL-1 in response to psychological stress challenge leads to cognitive, social, and sleep-deprivation. IL-1 induces many pathophysiological effects which can be inhibited by IL-37.

IL-1 can strongly affect neurotransmission in the

brain circuits after psychological stress and certainly induces hormonal changes. It strongly activates MCs to generate CRH, IL-1, IL-6 and TNF- α , which stimulate the adrenocorticotrophic hormone (ACTH).

It has been previously reported that human umbilical cord blood-derived mast cells (hCBMCs) and a human leukemic mast cell line (HMC-1) are activated by proinflammatory cytokine IL-1 to induce differential release of IL-6 without concomitant release of the preformed mediator tryptase (29).

IL-1 binds IL-1R1 and recruits the co-receptor IL-1R accessory protein (IL-1R3) which leads to MyD88 and TIR domain, a cascade that activates the transcription factor NF- κ B and AP-1, in addition to the mitogen-activated protein kinase (MAPK) pathways (30).

In psychological stress, IL-1 binds its receptor IL-1R1 and induces the upregulation of inflammatory molecules and elevated anxiety. These effects may be inhibited by IL-37, an IL-1 inhibitor which has proven to be beneficial in many inflammatory disorders.

IL-37 was formerly called IL-1 family member 7, with a molecular weight circa 45 kDa. IL-37 includes five different variants, namely IL-1F7a, b, c, d, which bind IL-18 receptor α -chain and does not act as a receptor antagonist. IL-1F7b is the largest protein which is cleaved by caspase-1 from IL-1F7b precursor into mature form. This cytokine is released by several cell types, including peripheral blood mononuclear cells (PBMC), monocytes, plasma cells, dendritic cells, and epithelial cells.

Activated Toll-like receptor (TLR) in macrophages, which is expressed only in inflamed tissues but not in healthy and physiological states, produces IL-37. This cytokine strongly inhibits the expression of several inflammatory cytokines including IL-1 α , IL-1 β , TNF, IL-23, IL-17, IL-18 and IFN- γ , and certain chemokines such as MIP-2/CXCL2, MCP-5/CCL12, BCA-1/CXCL13 and IL-8, in many different cell types (31). IL-37 may also inhibit inflammatory cytokines *in vivo*, and *in vitro* on MCs activated by LPS or IL-1 (32).

IL-37 inhibits pro-inflammatory cytokines generated by macrophages and mast cells, and down-regulates intracellular and extracellular immune responses, inflammation, and psychological stress

mediated by IL-1.

Here, we report for the first time that IL-1 family members take part in inflammatory psychological stress mediated by mast cells, an effect inhibited by IL-37.

These findings may have a therapeutic potentiality, although the use of IL-37 has in some points to be clarified, for example, the side effects and the therapeutic dose of IL-37 to use. For this reason, more studies are required to better define the role of IL-37 on mast cells mediating psychological stress. Furthermore, the screening of alexithymic traits and the administration of appropriate psychological and psychotherapeutic interventions should be integral parts of disease management programs. Supplying such interventions will probably help with prevention of the development of the disease and/or its exacerbation by improving the quality of life of alexithymic individuals.

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INFLAMMATION IN LUNG AFTER ACUTE MYOCARDIAL INFARCTION IS INDUCED BY DENDRITIC CELL-MEDIATED IMMUNE RESPONSE

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The present study was performed to describe the changes of lung tissues in mice with acute myocardial infarction (AMI) and also explain the cell mechanism involved in inflammation in lung. AMI was established by left coronary ligation in mice. Then mice were divided into three groups: control group, MW1 group (sampling after surgery for one week) and MW2 group (sampling after surgery for two weeks). Afterwards, measurement of lung weight and lung histology, cell sorting in bronchoalveolar lavage (BAL) fluid and detection of several adhesive molecules, inflammatory molecules as well as enzyme associated with inflammation were performed. Moreover, dendritic cells (DCs) were isolated from bone marrow of C57B/L6 mice. After incubating with necrotic myocardium, the expression of antigen presenting molecules, co-stimulatory molecules and inflammatory molecules were detected by flow cytometry or immunohistochemistry in DCs. We also detected T-cell proliferation after incubating with necrotic myocardium-treated DCs. AMI induced pathological changes of lung tissue and increased inflammatory cell amount in BAL fluid. AMI also increased the expression of several inflammatory factors, adhesive molecules and enzymes associated with inflammation. CD11c and TLR9, which are DC surface markers, showed a significantly increased expression in mice with AMI. Additionally, necrotic myocardium significantly increased the expression of co-stimulatory factors including CD83 and CD80, inflammatory cytokines including TNF- α , IFN- γ and NF- κ B in DCs. Furthermore, DCs treated with necrotic myocardium also significantly promoted T-cell proliferation. AMI induced inflammation in lung and these pathological changes were mediated by DC-associated immune response.

Myocardial infarction (MI) is the leading cause of cardiac death in Western countries (1). There has been a rising trend in the morbidity of MI in China in recent years, due to the increase in the aging population, changes in dietary habit and several other social and mental factors (2). MI is characterized by loss

of functioning cardiomyocytes caused by reduced blood supply from coronary artery to the heart, and myocardial inflammation induced by ischemia and hypoxia condition and increased ventricular wall tension, leading to further myocardial damage and possibly to heart failure (3). Heart failure represents

Key words: acute myocardial infarction, dendritic cells, inflammation, lung, coronary ligation

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a frequent cause of cardiovascular morbidity and mortality, affecting nearly 23 million people (4).

After years of study, acute or chronic heart failure induced by MI has been found to induce pulmonary edema and changes in lung tissues including alveoli. In clinical practice, increase in pulmonary capillary pressure has been found in patients with acute heart failure, posing the risk of pulmonary edema (5). In patients with pulmonary edema, the membrane of alveoli and capillary can be destroyed by increased pulmonary capillary pressure, leading to so-called “pulmonary capillary fractures” (6, 7). The injured alveoli and capillary membrane allow increased entry of liquid and proteins into alveoli, and the protein concentration in edematous fluid in alveoli is elevated in patients with heart failure. Further study on mice indicated that heart failure induced by MI increases the level of collagen and activity of fibroblast (8). In another research on guinea pigs, the changes in lung induced by heart failure after aorta ligation included thickening of alveolar interval and increased lung dry weight (9). Pulmonary edema may not occur in patients with chronic heart failure, which is interpreted as a protective mechanism (10–11), while there are also several changes in pulmonary vasculature and even irreversible pulmonary hypertension in chronic heart failure patients. Thus, we aimed to describe the changes of lung tissue in mice with acute myocardial infarction (AMI).

AMI is identified to induce changes in lung tissues and we supposed that these changes might be associated with inflammatory responses initiated by AMI in lung. T lymphocytes are the main participants in the inflammatory response. In the early stage of AMI, T cells serve as a protector against adverse ventricular remodeling and improve myocardial function via inhibiting inflammation (12). However, in the post-AMI, this response is largely exaggerated and the neutrophil infiltration into alveolar space is enhanced and other responses are induced (13). Wilson et al. have indicated that CD4⁺ and $\gamma\delta$ ⁺ T cells can promote the development of pulmonary fibrosis via secreting IL-17A inflammatory cytokine, and this process can also be enhanced by TGF- β (14). With the enhanced inflammatory signals, the macrophages in lung

tissue are activated (15), and the microenvironment of lung tissue is changed. Dependent on these alterations, recruitment, migration, proliferation and differentiation of fibroblasts are promoted and apoptosis is suppressed (16). Dendritic cells (DCs) functioning as professional antigen-presenting cells (APCs) play an important role in initiation of both innate and adaptive immune responses against foreign antigens (17). The immature DCs are highly efficient in capturing antigens. If they were triggered by appropriate signals, they would migrate to organized lymphoid tissues, differentiated into mature DCs. Then mature DCs present antigens to T cells and elicit T cell-mediated immune response (18, 19). Thus, DCs are well known to be involved in the pathogenesis of various inflammatory diseases. Therefore, in the present study, we also investigated the roles of DCs in lung changes induced by AMI after establishing an AMI model in mice. We believe our results could provide better interpretation of the changes in lung induced by AMI.

MATERIALS AND METHODS

Animals

Male C57B/L6 mice, 10–12 weeks old and weighing 18–22 g were purchased from the Experimental animal center, Shanghai Medical College of Fudan University. Animals were bred and kept in special pathogen-free (SPF) conditions, with free access to food and water. The ethics committee of Shanghai Medical College of Fudan University approved this study and all animal experiments were performed in accordance with institutional guidelines for animal experiments.

Surgical procedure

AMI in mice was induced by ligation of the left anterior descending coronary artery. Mice in the experimental group underwent coronary ligation by two skilled operators, as described previously (20, 21). After anesthesia, animals were placed in a supine position and intubated. Then mice underwent surgical opening of the chest and ligation of the proximal left coronary artery. Mice which had undergone coronary ligation were divided into two groups: MW1 group (sampling after surgery for one week) and MW2 group (sampling after surgery for

two weeks). Another 10 mice underwent a sham operation including every step except coronary ligation and were used as controls.

Serum and bronchoalveolar lavage (BAL) fluid collection

One or two weeks after surgery, blood was collected from the orbital sinus of mice by quickly removing the eyeball from the socket with tweezers. After centrifugation, blood samples were stored at -80°C .

Mice were sacrificed by cervical dislocation. BAL was performed on the isolated trachea and lung. After occlusion of the left main bronchus, a cannula was inserted into the right trachea. Sterile saline was instilled into the lungs and the BAL fluid was collected. Total cell counts were performed using a hemocytometer. Then cells were isolated with a cytospin, fixed and stained with Wright-Giemsa dye. Cell sorting was performed on at least 200 cells in each cytospin slide.

Changes of several inflammatory factors including IL-6, MCP-1, IL-12p70, TNF- α and IL-10 in serum and in BAL fluid were detected by flow cytometry (BD FACSCanto™).

Measurement of lung

After sacrifice, the right lungs were immediately ligated and isolated from the mice for weighing the wet weight. Next, lungs wrapped in silver paper were dried at 56°C and weighed for dry weight. Then wet weight/dry weight (W/D) was calculated.

Histologic examination

Lungs were infused via trachea with 4% neutral formalin and embedded in paraffin. Then samples were cut into 5- μm sections and stained with H&E and Masson's trichrome.

For immunohistochemistry, tissue sections were deparaffinized, rehydrated and blocked in goat serum. Then sections were incubated with mouse specific primary antibodies [anti-angiotensin converting enzyme (ACE), CD11c, CD68, TLR4, TLR9 and smooth muscle actin- α (α -SMA)] at a dilution of 1:200. As recommended by the manufacture, sections were visualized using a streptavidin-biotin complex (SABC) staining kit using diaminobenzidine as a chromogen and counterstained with hematoxylin. Positive cells were defined as the presence of staining in the cytoplasm and counted in at least 5 random

and non-overlapping fields at $400\times$ magnification under a Leica microscope by two independent researchers who were blinded to the experimental grouping design. Then the integral optical density (IOD) was measured within the area with positive cells using Image ProPlus 6.0 software (Media Cybernetics Inc., USA) and the effective area (Area) was measured based the marco-pathology of lung. The mean optical density (Mean Density) representing the protein expression was calculated as: Mean Density = IOD/Area.

Generation of DCs from bone marrow

DCs were isolated from bone marrow of C57B/L6 mice. The bone marrow was flushed from the femur and tibias of mice and incubated in serum-free medium for 24 h. After centrifugation, the cells were incubated in RPMI1640 medium containing 10% fetal bovine serum (FBS), granulocyte-macrophage colony stimulating factor (GM-CSF, 100 ng/ml) and interleukin-4 (IL-4, 100 ng/ml). Six or seven days after incubation, the colonies crowded with immature DCs were observed and these cells were used for further analysis.

Generation of necrotic myocardium

HL-1 (mouse myocardial cells) were subjected to free/thaw cycles ($-80^{\circ}\text{C}/37^{\circ}\text{C}$ cycles) for 5 times. When 90% of cells were destroyed, confirmed by trypan blue staining, necrotic myocardium was successfully prepared. Then these cells were added into the cultured DCs. The levels of MHC-II, CD40, CD80, CD83, CD86, TNF- α and IFN- γ in DCs were detected by a flow cytometry. DCs treated with RPMI1640 medium were used as controls.

CD4⁺ T cells

Spleen was collected from sacrificed mice and crushed using forceps. After washing with PBS and centrifugation, cells were collected, counted and resuspended with complete medium. Then the cells were isolated using CD4⁺ T cells Isolation Kit II, and the unlabeled CD4⁺ T cells were collected. Afterwards, DCs treated with necrotic myocardium were mixed with CD4⁺ T cells at a ratio of 1:10. At days 5, 6 and 7 after incubation, this mixture was stained with carboxy-fluorescein diacetate succinimidyl ester (CFSE) dye, and the proliferation of CD4⁺ T cells was evaluated using a flow cytometry.

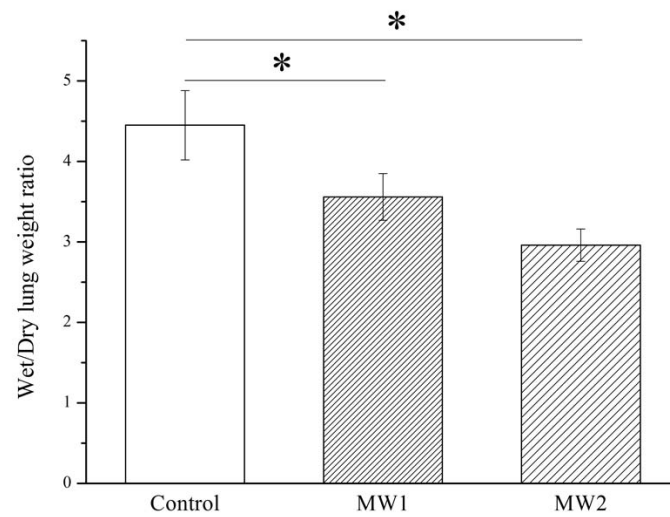


Fig. 1. Changes in wet/dry lung weight in mice after acute myocardial infarction. MW1 group, sampling after surgery for one week; MW2 group, sampling after surgery for two weeks; Control group, sham-operated mice. * $P < 0.05$, compared to the control group.

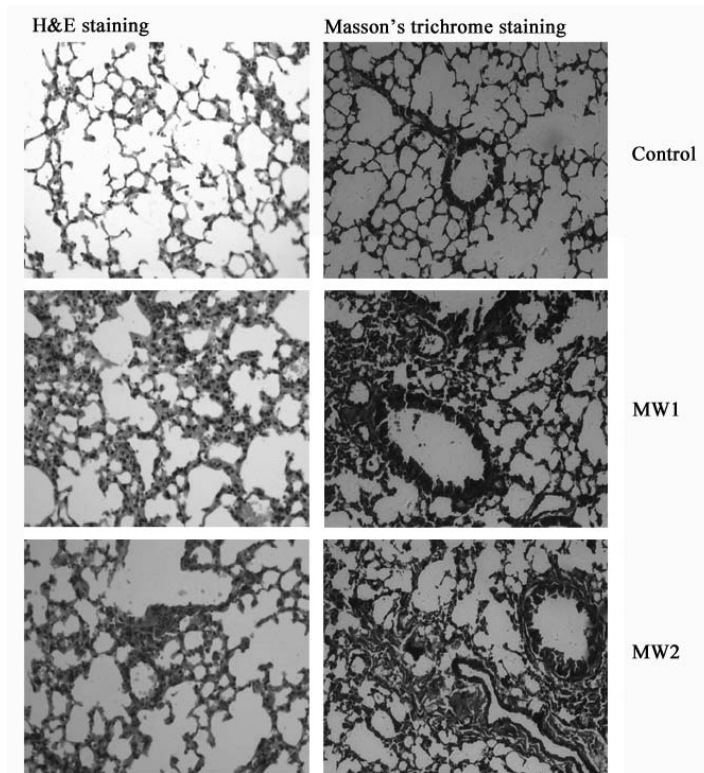


Fig. 2. Histological changes of lung in mice with acute myocardial infarction detected by H&E staining and Masson's trichrome staining. MW1 group, sampling after surgery for one week; MW2 group, sampling after surgery for two weeks; Control group, sham-operated mice.

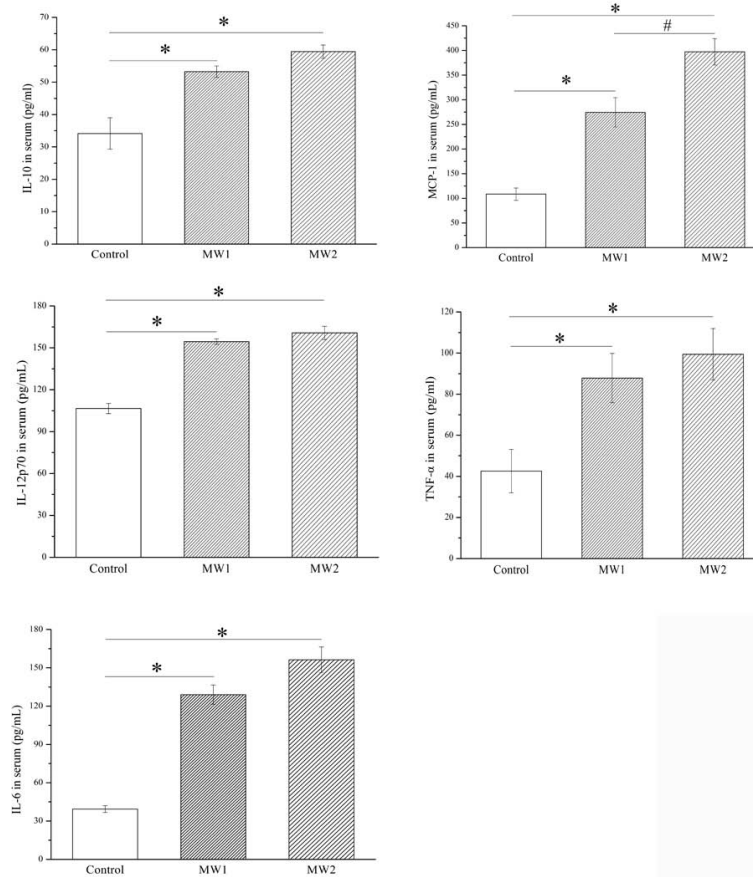


Fig. 3. Changes in inflammatory factors in serum in mice with acute myocardial infarction detected by a flow cytometry. MW1 group, sampling after surgery for one week; MW2 group, sampling after surgery for two weeks; Control group, sham-operated mice. * $P < 0.05$, compared to the control group. # $P < 0.05$, compared to the MW1 group.

Western blot

DCs with or without addition of necrotic myocardium were lysed with cell lysis buffer (1% NP-40, 50 mmol/L Tris, 150 mmol/L NaCl, 1% SDS, 5% deoxycholate acid, 0.1 mmol/L PMSF and 1mg/L Aprotinin). Total cell proteins were quantified using a standard bicinchoninic acid assay (Pierce, Rockford, IL, USA). Then proteins were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto a polyvinylidene difluoride membrane. After incubation in 5% milk in 0.1% Tris-buffered saline-Tween20 (TBST) at room temperature, membranes were incubated with mouse anti-NF- κ B (p65) primary antibodies at 4°C overnight at a dilution of 1:1000, followed by incubation with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse secondary antibodies (Abcam, Cambridge, MA, USA) at a dilution of 1:5000. Membranes incubated with GADPH primary antibody were set as controls.

Finally, membranes were washed and developed with enhanced chemiluminescence reagent and visualized using a Fujifilm Luminescent Image Analyser LAS-3000.

Statistical analysis

Statistical analysis in our study was performed using SPSS 19.0 software. Data were presented as mean $\pm$ standard deviation (SD) and compared using independent t -test. A value of $P < 0.05$ was considered statistically significantly different.

RESULTS

AMI established by coronary ligation induced acute lung inflammation in mice

Cell sorting in BAL fluid

There was a significant increase in total cell counts

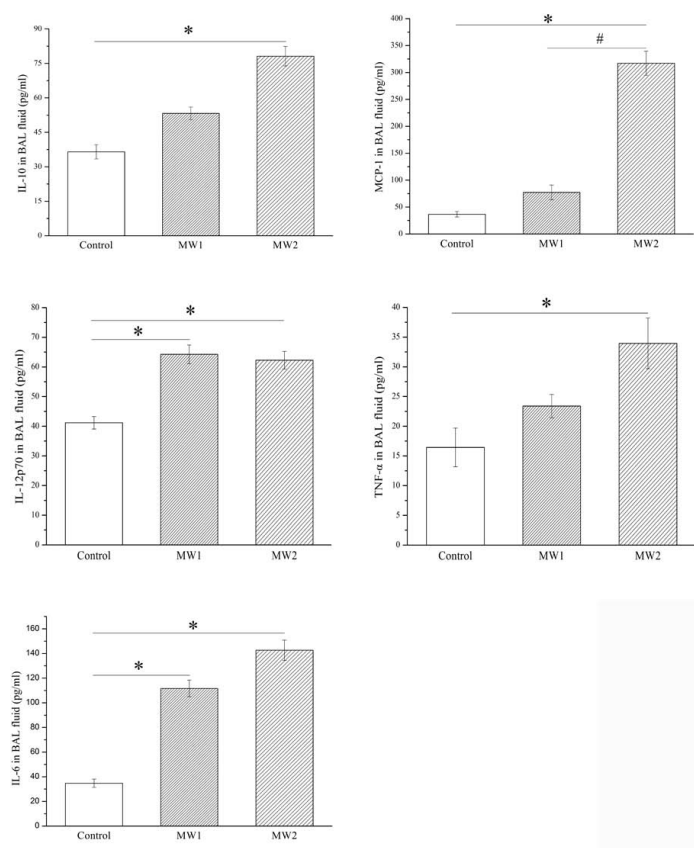


Fig. 4. Changes in inflammatory factors in bronchoalveolar lavage (BAL) fluid in mice with acute myocardial infarction detected by a flow cytometry. MW1 group, sampling after surgery for one week; MW2 group, sampling after surgery for two weeks; Control group, sham-operated mice. * $P < 0.05$, compared to the control group. # $P < 0.05$, compared to the MW1 group.

in BAL fluid in mice which had undergone coronary ligation compared to those which had undergone sham operation (MW1 vs Control: 167.46 ± 18.22 vs 99.32 ± 20.64 , MW2 vs Control: 153.84 ± 19.18 vs 99.32 ± 20.64 , $P < 0.05$). For cell sorting, the number of macrophagocyte decreased in the MW1 and MW2 groups compared to that in the control group, while the number of neutrophils increased dramatically when compared to the control group (MW1 vs Control: 61.44 ± 0.49 vs 40.81 ± 0.13 , MW2 vs Control: 55.74 ± 0.50 vs 40.81 ± 0.13 , $P < 0.05$).

Measurement of W/D

The ratio of wet/dry lung weight was also analyzed after the mice were sacrificed. As shown in Fig. 1, W/D decreased obviously in mice with AMI compared to that in sham-operated mice (MW1 vs Control: 3.56 ± 0.29 vs 4.45 ± 0.43 , MW2

vs Control: 2.96 ± 0.20 vs 4.45 ± 0.43 , $P < 0.05$).

Detection of changes in lung histology

Changes in lung histology in mice which had undergone coronary ligation were detected by H&E and Masson's staining (Fig. 2). Compared to the sham-operated mice, a lung phenotype of thickening of alveolar interval, pulmonary congestion, distortion of lung structure, pulmonary interstitial edema and fibrosis induced by coronary ligation (both in MW1 and MW2 groups) was viewed. In addition, there was an increase of fibrosis when compared to the mice in the MW1 group to that in the MW2 group.

Measurement of cytokines in serum and in BAL fluid

We also detected the changes of several factors including IL-6, MCP-1, IL-12p70, TNF-α and IL-10 in both serum and BAL fluid in mice with AMI

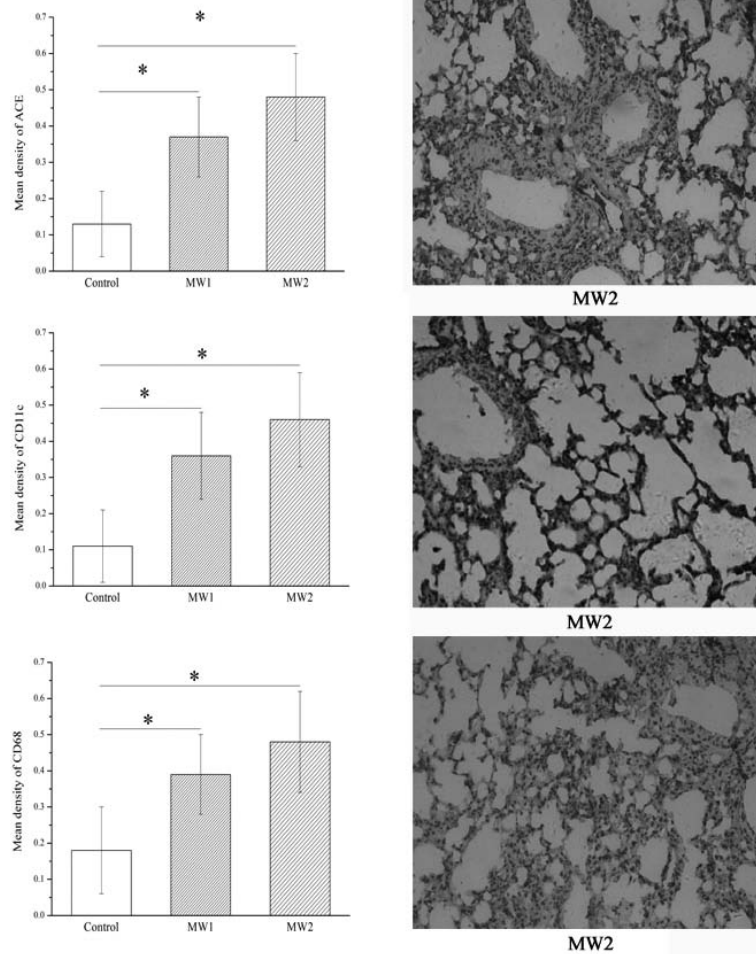


Fig. 5. Changes in anti-angiotensin converting enzyme (ACE), CD11c and CD68 in lung tissue in mice with acute myocardial infarction detected by immunohistochemical staining. MW1 group, sampling after surgery for one week; MW2 group, sampling after surgery for two weeks; Control group, sham-operated mice. * $P < 0.05$, compared to the control group.

by flow cytometry. The results are shown in Figs. 3 and 4. Compared to the sham-operated mice, the mice in the MW1 and MW2 groups had a significant increase in terms of IL-10, MCP-1, IL-12p70, TNF- α and IL-6 ($P < 0.05$) in serum. In addition, there were also significant increases of IL-10, MCP-1, IL-12p70, TNF- α and IL-6 levels in BAL fluid in mice with AMI compared to the mice in the control group ($P < 0.05$). Furthermore, the levels of these cytokines both in serum and in BAL fluid of mice in the MW2 group were higher than those of the MW1 group and differences in

several indexes between these two groups were statistically significant.

Measurement of factors associated with inflammation and fibrosis

Factors associated with inflammation and fibrosis, including ACE, CD11c, CD68, TLR4, TLR9 and α -SMA were measured by immunohistochemistry and the results are shown in Figs. 5 and 6. Compared to the mice in the control group, the levels of these molecules increased significantly in mice both in the MW1 and the MW2 groups, indicating coronary

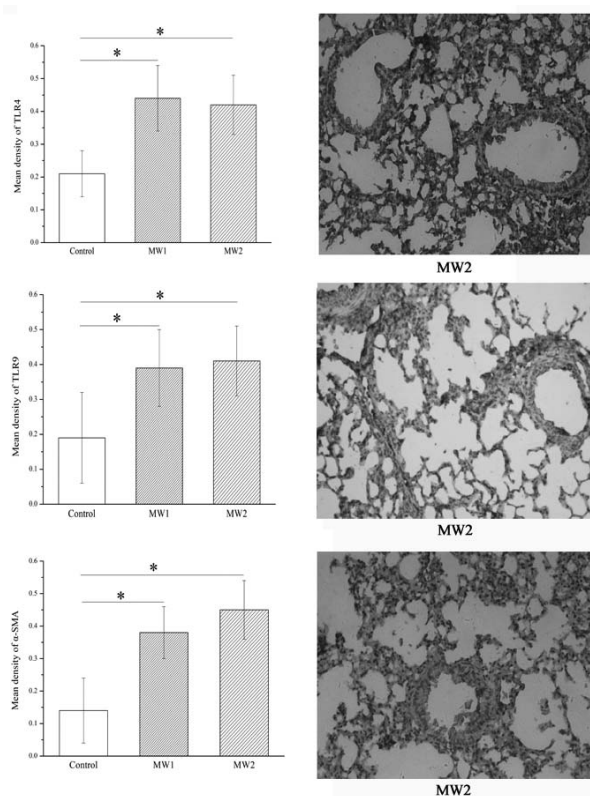


Fig. 6. Changes in TLR4, TLR9 and smooth muscle actin- α (α -SMA) in lung tissue in mice with acute myocardial infarction detected by immunohistochemical staining. MW1 group, sampling after surgery for one week; MW2 group, sampling after surgery for two weeks; Control group, sham-operated mice. * $P < 0.05$, compared to the control group.

ligation induced inflammatory reaction and fibrosis in lung of mice.

Necroticmyocardium induced changes in the phenotype of DCs

The phenotype changes of DCs were evaluated by detecting the expression of several biomarkers for DCs, including MHC-II, CD40, CD80, CD83 and CD86 by a flow cytometry and the results are displayed in Table I. After addition of necroticmyocardium, there were significantly increased expressions of CD83 and CD80 in DCs compared to the controls ($P < 0.05$), while the effects of necroticmyocardium on other molecules were not statistically significant.

Necroticmyocardium increased the activity of TNF- α and IFN- γ

As presented in Table II, the levels of TNF- α and

IFN- γ were significantly higher in DCs added with necrotic myocardium compared to the control DCs ($P < 0.05$), indicating an elevated secretion of these cytokines from DCs.

Necroticmyocardium increased the expression of NF- κ B (p65)

After addition of necrotic myocardium, there was an obviously up-regulated expression of NF- κ B (p65) in DCs compared to the control group (Fig. 7, $P < 0.05$), indicating that addition of necrotic myocardium to DCs activates the activity of NF- κ B in DCs.

Necroticmyocardium promoted CD4⁺ T cell proliferation

Detection of CD4⁺ T cell proliferation induced by DCs is one of the most important indicators for detection of immune response of DCs, which was performed by CFSE staining. As shown in the results in Figs. 8 and 9, there was a trend of increased percentage of proliferative T cells in DCs after addition of necrotic myocardium. At day 7, the percentage of proliferative T cells was significantly higher in DCs with necrotic myocardium than that in control DCs ($P < 0.05$), indicating that addition of necrotic myocardium to DCs promotes CD4⁺ T cell proliferation.

DISCUSSION

As described previously, the inflammation in myocardial tissue and in the whole body after AMI may lead to deterioration of ventricular function and the development of heart failure (22). Necrotic myocardium trigger the activation of complement system, the release of cytokines and chemotaxis and infiltration of immune cells. Immune-mediated inflammation induced by AMI can exacerbate myocardial injury and cause infarct expansion (23). Previous studies indicated the presence of pulmonary structural remodeling in rats with MI induced by coronary ligation (8). We aimed to explore the changes of lung tissue in mice after AMI. Following the results of our study, the increased cell counts in BAL fluid detected by flow cytometry and the pathological changes in lung tissue

detected by H&E and Masson staining revealed the activation of inflammatory response in lung in mice after AMI. In addition, based on the results from immunohistochemical staining for α -SMA, and the changes in W/D, there was an increase in fibroblasts and collagenous fiber in lung tissue in mice after AMI, indicating the development of pulmonary fibrosis.

Furthermore, CD11c and TLR9-positive cell amount in lung tissue increased significantly in mice with AMI. TLR9 is mainly expressed in APCs, such as B cells and DCs (24). CD11c is one of the highest cell surface markers of DCs (25). DCs are the most potent APCs and also the only APC that can induce initial immune response. After being triggered appropriately by several cytokines and chemokines, the immature DCs are recruited from the blood into the inflammatory sites to intake and process antigen (26). Meanwhile, they will differentiate into mature DCs and elicit T-cell mediated immune

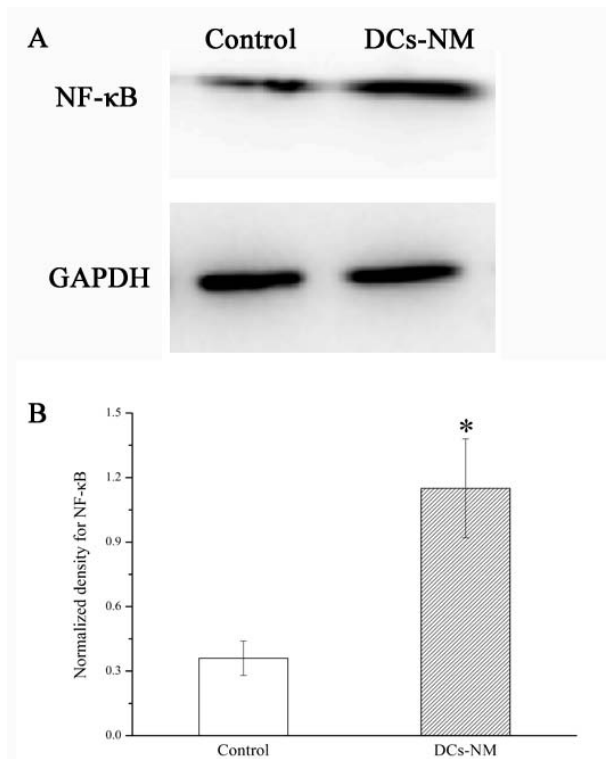


Fig. 7. Changes in NF- κ B (p65) expression in dendritic cells after addition of necrotic myocardium. DCs-NM: dendritic cells treated with necrotic myocardium. * $P < 0.05$, compared to the control group.

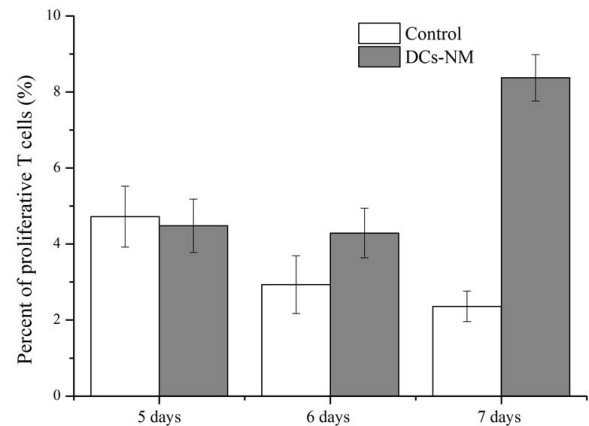


Fig. 8. Changes in T cell proliferation induced by necrotic myocardium treated dendritic cells. DCs-NM: dendritic cells treated with necrotic myocardium.

response. Moreover, previous study indicated that several inflammatory factors including TNF- α , IL-1, IL-6 and IL-12 increased significantly in serum in patients with AMI (27). In our study, the activity of these factors also significantly increased in both serum and BAL fluid. Thus, we hypothesized that the inflammatory response in lung is initiated by AMI-induced DC activation. Activated immature DCs undergo a maturation process with up-regulation of several antigen presenting molecules, adhesion molecules and co-stimulatory molecules, then eliciting T cell-mediated immunity and local inflammatory response. Along with the migration of inflammatory factors and cells through blood to lung, inflammation is observed in lung in patients with AMI. In addition, we can obviously identify that the inflammatory phenotype was more severe in the MW2 group than the MW1 group. Considering the influence of inflammation on pulmonary fibrosis, we supposed that development of inflammation is closely related with the illness course. With the development of AMI, the inflammatory condition was significantly aggravated, and the speed and level of inflammatory infiltration might also be increased. Hence, the inflammatory levels in both blood and lung tissue were severer in the MW2 group than the MW1 group.

In order to verify our theory, we tried to explore the immune regulatory effects of DCs after AMI by adding necrotic myocardium into DCs. Following

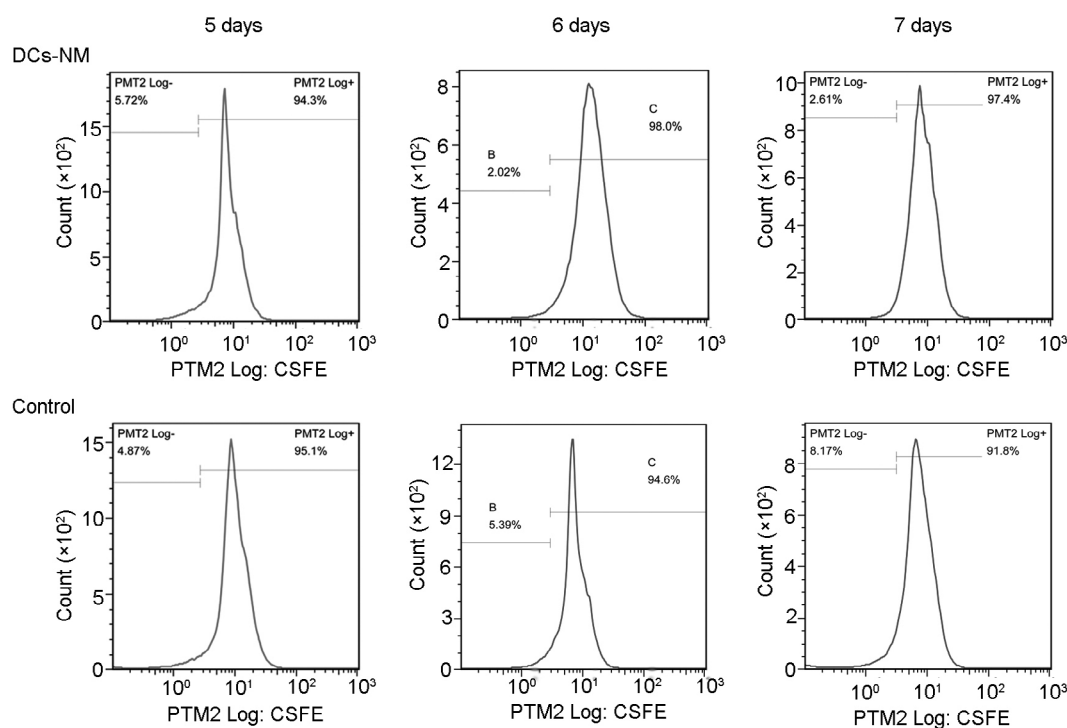


Fig. 9. Changes in T cell proliferation induced by necrotic myocardium treated dendritic cells showed by flow cytometry charts. DCs-NM: dendritic cells treated with necrotic myocardium.

the results of our study, there was a significant increase of co-stimulatory factors including CD83 and CD80 after addition of necrotic myocardium into DCs, indicating that DCs were activated by necrotic myocardium. Moreover, the activity of inflammatory factors, including TNF- α and IFN- γ , was also increased in DCs incubated with necrotic myocardium. Moreover, DCs after addition of necrotic myocardium also significantly promoted T cell proliferation. These results indicated that immune-mediated inflammation was triggered by necrotic myocardium.

In addition, we also found NF- κ B (p65) was significantly up-regulated in DCs incubated with necrotic myocardium. NF- κ B is one of the most important regulatory factors involved in inflammation and its activated form can initiate the transcription of a series of targeted genes, mainly including several cytokines, adhesive molecules and enzymes associated with inflammation (28). NF- κ B plays an important role in inflammation in lung (29). Our results further indicated that inflammatory

response in lung was activated by DCs after AMI.

In conclusion, increase of inflammation with inflammatory cell infiltration and inflammatory factors in lung tissues was found in mice with AMI induced by coronary ligation. We also demonstrated that DCs play an important role in immune-mediated inflammation in lung.

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KNOCKDOWN OF HMGB1 INHIBITS CELL PROLIFERATION AND INDUCES APOPTOSIS IN HEMANGIOMA VIA DOWNREGULATION OF AKT PATHWAY

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The high mobility group box 1 (HMGB1) as a conserved non-histone nuclear protein has been involved in a variety of biological processes of cancer, such as cell proliferation, apoptosis, angiogenesis and metastasis. Despite the increased expression of HMGB1 in many malignant tumors, the functions and molecular mechanisms by which HMGB1 contributes to the formation of hemangioma (HA) remain unclear. In the present study, immunohistochemistry was used to detect the expression levels of HMGB1 in different phases of human HAs. Cell function experiments, including MTT, cell colony formation and flow cytometry analysis were performed to evaluate the effects of HMGB1 knockdown on cell proliferation and apoptosis in HA CRL-2586 EOMA cells. As a consequence, we found that HMGB1 expression was significantly increased in proliferating phase HAs compared with the involuting phase HAs and normal skin tissues ($P<0.01$). Moreover, knockdown of HMGB1 gene *in vitro* suppressed EOMA cell proliferation and colony formation and induced cell apoptosis and cycle arrest at G0/G1 phase by downregulation of PCNA, CyclinD1, p-AKT and upregulation of p53 and cleaved PARP. Taken together, our findings demonstrate that HMGB1 may be implicated in the formation of HA through upregulation of AKT pathway, and represent a potential therapeutic target for treating HA.

Hemangiomas (HAs) belong to the benign tumors caused by the proliferating endothelial cells of blood vessels, and the life cycle of HA is divided into proliferating phase HA and involuting phase HA (1). HAs are regarded as one of the most common infantile tumors that occur in 5%-10% of infants and mainly lie in the head and neck (2). It has been reported that the activation of many key signaling pathways participates in the genesis of HA and leads to the development of HAs (3). Therefore, identification of the effective biomarkers required for HA progression may provide insight into therapeutic strategies.

High mobility group box 1 (HMGB1) as a nuclear and extracellular protein participates in various tumor formations and progression. It is identified as a protein overexpressed in ovarian cancer (4), osteosarcoma (5), and colorectal cancer (6) and is correlated with poor survival and metastasis (4-6), and overexpression of HMGB1 promotes tumorigenesis, invasion, and metastasis in osteosarcoma and gastric cancer cells via upregulation of MMP9 and CyclinD1 and downregulation of caspase-3 (6, 7), indicating a potential biomarker and a therapeutic target for cancer patients (4-6). In addition, HMGB1 increases autophagy and chemo-resistance in leukemia and

Key words: HMGB1, proliferation, apoptosis, hemangioma

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lung adenocarcinoma via upregulation of PI3K/AKT/mTORC1 pathway (8, 9), and autophagy-mediated HMGB1 release enhances gastric cancer and neuroblastoma proliferation via upregulation of TLR4 and RAGE/extracellular signal-regulated kinases 1/2 (ERK1/2) pathway (10, 11). Therefore, HMGB1 may play a key role in regulation of cancer cell development and progression and provide a therapeutic target for tumor therapy.

Moreover, HMGB1, as a signaling molecule, acts not only in cancer progression but also in angiogenesis. HMGB1 promotes cell proliferation of pulmonary arterial smooth muscle cells and primary human arterial endothelial cells, leading to vascular remodeling processes and pulmonary hypertension pathogenesis via p38 MAPK, ERK and c-Jun N-terminal kinase (JNK) pathways (12). Additionally, PI3K/AKT pathway is involved in inducing cell growth, apoptosis decrease and invasive potential in hemangioendothelioma cells (13), but blockade of AKT/mTOR pathway inhibits cell proliferation and induces apoptosis and cycle arrest in proliferative phase HA (14). However, the role and molecular mechanisms of HMGB1 in regulating HA have not been elucidated. In the present study, we found that the expression of HMGB1 was significantly increased in proliferating phase HAs compared with the involuting phase HAs and normal skin tissues, and knockdown of HMGB1 suppressed EOMA cell proliferation and colony formation and induced cell apoptosis and cycle arrest at G0/G1 phase followed by the downregulation of PCNA, CyclinD1, p-AKT and upregulation of p53. HMGB1 may be implicated in the formation of HA through regulation of AKT pathway, and represent a potential therapeutic target for treating HAs.

MATERIALS AND METHODS

Materials

The HA CRL-2586 EOMA cell line used in this study was from the Institute of Biochemistry and Cell Biology (Shanghai, China). The lentivirus vector Lv-shHMGB1, negative control vector and virion-packaging elements were from Genechem (Shanghai, China). The primer of HMGB1 was synthesized by ABI (USA); HMGB1

antibody was from Cell Signaling (USA) and other antibodies were from Santa Cruz Biotechnology (USA).

Drugs and reagents

Dulbecco's Modified Eagle medium (DMEM) and fetal bovine serum (FBS) were from Thermo Fisher Scientific Inc. (Waltham, MA, USA); TRIzol Reagent was from Invitrogen (Carlsbad, CA, USA); M-MLV Reverse Transcriptase was from Promega (Madison, WI, USA); SYBR Green Master Mixture was from Takara (Otsu, Japan). ECL-PLUS/Kit was from GE Healthcare (Piscataway, NJ, USA). Cell apoptosis kit (Propidium Iodide (PI), RNase A, Annexin V-FITC) was from KeyGEN biology (Nanjing, China).

Tissue samples

Fifteen freshly resected human HA samples were collected at the Department of General Surgery, and were classified according to Mulliken criteria. Tissues and clinical information were obtained as part of an approved study at Shanghai Jiao Tong University School of Medicine. There were 8 cases of proliferating phase HAs, 7 cases of involuting phase HAs and 5 cases of normal skin tissues. A portion of each tissue sample was fixed with 10% formalin for histopathological and immunohistochemical examination. All HA tissues were diagnosed by two independent pathologists.

Immunohistochemical staining

Immunohistochemical (IHC) staining for HMGB1 using monoclonal antibodies was performed. Unstained sections were deparaffinized and incubated overnight at 4°C with primary antibodies against the proteins, then with biotinylated secondary antibody (1:200) at room temperature for 1 h, followed by incubation with ABC peroxidase and 3,3'-diaminobenzidine. In each section of each group, three 200-fold fields were randomly selected for taking pictures, and Plus 6.0 software was used to select the uniform standard for positive expression, and each photo was analyzed for integral optical density (IOD) values.

Cell culture and transfection

The HA CRL-2586 EOMA was cultured in DMEM medium supplemented with 10% heat-inactivated FBS, 100U/ml of penicillin and 100µg/ml of streptomycin. They

were all placed in a humidified atmosphere containing 5% CO₂ at 37°C. Lv-shHMGB1 and negative control virus were transfected into proliferating phase EOMA. Positive stable transfectants were selected and expanded for further study. The clone in which the Lv-shHMGB1 vectors transfected was named as shHMGB1 group, the negative control vectors transfected was named as NC group.

Quantitative real-time PCR

To quantitatively determine the mRNA expression level of HMGB1 in CRL-2586 EOMA, Real-time PCR was used. Total RNA of each clone was extracted with TRIzol according to the manufacturer's protocol. Reverse-transcription was carried out using M-MLV and cDNA amplification was carried out using SYBR Green Master Mix kit according to the manufacturer's protocol. HMGB1 gene was amplified using specific oligonucleotide primer and human GAPDH gene was used as an endogenous control. The PCR primer sequences of HMGB1 gene were as follows: 5'-ATATGGCAAAAGCGGACAAG-3' and 5'-AGGCCAGGATGTTCTCCTTT-3'; GAPDH, 5'-CAACGAATTTGGCTACAGCA-3' and 5'-AGGGGTCTACATGGCA ACTG-3'. Data were analyzed using the comparative Ct method (2-Ct). Three separate experiments were performed for each clone.

Western blot assay

The CRL-2586 EOMA was harvested and extracted using lysis buffer. Cell extracts were boiled for 5 min in loading buffer and then equal amount of cell extracts were separated on 15% SDS-PAGE gels. Separated protein bands were transferred into polyvinylidene fluoride (PVDF) membranes and the membranes were blocked in 5% skim milk powder. The primary antibodies against HMGB1, p-AKT, AKT, PCNA, cyclinD1, p53, PARP and cleaved PARP were diluted according to the instructions for antibodies and incubated overnight at 4°C. Then, horseradish peroxidase-linked secondary antibodies were added at a dilution ratio of 1:1000, and incubated at room temperature for 2 h. The membranes were washed with PBS and the immunoreactive bands were visualized using ECL-PLUS/Kit according to the manufacturer's instructions.

Cell viability assay

Cell viability was analyzed with the MTT assay. Briefly,

4×10³ cells infected with Lv-shHMGB1 were incubated in 96-well-plates with DEME medium supplemented with 10% FBS. Cells were treated with 10 µl MTT dye on each day and then incubated with 100 µl of DMSO for 15 min. The color reaction was measured at 570 nm with enzyme immunoassay analyzer (Bio-Rad, American).

Colony formation assay

The cells transfected with Lv-shHMGB1 were cultured in a 6-well plate at 37°C under 5% CO₂ for 7 days. Surviving colonies were counted and photographed with a Qimaging Micropublisher 5.0 RTV microscope camera (Olympus, Tokyo, Japan).

Cell apoptosis analysis

To detect cell apoptosis, CRL-2586 EOMA was trypsinized, washed with PBS and resuspended in binding buffer according to the instructions of the apoptosis kit. FITC-AnnexinV and PI were added to the fixed cells for 15 min in darkness at room temperature. Then, Annexin V binding buffer was added to the mixture before the fluorescence was measured on a FACsort flow cytometer.

Cell cycle analysis

The fixed cells were stained with PI in the presence of RNase A for 30 min at room temperature in darkness. Each sample was filtered through a 50 µm nylon filter to obtain single-cell suspension. The samples were then analyzed on a FACsort flow cytometer (Becton Dickinson, Mountain View, CA, USA).

Statistical analysis

SPSS 15.0 was used for the statistical analysis. T-test and Chi-squared were used to analyze the differences between groups. Statistical significance was P<0.05.

RESULTS

The expression of HMGB1 in different phases of human HAs

The protein expression of HMGB1 in human HAs was evaluated by IHC staining. As shown in Fig. 1, the positive expression of HMGB1 protein was mainly localized in the cell nucleus of HA tissue cells. HMGB1 expression level was increased in proliferating phase HAs compared with the

involuting phase HAs or the normal skin tissues (** $P<0.01$).

The effect of HMGB1 knockdown on the expression of AKT pathway

To confirm whether the expression of HMGB1 was knocked down by shRNA-mediated Lv-shHMGB1 in CRL-2586 EOMA, we detected the mRNA and protein expression levels of HMGB1 by Real-time PCR and Western blot assays. As shown in Fig. 2 A,B, HMGB1 expression was significantly downregulated in shHMGB1 group compared to the NC group (** $P<0.01$). As for the protein expression levels of AKT and p-AKT, HMGB1 knockdown downregulated the expression of p-AKT (Fig. 2D), but had no effect on AKT expression (Fig. 2C).

The effect of HMGB1 knockdown on cell growth

To determine the effects of HMGB1 knockdown on cell growth of CRL-2586 EOMA, we measured cell proliferation viability by MTT and colony

formation assays. We found that knockdown of HMGB1 markedly decreased cell proliferation viability in a time-dependent manner and colony formation compared with the NC group ($P<0.01$, Fig. 3 A, B). In addition, whether HMGB1 knockdown reduced the endogenous expression of PCNA from translational repression was undefined. The protein expression of PCNA was detected by Western blot assay, indicating a decrease in the hHMGB1 group compared with the NC group (Fig. 3C).

The effect of HMGB1 knockdown on cell apoptosis

To verify whether HMGB1 exerted the effect on apoptosis of CRL-2586 EOMA, we conducted flow cytometric analysis of cell apoptotic index, which was significantly increased in the shHMGB1 group compared to the NC group ($P<0.01$) (Fig. 4 A, B). To determine whether HMGB1 affected the expression of p53, PARP and cleaved PARP from translational level, we measured their expression by Western blot, indicating that p53 and cleaved PARP had increased

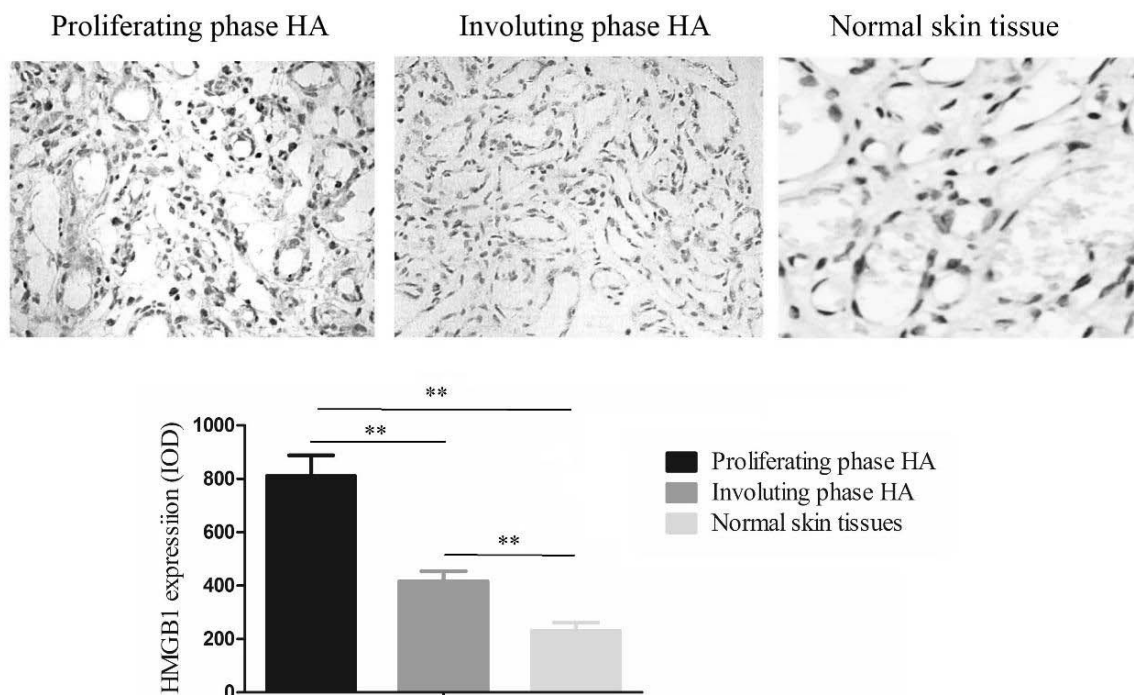


Fig. 1. The expression of HMGB1 in different phases of HAs. IHC staining was used for identifying the differential expression of HMGB1 between proliferating phase HAs, involuting phase HAs and normal skin tissues according to the IOD values. ** $P<0.01$.

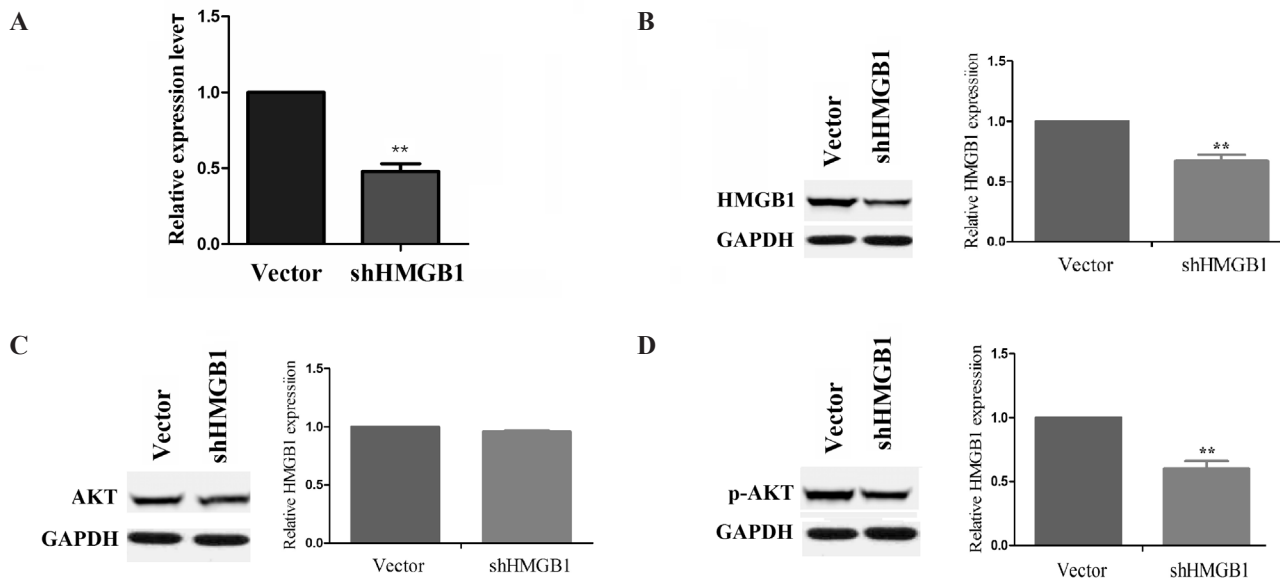


Fig. 2. HMGB1 knockdown downregulates the expression of p-AKT in HA CRL-2586 EOMA cells. Real-time PCR (A) and Western blot assays (B) were performed for detecting the expression of HMGB1 in EOMA cells after Lv-shHMGB1 transfection for 24 h. The expression of AKT (C) and p-AKT (D) was evaluated by Western blot assay. ** $P < 0.01$.

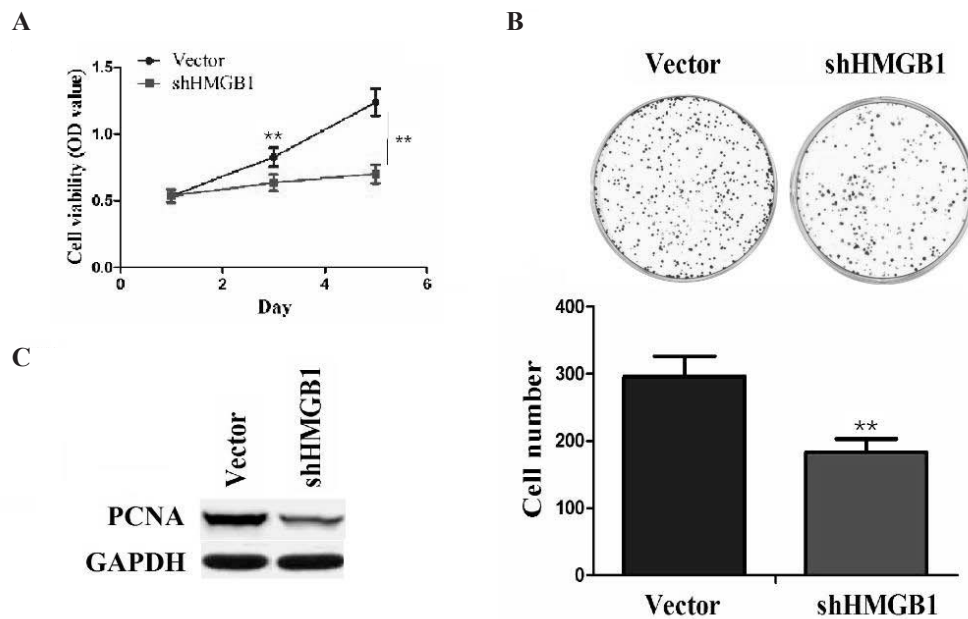


Fig. 3. HMGB1 knockdown inhibits EOMA cell growth. A) Cell proliferation viability was measured by MTT assay. B) Cell colony formation ability was assessed by colony formation assay. C) The expression of PCNA was examined by Western blot assay. ** $P < 0.01$.

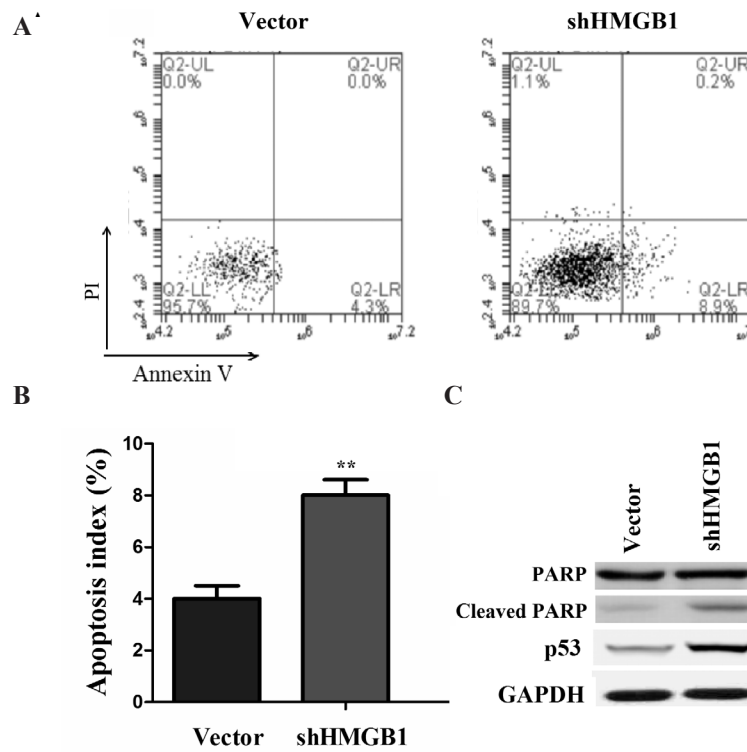


Fig. 4. HMGB1 knockdown induces EOMA cell apoptosis. **A, B)** Cell apoptosis index was analyzed by flow cytometry. **C)** The expression of p53, PARP and cleaved PARP was examined by Western blot assay. ** $P < 0.01$.

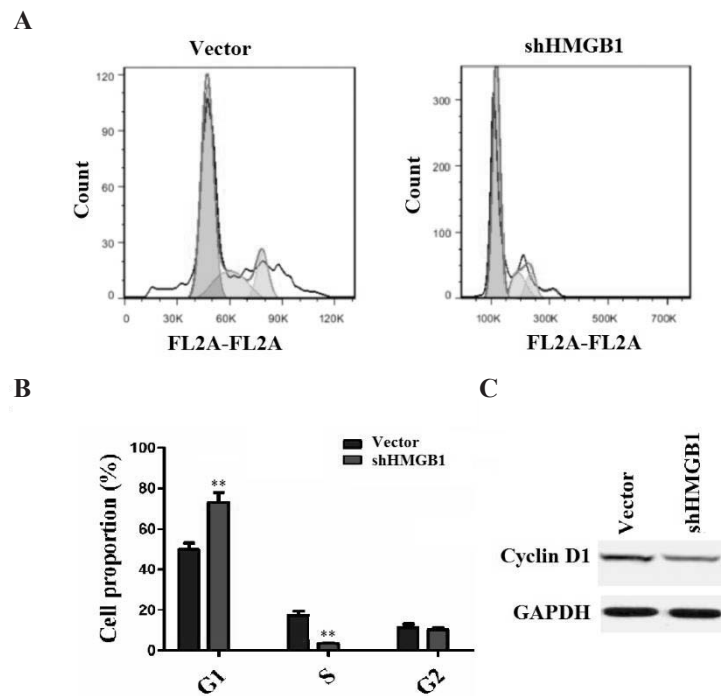


Fig. 5. HMGB1 knockdown induces EOMA cell cycle arrest. **A, B)** Cell cycle distribution was analyzed by flow cytometry. **C)** The expression of cyclinD1 was examined by Western blot assay. ** $P < 0.01$.

expression, but PARP had no differential expression in the shHMGB1 group compared with the NC group (Fig. 4C).

The effect of HMGB1 knockdown on cell cycle distribution

To observe whether HMGB1 influenced cell cycle distribution of CRL-2586 EOMA, we performed flow cytometric analysis of cell cycle distribution. The results showed that Lv-shHMGB1 transfected cells were increased in G0/G1 phase but were decreased in S phase in comparison with the NC group ($P < 0.01$, Fig. 5 A, B). To determine whether HMGB1 affected the expression of cyclinD1 from translational level, we measured the protein expression of cyclinD1 by Western blot, indicating a marked decrease in the shHMGB1 group compared with the NC group (Fig. 5C).

DISCUSSION

HMGB1 as a chromatin-binding protein facilitates the pathogenesis of angiogenic diseases via induction of proangiogenic cytokine release and activation of endothelial cells leading to vessel formation (15). HMGB1 accelerates angiogenesis and apoptosis reduction in ovarian cancer (16) and osteosarcoma, and Emodin administration inhibits HMGB1-induced angiogenesis *in vivo* and *in vitro* (17). Here, we investigated the expression of HMGB1 in human HAs, and found that HMGB1 expression level was significantly increased in proliferating phase HAs but decreased in involuting phase HAs, suggesting that HMGB1 might act a role in the formation of HAs.

Functionally, knockdown of HMGB1 inhibits cell growth and metastasis of cancer cells (18, 19) and induces cell apoptosis and cycle arrest (18, 20), and HMGB1 deficiency induces inhibition of cell proliferation in melanoma via p21 (21) and in pancreatic cancer via reduction of adenosine triphosphate release (22), enhances anti-tumor T-cell response caused by peptide vaccination (23), and increases the chemotherapy sensitivity via cell autophagy and apoptosis (24). Herein, we observed the role of HMGB1 in HA CRL-2586 EOMA cells, and found that knockdown of HMGB1 inhibited cell proliferation and colony formation of EOMA cells

and induced cell apoptosis and cycle arrest in G0/G1 phase, indicating that HMGB1 may be implicated in the development and progression of HAs.

Mechanically, HMGB1 regulates cell apoptosis and autophagy, enhances cell proliferation via HMGB1-RAGE-Erk1/2 (25) or RAGE/TLR4-dependent signal pathways (26) and promotes precancerous lesions of lung cancer via RAGE/JNK/NF- κ B pathway (27). Moreover, PTEN/PI3K/AKT/VEGF signaling is involved in angiogenesis (28) and propranolol-induced regression of HA cells via inhibition of cell cycle progression, invasion, and tube formation (29). However, whether HMGB1 modulates AKT signaling involving HA formation is poorly understood. Thus, we found that HMGB1 knockdown downregulated the expression of p-AKT pathway as well as PCNA and cyclinD1 and upregulated the p53 and cleaved PARP expression, suggesting that HMGB1 may regulate HA progression via the AKT pathway.

In conclusion, HMGB1 expression is significantly increased in proliferating phase HAs compared with the involuting phase HAs, and knockdown of HMGB1 suppresses EOMA cell growth and induces cell apoptosis via inhibition of AKT pathway, and represents a potential therapeutic target for treating HA.

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JAK2 TYROSINE KINASE INHIBITOR AG490 SUPPRESSES CELL GROWTH AND INVASION OF GALLBLADDER CANCER CELLS VIA INHIBITION OF JAK2/STAT3 SIGNALING

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The Janus kinase-signal transducers and activators of transcription signaling pathway (JAK/STAT pathway) have displayed a critical role in tumor development and progression in multiple malignancies. Previous studies showed that inhibition of JAK/STAT signaling blocked cell growth and metastasis in cancer cells, however, the antitumor effects of JAK inhibitor AG490 on gallbladder cancer (GBC) have not been reported. Our present study aimed to investigate the effects and associated mechanisms of JAK inhibitor AG490 on cell growth, invasive potential and apoptosis in GBC cells (GBC-SD and SGC-996) indicated by MTT, cell colony formation, Transwell and flow cytometry. As a consequence, we found that JAK2 inhibitor AG490 inhibited cell growth and invasion, and induced cell apoptosis and cycle arrest in GBC-SD and SGC-996 cells. Furthermore, the expression levels of p-JAK2, p-STAT3, VEGFC/-D and cyclinD1 were downregulated, while p53 expression was upregulated in AG490-treated GBC cells indicated by Western blot assay. Therefore, our findings demonstrate that JAK inhibitor AG490 inhibits growth and invasion of GBC cells via blockade of JAK2/STAT3 signaling and provides the potential therapeutic strategy for the treatment of GBC patients.

Gallbladder cancer (GBC) is one of the most common and aggressive malignancies of the biliary tract (1). The past decades have witnessed the rapid development of comprehensive treatment measures, including chemotherapies and radiotherapy for GBC, but surgical resection remains the main treatment strategy for treatment of GBC patients (2, 3). Unfortunately, most GBC patients diagnosed are found at an advanced stage and have a poor survival outcome due to the metastatic tumor sites, which express pro-angiogenic and adhesion molecules required for tumor growth and invasion (4). Molecular alterations in relevant intracellular signaling pathways are involved in promoting the

development of GBC, and understanding these molecular alterations may provide great promise for effective treatment of GBC (5).

Activation of JAK/STAT3 signaling is implicated in promoting cancer hallmarks in tumor environment including proliferation, invasion and angiogenesis (6). JAK or STAT3 is constitutively activated by interleukins and other growth factors in Hodgkin cells (7), gastrointestinal stromal tumors (8), prostate cancer (9) and lung adenocarcinoma (10), contributing to cell proliferation and apoptosis inhibition in gastric cancer (11), Hodgkin lymphoma (12), diffuse large B-cell lymphoma (13) and myeloproliferative neoplasms (14), and causing cell

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survival, invasion and metastasis in lymphoma (15), osteosarcoma (16) and breast cancer (17). Therefore, targeting JAK/STAT signaling pathway may be a potential therapeutic strategy for cancer therapy.

In terms of the important role of JAK/STAT signaling in cancer progression, pharmacological inhibition of this signaling suppresses cell proliferation, migration and invasion (18-20), induces cell apoptosis (20) and reverses chemotherapy resistance (21, 22) in some cancers. Thus, many chemical drugs, small molecular inhibitors or herbal extracts are developed to exert antitumor activity via targeted inhibition of JAK/STAT signaling, such as resveratrol (23), ginsenoside Rg1 (24), evodiamine (25) and embelin (26), of which JAK2 tyrosine kinase inhibitor AG490 inhibits inflammation, proliferation and migration, and induces cell apoptosis in cancer cells (27-30), indicating that AG490 may represent a novel therapeutic strategy for cancer therapy.

Constitutive activation of JAK/STAT signaling also occurs in v-Src-transformed human GBC cells (31), however, the antitumor activity of JAK inhibitor AG490 on human GBC has not been elucidated. In our study, we found that JAK inhibitor AG490 inhibited growth and invasion of GBC cells and induced cell apoptosis and cycle arrest via blockade of JAK2/STAT3 signaling and may provide a potential therapeutic strategy for the treatment of GBC patients.

MATERIALS AND METHODS

Materials

The GBC cell lines (GBC-SD and SGC-996) were purchased from Cell Resource Center, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The antibodies of JAK2, p-JAK2, STAT3 and p-STAT3 were from Cell Signaling Technology (USA) and other antibodies such as VEGFC, VEGFD, p53 and cyclinD1 were from Santa Cruz Biotechnology (USA).

Drugs and reagents

AG490 and MTT used in this study were from Sigma (Louis, MO, USA). Dulbecco's Modified Eagle medium

(DMEM) and fetal bovine serum (FBS) were from Thermo Fisher Scientific Inc (Waltham, MA, USA); Cell apoptosis and cycle kits were from BD Biosciences (San Diego, CA, USA).

Cell culture and drug treatment

The GBC cell lines (GBC-SD and SGC-996) were cultured in DMEM medium supplemented with 10% heat-inactivated FBS, 100U/ml of penicillin and 100µg/ml of streptomycin. They were all placed in a humidified atmosphere containing 5% CO₂ at 37°C. After cell culture for 24 h, they were exposed to different concentrations of AG490 (0, 50 µM and 100 µM) and AG490 (0 µM) or PBS was regarded as the control group.

Western blot assay

The GBC cell lines (GBC-SD and SGC-996) were harvested and extracted. Separated protein bands were transferred into polyvinylidene fluoride (PVDF) membranes and the membranes were blocked in 5% skim milk powder. The primary antibodies against JAK2, p-JAK2, STAT3, p-STAT3, VEGFC, VEGFD, p53 and cyclinD1 were diluted according to the instructions for antibodies and incubated overnight at 4°C. Then, horseradish peroxidase-linked secondary antibodies were added at a dilution ratio of 1:1000, and incubated at room temperature for 2 h. The membranes were washed with PBS and the immunoreactive bands were visualized using ECL-PLUS/Kit according to the kit's instructions.

Cell viability assay

Cell viability was analyzed with the MTT assay. Briefly, cells exposed to AG490 were incubated in 96-well-plates with DME medium supplemented with 10% FBS. Cells were treated with 15 µl MTT dye on each day and then incubated with 150 µl of DMSO for 15 min. The color reaction was measured at 570 nm with enzyme immunoassay analyzer (Bio-Rad, American).

Transwell invasion assay

Transwell filters were coated with Matrigel on the upper surface of a polycarbonic membrane. After incubating at 37°C for 30 min, the Matrigel solidified and served as the extracellular matrix for analysis of tumor cell invasion. The number of cells invading through the Matrigel was counted in three randomly selected visual

fields from the central and peripheral portion of the filter using an inverted microscope (200×magnification). Each assay was repeated three times.

Cell apoptosis analysis

To detect cell apoptosis, GBC cell lines (GBC-SD and SGC-996) were trypsinized and suspended in binding buffer according to the manufacturer's. FITC-AnnexinV and PI were added to the fixed cells for 15 min in darkness at room temperature. Then, Annexin V binding buffer was added to the mixture before the fluorescence was measured on FACSsort flow cytometer.

Cell cycle analysis

The fixed cells were stained with PI in the presence of RNase A for 30 min at room temperature in the dark. Each sample was filtered through a 50- μ m nylon filter to obtain single-cell suspension. The samples were then analyzed on FACSsort flow cytometer (Becton Dickinson, Mountain View, CA, USA).

Statistical analysis

SPSS 20.0 was used for the statistical analysis. One-way analysis of variance (ANOVA) was used to analyze the differences between groups. The LSD method of multiple comparisons was used when the probability for ANOVA was statistically significant. Statistical significance was $P < 0.05$.

RESULTS

The effects of AG490 administration on JAK2/STAT3 pathway

To define whether JAK2 inhibitor AG490 exerts an effect on JAK2/STAT3 signaling pathway, we treated GBC-SD and SGC-996 cells with different concentrations of AG490, and found that AG490 administration downregulated the protein expression of p-JAK2 and p-STAT3 ($P < 0.01$), but had no effect on JAK2 and STAT3 expression in GBC cells (Fig. 1).

The effects of AG490 administration on cell proliferation

To evaluate the effects of AG490 administration on cell growth in GBC-SD and SGC-996 cells, we examined cell proliferation activity by MTT assay. It was found that AG490 administration significantly reduced cell proliferation viability in a time-dependent manner ($P < 0.01$, Fig. 2A). Additionally, whether AG490 administration inhibits the endogenous expression of VEGFC from translational level is unclear. The protein expression of VEGFC was examined by Western blot assay, indicating a marked decrease of VEGFC by AG490 administration in GBC cells ($P < 0.01$, Fig. 2B).

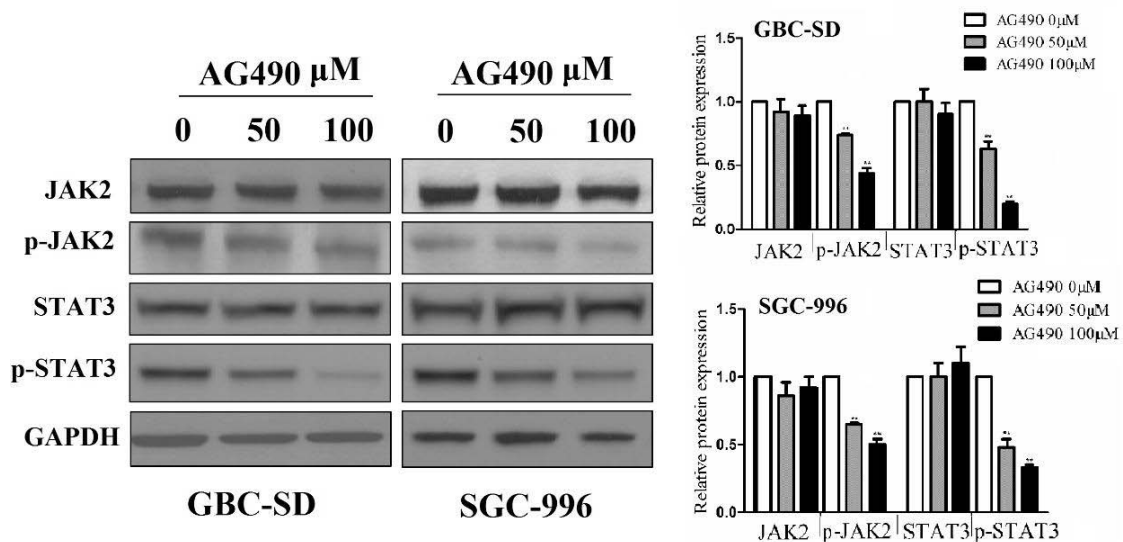


Fig. 1. The effects of AG490 on the expression of JAK2/STAT3 signaling in GBC cells. Western blot assay was used to quantitatively detect the expression of JAK2, p-JAK2, STAT3 and p-STAT3 in GBC-SD and SGC-996 cells ** $P < 0.01$.

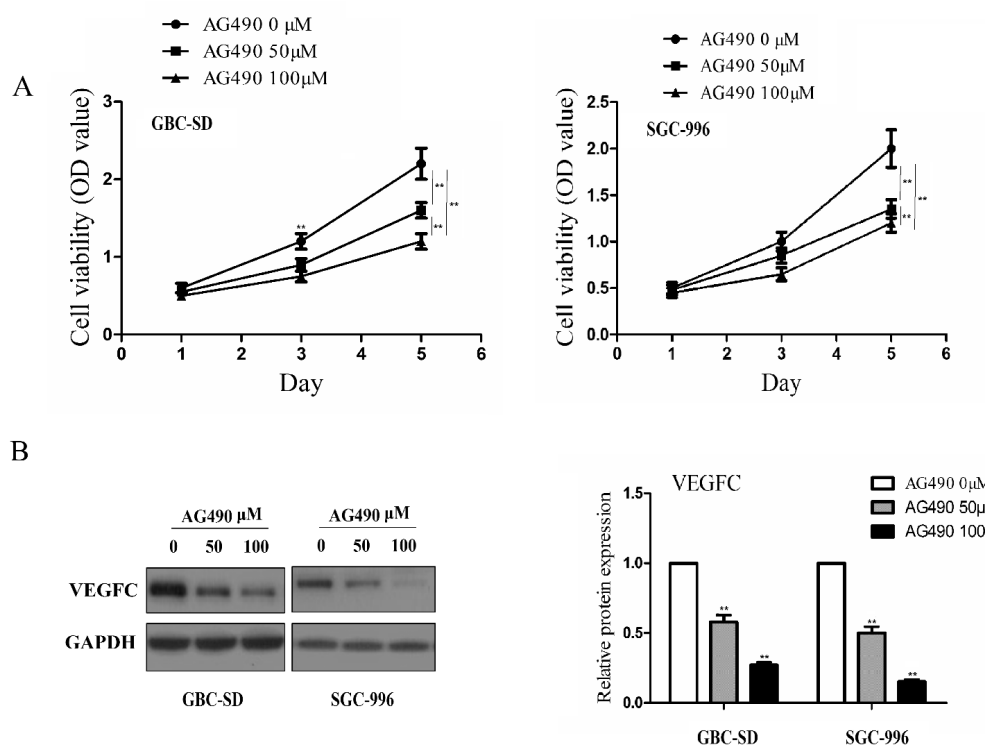


Fig. 2. The effects of AG490 administration on cell proliferation in GBC cells. A) MTT was used to examine the cell proliferation viability in GBC cells. B) Western blot was used to quantitatively evaluate the expression of VEGFC in GBC-SD and SGC-996 cells $**P < 0.01$.

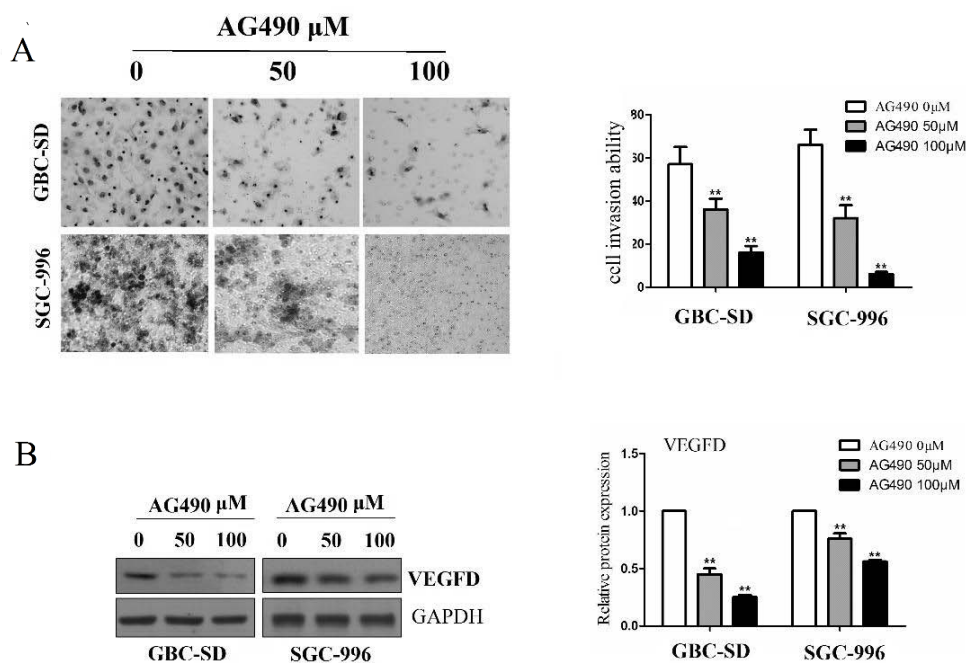


Fig. 3. The effects of AG490 administration on cell invasive potential in GBC cells. A) Transwell was used to assess the cell invasive potential in GBC cells. B) Western blot was used to quantitatively detect the expression of VEGFD in GBC-SD and SGC-996 cells $**P < 0.01$.

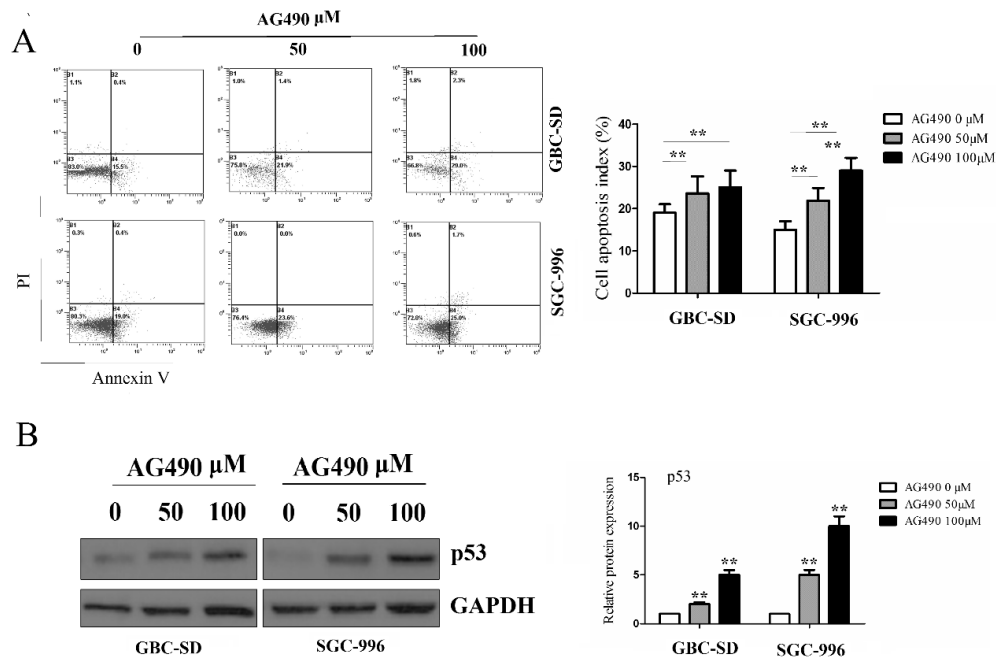


Fig. 4. The effects of AG490 administration on cell apoptosis in GBC cells. A) Flow cytometry was used to estimate the cell apoptosis in GBC cells. B) Western blot was used to quantitatively detect the expression of p53 in GBC-SD and SGC-996 cells $**P < 0.01$.

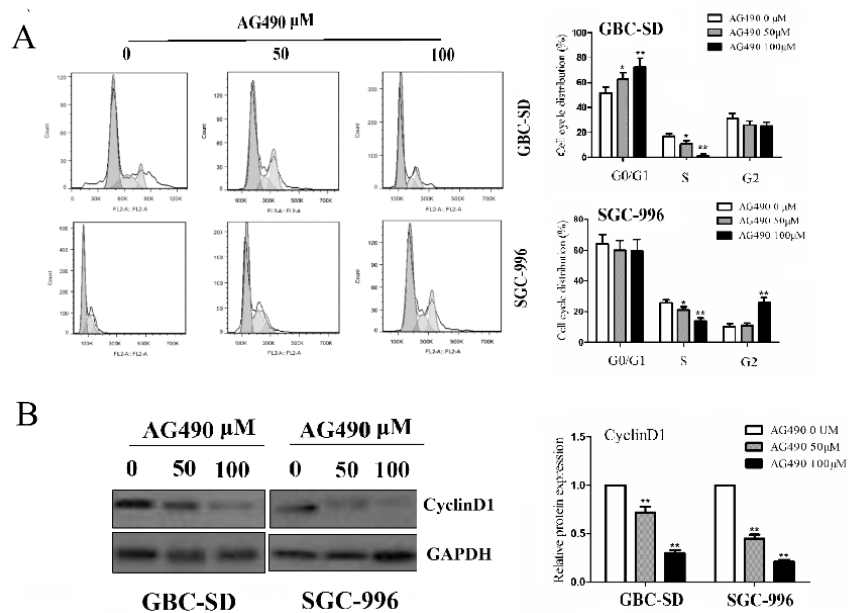


Fig. 5. The effects of AG490 administration on cell cycle distribution in GBC cells. A) Flow cytometry was used to assess the cell cycle distribution in GBC cells. B) Western blot was used to quantitatively detect the expression of cyclinD1 in GBC-SD and SGC-996 cells. $*P < 0.05$, $**P < 0.01$.

The effects of AG490 administration on cell invasion

To verify whether AG490 administration affects cell invasive potential in GBC-SD and SGC-996 cells, Transwell invasion assay was performed. After GBC cells were exposed to AG490, cell invasive potential was significantly lowered by AG490 administration ($P < 0.01$, Fig. 3A). To determine whether AG490 administration impacts VEGFD expression from translational level, the protein expression of VEGFD was detected by Western blot assay, which indicated a significant decrease of VEGFD expression caused by AG490 administration ($P < 0.01$, Fig. 3B).

The effects of AG490 administration on cell apoptosis

To confirm whether AG490 administration has an effect on cell apoptosis, flow cytometric analysis was performed. After GBC-SD and SGC-996 cells were exposed to AG490, cell apoptotic index was significantly increased by AG490 ($P < 0.01$, Fig. 4A). To determine whether AG490 administration influences p53 expression from translational level, the protein expression of p53 was detected by Western blot assay, which indicated a significant increase of p53 expression induced by AG490 administration ($P < 0.01$, Fig. 4B).

The effects of AG490 administration on cell cycle distribution

To observe the effects of AG490 administration on cell cycle distribution of GBC-SD and SGC-996 cells, flow cytometric analysis was performed. The results showed that after AG490 treatment for 24 h, cell distribution decreased in S phase, but increased in G0/G1 phase or G2 phase ($P < 0.05$, Fig. 5A). To investigate the effects of AG490 administration on cyclinD1 expression from translational level, the protein expression of cyclinD1 was measured by Western blot, indicating a marked decrease of cyclinD1 caused by AG490 administration ($P < 0.01$, Fig. 5B).

DISCUSSION

Accumulating evidence shows that JAK/STAT signaling plays a key role in cancer tumorigenicity and progression (11-17) and targeted inhibition of JAK by AG490 has antitumor activity in a variety

of cancers (27-30). Moreover, activation of JAK/STAT signaling has been reported in human GBC cells. However, whether JAK inhibitor AG490 has inhibitory effects on human GBC was not known. In the present study, we pretreated GBC GBC-SD and SGC-996 cells with different concentrations of JAK inhibitor AG490, and found that AG490 administration reduced GBC cell proliferation and invasive potential and induced cell apoptosis and cycle arrest. Our findings corroborate the previously reported studies on cancer cells (27-30) and suggest AG490 as the JAK inhibitor displays antitumor effects on GBC cells.

Signal transducer and activator of transcription 3 (STAT3), a member of JAK/STAT signaling pathway, is involved in promoting invasion and metastasis in human tumors. Inhibition of STAT3 signaling by AG490 weakens cell invasion via downregulation of VEGF and MMP-2 expression in pancreatic cancer (32), reduces cell proliferation (33), and enhances doxorubicin sensitivity in breast cancer (34). Intriguingly, we also found that AG490 as the JAK inhibitor downregulated the expression of p-STAT3, indicating that AG490 might inhibit the development of GBC cells through inhibition of JAK/STAT signaling.

VEGFC and VEGFD act as the VEGF family ligands for receptor tyrosine kinases and participate in the angiogenesis and lymphatic metastasis in cancer progression (35). P53 and cyclinD1 expression are regarded as the detected biomarkers used for investigating cell apoptosis and cycle distribution (36). Moreover, JAK-STAT3-VEGF-C signaling promotes growth, invasion and lymphangiogenesis, and AG490 decreases the expression of JAK-STAT3-VEGF-C signaling in gastric cancer (37). In our study, we also found that AG490 downregulated the expression of VEGF-C/D and CyclinD1 and upregulated p53 expression in GBC cells, suggesting that AG490 may inhibit JAK/STAT signaling-mediated GBC tumorigenicity through modulation of VEGF-C/D, CyclinD1 and p53 expression.

In conclusion, our findings show that JAK inhibitor AG490 inhibits growth and invasion of GBC cells and induces cell apoptosis and cycle arrest via blockade of JAK2/STAT3 signaling and provides

a potential therapeutic strategy for the treatment of GBC patients.

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CYTOKINE MODULATION IN PATIENTS WITH IDIOPATHIC PULMONARY FIBROSIS UNDERGOING TREATMENT WITH STEROIDS, IMMUNOSUPPRESSANTS, AND IFN- γ 1b

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Idiopathic pulmonary fibrosis (IPF) is a chronic lung disease of unknown etiology and pathogenic mechanisms. From an etiopathogenic point of view, alveolar macrophages play a key role in accumulation of fibroblasts and deposition of collagen and extracellular matrix by releasing specific cytokines and inflammatory mediators. IPF seems to be also associated with circulating fibrocytes, which might be involved with an abnormal pulmonary vascular repair and remodeling. Based on its hypothesized pathologic mechanisms, anti-inflammatory, anti-fibrotic and immunosuppressive therapies are often used. For these reasons, Interferon- γ (IFN- γ) has been used to exploit its activity on macrophages and fibroblasts. The aim of this study was to investigate the response to corticosteroids and/or IFN- γ 1b treatments based on pulmonary function tests and on inflammatory cytokine patterns of expression on bronchoalveolar lavage (BAL), at baseline and during and after the therapies. Unlike previous studies, we analyzed a period of therapy longer than 1 year. Our results demonstrated the effectiveness of IFN- γ in a group of IPF patients in whom the treatment was prolonged for over a year. These data suggest a positive role of IFN- γ treatment in patients in the initial stage of the disease.

Idiopathic pulmonary fibrosis (IPF) is a chronic lung disease of unknown etiology and pathogenic mechanisms (1), and is characterized by parenchymal fibrosis, which leads to progressive dyspnea, worsening pulmonary functions and respiratory failure (2). The median survival of patients with IPF is between 3 and 5 years (3). As shown by several studies (4), alveolar macrophages play a key role in accumulation of fibroblasts and deposition of collagen and extracellular matrix by releasing specific cytokines and inflammatory mediators. In particular,

macrophages are classified as type 1, which are involved in Th1 response and release inflammatory cytokines (IL-1 β , TNF- α , IL-6), and type 2, which are associated with Th2 immune response and are also believed to have a pivotal role in the control of cell growth and collagen deposition (5). IPF disease shows epithelial injury at the initial stage with release of inflammatory cytokines (6). Thus, a Th2 response seems to be most active in IPF patients and may be responsible for the strong, progressive and uncontrolled fibro-proliferative response (7). IPF seems also to be

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associated with circulating fibrocytes, which might be involved with an abnormal pulmonary vascular repair and remodeling (8, 9). Based on its hypothesized pathologic mechanisms, anti-inflammatory, anti-fibrotic and immunosuppressive therapies are often used (10). Specifically, treatment of the patients with corticosteroids and immunosuppressive drugs has proved to be ineffective in improving survival and quality of life (11). For several years, Interferon- γ (IFN- γ) has been used to exploit its activity on macrophages and fibroblasts: in lung injury. IFN- γ may shift the Th2 to Th1 immune response and thus it can down-regulate fibrotic and angiogenetic markers (12). Moreover, *in vitro* and murine models have shown IFN- γ dose-dependent inhibition of fibroblast proliferation, collagen-matrix synthesis and deposition (13). In fact, several reports on the pathogenesis of pulmonary fibrosis have investigated a bleomycin (BLM)-induced lung fibrosis model in mice, rats, hamsters, dogs, and other species (14). This model presents some limitations: especially, intra-tracheal instillation of BLM leads to pulmonary fibrosis, which is reversible with steroids, whereas the progression of human IPF is only partly influenced by steroids and immunosuppressive treatments. Despite this fact, in this model of lung fibrosis, an IFN- γ dependent down-regulation of the transcription of transforming growth factor- (TGF- β) was demonstrated (15). In addition, clinical studies reported that IFN- γ 1b treatment of IPF was effective in patients resistant to three months of high doses of glucocorticoids (16). Conversely, a large clinical trial in USA failed to demonstrate an IFN- γ 1b effectiveness on progression-free survival, pulmonary function and quality of life in patients treated for 1 year (17). The aim of this study was to investigate the response to corticosteroids and/or IFN- γ 1b treatments based on pulmonary function tests and on inflammatory cytokine patterns of expression on bronchoalveolar lavage (BAL), at baseline and during and after the therapies. Unlike previous studies (10), we analyzed a period of therapy longer than 1 year.

MATERIALS AND METHODS

Study subjects

The Medical Research Ethics Committee of the

SS Annunziata University Hospital of Chieti, Italy approved the study and the patients gave their written informed consent. Idiopathic pulmonary fibrosis was diagnosed according to previously described clinical, radiologic and histologic criteria (18). Eligible patients had shown clinical symptoms of IPF for ≥ 3 months, and had a forced vital capacity (FVC) of $\geq 55\%$ and $\leq 90\%$ of the predicted value, a transfer factor of the lung for carbon monoxide (TLCO) of $\geq 35\%$ and an arterial oxygen tension (PO₂) of > 7.3 kPa while breathing room air at rest. Exclusion criteria were active infection within one week before enrollment, unstable cardiovascular or neurological disease, uncontrolled diabetes, pregnancy, lactation, or any active malignancy, exposure to any fibrogenic agents (e.g., birds, asbestos, and iatrogenic drugs), as an alternative cause of interstitial lung disease.

Study design

In this study 11 newly diagnosed patients with IPF (9 males and 2 females), mean age 66 (SD ± 6.6) years, were enrolled (stage T00). The study was designed in consecutive steps (Fig. 1). All eligible patients received 600 mg X 3/die of N-acetyl cysteine for 30 days (stage T0). Subsequently, patients were randomly assigned (1:1) to receive 0.8 mg/Kg/die oral methylprednisolone for 1 month, after reducing the dosage to a maintenance dose of 12-16 mg/die and azathioprine 1-3 mg/Kg/die, increasing the dose every 2-4 weeks up to a maximum dose of 250 mg/die with or without 600 mg X 3/die of N-acetyl cysteine for a further 90 days (stages T1A and T1B). IPF patients with a worsening in lung function, documented by a decrease of TLC or CV $\geq 10\%$, of DLCO $\geq 15\%$, a reduction of SaO₂ during 6' WT $\geq 4\%$ or evidence of worsening disease shown by a chest radiograph, or of worsening dyspnea at rest or on exertion despite treatment with glucocorticoids, azathioprine w/out NAC, were eligible for IFN- γ 1b treatment. In fact, if the glucocorticoid treatment was ineffective, patients received 200 μ g subcutaneously three times/week of IFN- γ 1b for a total period of 360 days divided into four consecutive steps (T2, T3, T4 and T5). No other treatments for IPF were allowed during the study.

H₂O₂ determination

The subjects breathed through a mouthpiece at a normal frequency rate for about 10 min and the expired air through a 50-cm double-jacketed glass tube cooled to a temperature of 0°C. Exhaled air condensate, at least 1 ml, was collected on ice. The concentration of H₂O₂ was determined by means a fluorimetric assay based on the reaction of H₂O₂ with horseradish peroxidase (Boehringer, Mannheim, Germany) to form a compound, which oxidizes p-hydroxyphenilacetic acid to a fluorescent product (19). In order to assess the fluorescent product of the condensate and of standard solutions of H₂O₂, a spectrofluorimetric analysis (SPEX Fluorolog-2, Edison, NJ, USA; excitation wavelength 295 nm, emission wavelength 405 nm) was used. Concentrations of H₂O₂ were obtained by linear interpolation of a standard curve.

Pulmonary function tests

A spirometer computerized system (Vmax, Sensor Medics) was used for evaluating the following parameters: diffusing capacity for carbon monoxide (DLCO), DLCO%, DLCO/VA, DLCO/VA%, total lung capacity (TLC), TLC%, vital capacity (CV), CV%, 6 minutes walking test (6' WT), 6' WT sat%, 6' WT dyspnea. Moreover, in order to determine PO₂, PCO₂ and O₂ saturation (%) an arterial blood gas determination was performed at rest using an arterial blood gas analyzer (Radiometer Medical, Copenhagen, Denmark).

Bronchoalveolar lavage and cytokine determination

Bronchoalveolar lavage (BAL) was performed according to the technical recommendations and guidelines for the standardization of BAL procedures (20). Briefly, BAL was performed with a flexible bronchoscope (Exera II, Olympus) wedged into

Study design

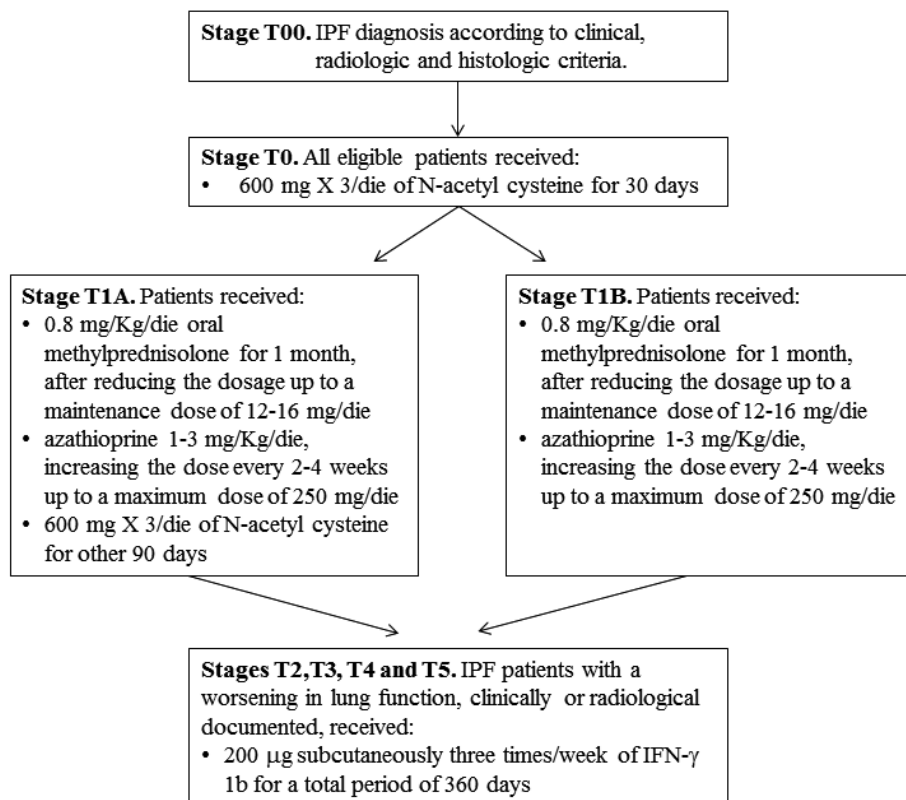


Fig. 1. Study design.

segmental or sub-segmental middle lobe bronchus. Four 50 ml aliquots of sterile 0.9% saline solution were instilled and then aspirated. In order to analyze BALF specimens, a FACSCalibur cytometer (Becton-Dickinson, CA) was used. Specifically, the first two doses were processed for differential cell counts, with the use of May-Grunwald and Giemsa-Romanowsky stains. The third dose of BALF underwent cytometric investigation. Mononuclear cells were stained with a commercially available conjugated and unconjugated mAbs that belonged to the Becton Dickinson series. They included: CD3, CD4, CD8, CD19, and CD45. The fourth dose of BALF was immediately centrifuged at 250x g for 5 min at room temperature to remove most of the cells. The supernatant was centrifuged again at 500xg for 10 min at room temperature to produce completely cell-free fluid. Then the supernatants were frozen at 80°C and they were later processed together for cytokine determination. All antibodies and cytokines were from PeproTech (Rocky Hill, NJ, USA). The concentration of cytokines released in the supernatants was determined using enzyme immunoassays. A blocking buffer containing PBS (pH 7.4) supplemented with 0.05% Tween 20 and 5% dry milk was added to the wells and incubated at 37°C for 60°C. After blocking, primary antibodies were added to the wells and the plate was incubated at 37°C for 2.5 h. A series of polyclonal rabbit anti-mouse antibodies were used. All antibodies and cytokines were from PeproTech (Rocky Hill, NJ, USA). Then, secondary antibodies (all anti-rabbit goat IgG conjugated with biotin; Stress Gen, Canada) were added and the plate was incubated at 37°C for 1 h, whereupon a solution containing horseradish peroxidase-conjugated biotin (IMTEK, Russia) was added. A panel of 10 different cytokine antibodies (IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IFN- γ and TNF- α) was used and the different levels of induction were determined.

RNAse protection assay

For Ribonuclease Protection Assay, a multistep template construct corresponding to a set of inflammatory and anti-inflammatory cytokine coding sequences was used and treated *in vitro* with T7 RNA

polymerase in 10 μ l transcription buffer, containing ribonucleotides (NTPs), Rnasin, dithiothreitol as a reducing agent and a 5 mCi of [α -³²P]UTP as a tracer. The transcription reaction was carried at 37°C for 1 h. Then, it was stopped by adding 26 μ l of 20 mM EDTA; then 2 μ l of 10 μ g/ μ l yeast tRNA were added to each sample, phenol/chlorophorm extracted and subsequently precipitated using 2 vol of cold 100% Ethanol in dry ice for 10 min. The radiolabelled transcribed RNA was centrifuged, washed with 70% ethanol, dried in a speed vac and resuspended in a hybridization buffer. Different aliquots were then incubated overnight at 37°C with 10 μ g of cold RNA corresponding to PBLs of target patients. On the next day, the samples were treated with RNase A in order to remove the non-hybridizing portions of the RNA, and the protected radiolabeled hybrids were again treated with phenol/chlorophorm, ethanol precipitated and resuspended in 1X loading buffer for an acrylamide run electrophoresis. Dry gels were the exposed for autoradiography and bands analysed using densitometry for determining quantitative expression of cytokine RNAs.

Statistical analysis

Data are expressed as means $\pm$ SEM. Student *t*-test or ANOVA followed by the appropriate post-hoc test was used (Stat View 4.0 software, Abacus Concepts, Berkeley, CA, USA). P values < 0.05 were considered statistically significant, with a confidence interval of 95%. In addition, in order to evaluate the trend of pulmonary function tests and cytokine release a correlation study was performed.

RESULTS

Clinical features and BAL

Baseline clinical characteristics of the study population at entry are shown in Table I. Before IPF treatment, a bronchoalveolar lavage for each patient was performed. As depicted in Table II, BAL at baseline highlighted an increase in the percentage of neutrophils (2.4 $\pm$ 0.5) and eosinophils (4 $\pm$ 3.24) and normal percentage values of alveolar macrophages (88.8 $\pm$ 3.4) and lymphocytes (4.8 $\pm$ 0.4), as commonly observed in IPF patients. BALs performed in the

later stages did not show any significant differences (data not shown).

Oxidative stress at baseline and during IPF treatment

H₂O₂ determinations, measured to evaluate oxidative stress, showed a constant increase during IPF treatment, probably correlated with the stage of the disease. The only reduction of oxidative stress was documented in patients treated with corticosteroids, azathioprine and NAC (Anova $p < 0.05$; Bonferroni test vs T3 C and T4 C, $p < 0.05$). In addition, the increase observed respectively in T3 C and T4 C groups was significantly higher compared to oxidative stress measured at baseline (T00; $p < 0.05$) and to T2 C phase ($p < 0.05$). This data pointed out that treatment with corticosteroids, azathioprine and NAC was effective in reducing oxidative stress,

instead therapy with NAC alone or IFN- γ was unable to down-regulate H₂O₂ production (Fig. 2).

Clinical parameters modified during IPF treatment

Patients with IPF treated with corticosteroids and azathioprine w/out NAC or a short-term therapy of IFN- γ showed a decrease in 6' WT and a slight increase of 6' WT dyspnea, in respect to the baseline. Surprisingly, after a prolonged treatment with IFN- γ (T4 C), we observed an improvement of 6' WT in respect to a short therapy with the same drug (T2 C) and to the treatment with NAC w/out corticosteroids and azathioprine (T1 A and T1 B) (Anova $p < 0.05$; Bonferroni test T4 C vs T2 C, T1 A and T1 B, $p < 0.05$). Furthermore, the evaluation of 6' WT dyspnea showed lower levels in patients at baseline (T00) and in T4 C phase compared to patients treated with corticosteroids and azathioprine w/out NAC (Anova $p < 0.05$; Bonferroni test, T00 and T4 C vs T1 A and T1 B, $p < 0.05$) (Fig. 3).

In addition, as shown in Fig. 4, we demonstrated an improvement in the following parameters during IFN- γ treatment: H₂O₂ ($R^2 = 0.9$), DLCO ($R^2 = 0.95$), TLC ($R^2 = 0.93$), TLC % ($R^2 = 0.99$), CV ($R^2 = 0.95$), 6' WT and sat% ($R^2 = 0.89$), 6' WT dyspnea ($R^2 = 0.94$), PO₂ ($R^2 = 0.99$), sat% O₂ ($R^2 = 0.96$). These data pointed out that

Table I. Clinical features at baseline. Characteristics of study population at entry.

Baseline parameter	Mean $\pm$ SD
Age (years)	67 $\pm$ 6.6
Height (cm)	166.5 $\pm$ 12.5
Weight (Kg)	79.8 $\pm$ 16.9
BMI	27.9 $\pm$ 2.97
CV (L)	2.85 $\pm$ 0.9
CV %	87 $\pm$ 24.9
DLCO (ml/min/mmHg)	16.4 $\pm$ 4.99
DLCO %	69.4 $\pm$ 17.7
TLC (L)	4.23 $\pm$ 1.15
TLC %	74 $\pm$ 24.8
6' WT (m)	398.4 $\pm$ 144.9
PO ₂ (mmHg)	79.7 $\pm$ 10.6
PCO ₂ (mmHg)	38.9 $\pm$ 3.6
SaO ₂ %	96.1 $\pm$ 2.1

BMI: body mass index; CV: vital capacity; DLCO: carbon monoxide diffusing capacity; TLC: total lung capacity; 6' WT: 6 minutes walking test; PO₂: partial pressure of oxygen; PCO₂: partial pressure of carbon dioxide; SaO₂: oxygen saturation.

Table II. Bronchoalveolar lavage. Total and differential cell count.

Cell count	Mean $\pm$ SD
Cells/ml ($\times 10^4$)	6.9 $\pm$ 3.9
Alveolar Macrophages (%)	88.8 $\pm$ 3.4
Neutrophils (%)	2.4 $\pm$ 0.5
Lymphocytes (%)	4.8 $\pm$ 0.4
Eosinophils (%)	4 $\pm$ 3.2
CD4/CD8	2.5 $\pm$ 1.4

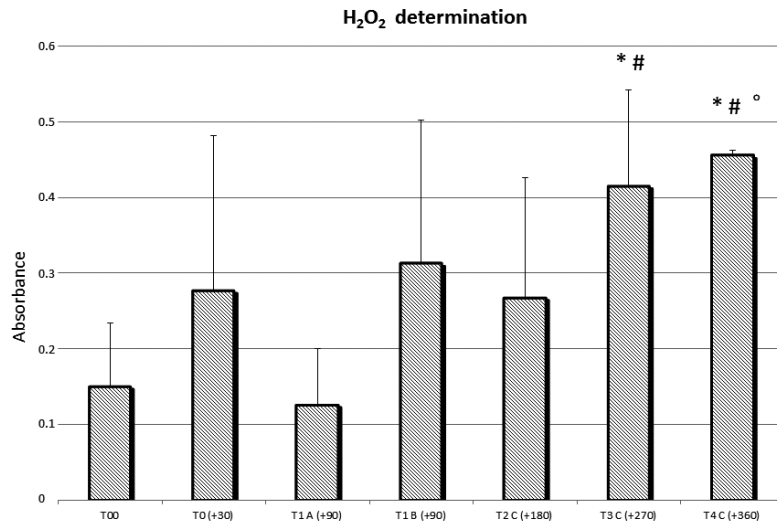


Fig. 2. Oxidative stress at baseline and during IPF treatment. H₂O₂ determination. T00: patients at baseline; T0: treatment with NAC; T1 A: corticosteroids, azathioprine and NAC; T1 B: corticosteroids and azathioprine; T2, T3, T4 C: IFN- γ . *: $p < 0.05$ vs T00; #: $p < 0.05$ vs T1 A (+90); °: $p < 0.05$ vs T2 C (+180).

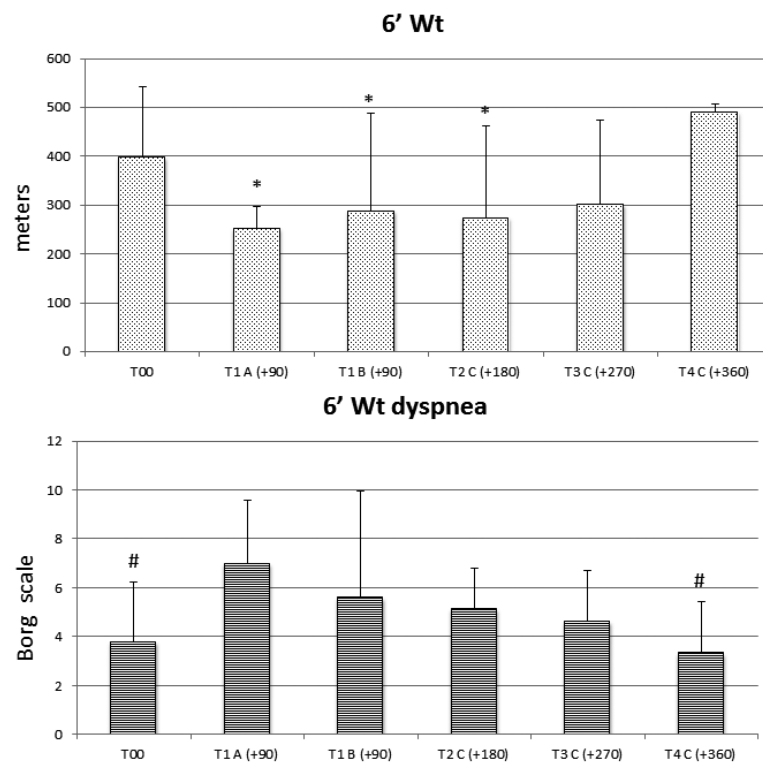


Fig. 3. Clinical parameters modified during IPF treatment. 6' WT: 6 min walking test; 6' WT dyspnea: 6 min walking test dyspnea.

*: $p < 0.05$ vs T4 C (+360); #: $p < 0.05$ vs T1 A and T1 B (+90).

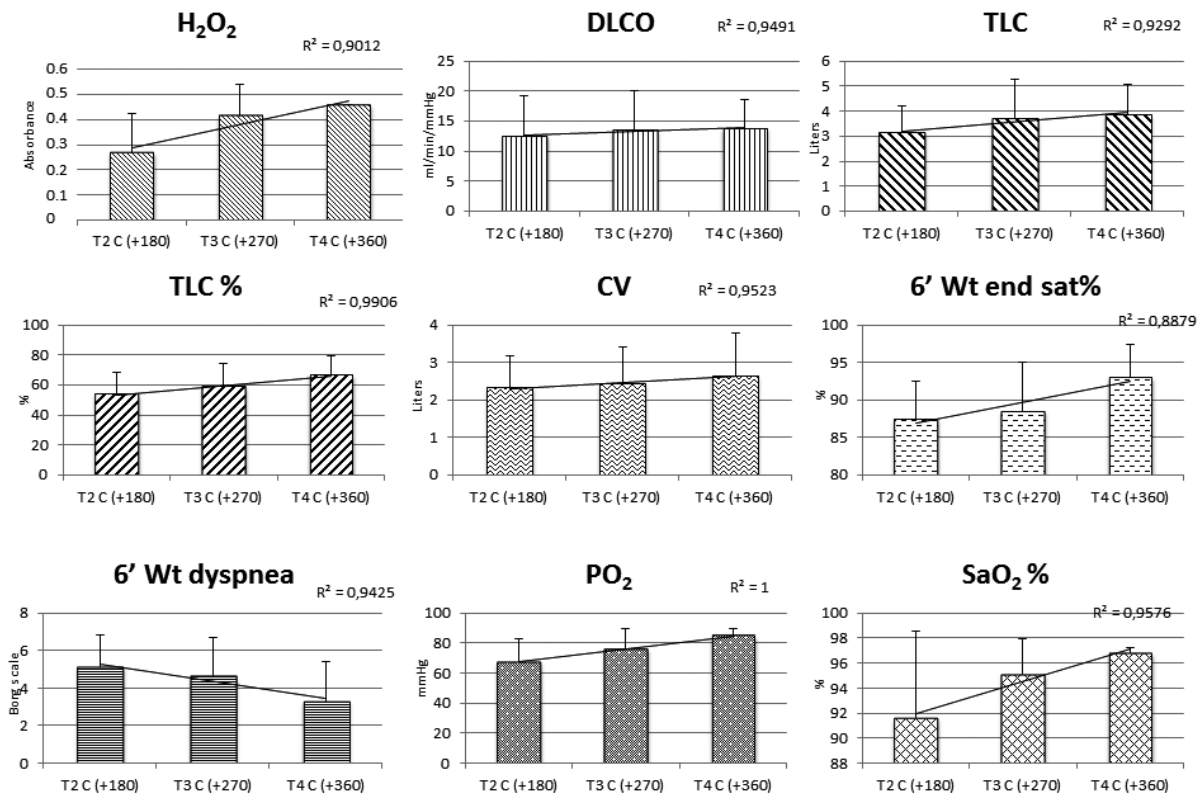


Fig. 4. Parameters modified during IFN- γ 1b treatment.

corticosteroids and azathioprine w/out NAC, unlike IFN- γ therapy, were ineffective to improve the clinical parameters and the quality of life of IPF patients.

Cytokine modulations during IPF treatment

The evaluation of cytokine modulations showed, as expected, a significant increase of IFN- γ during IFN- γ treatment, and this evidence was strongly correlated with a reduction of IL-12, IL-1, IL-6 and TNF- α (Fig. 5. Anova, $p < 0.05$; for IFN- γ Bonferroni test: T2 C [mean $\pm$ SD: 23.3 $\pm$ 16 pg/ml], T3 C [27.5 $\pm$ 10.1 pg/ml], T4 C [31.2 $\pm$ 10.8 pg/ml] and T5 C [30.7 $\pm$ 11.8 pg/ml] vs T0 [1.9 $\pm$ 1.3 pg/ml] and T1 [2.2 $\pm$ 1.6 pg/ml], $p < 0.05$; for IL-12 Bonferroni test: T2 C [53.6 $\pm$ 12 pg/ml], T3 C [40.5 $\pm$ 5.8 pg/ml], T4 C [34.9 $\pm$ 6.1 pg/ml] and T5 C [30.1 $\pm$ 2.2 pg/ml] vs T0 [81 $\pm$ 13.3 pg/ml] and T1 [73.5 $\pm$ 17.5 pg/ml], $p < 0.05$; T4 C and T5 C vs T2 C, $p < 0.05$; for IL-1 Bonferroni test: T3 C [210.4 $\pm$ 30.7 pg/ml], T4 C [182.8 $\pm$ 44.3 pg/ml] and T5 C [155.4 $\pm$ 50.8 pg/ml] vs T0 [311.5 $\pm$ 33.7 pg/ml] and T1 [286.2 $\pm$ 50.7 pg/ml], $p < 0.05$; T5

C vs T2 C [234.1 $\pm$ 30.5 pg/ml], $p < 0.05$; for IL-6 Bonferroni test: T4 C [11.6 $\pm$ 0.96 pg/ml] and T5 C [14 $\pm$ 2.6 pg/ml] vs T0 [25.9 $\pm$ 7.8 pg/ml], $p < 0.05$; T5 C vs T1 [24 $\pm$ 9 pg/ml], $p < 0.05$; for TNF- α Bonferroni test: T2 C [26.7 $\pm$ 9.7 pg/ml], T3 C [16.1 $\pm$ 7.2 pg/ml], T4 C [8.6 $\pm$ 7.6 pg/ml] and T5 C [3.3 $\pm$ 0.6 pg/ml] vs T0 [43.1 $\pm$ 12.8 pg/ml], $p < 0.05$; T3 C, T4 C and T5 C vs T1 [40.2 $\pm$ 19.3 pg/ml], $p < 0.05$; T4 C and T5 C vs T2 C, $p < 0.05$). Conversely IL-2, IL-4, IL-5, IL-8 and IL-12 did not show a significant modulation during IPF treatments. In addition, the modulation of these cytokines seems to take place in a time-dependent manner. In fact, the correlation between cytokine concentrations and time of treatment were the following: IFN- γ , $R^2 = 0.83$; IL-12, $R^2 = 0.95$; IL-1, $R^2 = 0.99$; IL-6, $R^2 = 0.89$; TNF- α , $R^2 = 0.98$ (Fig. 5). Conversely, patients treated with corticosteroids and azathioprine w/out NAC (T1 A and B were clustered as T1 group) did not show a striking cytokine modulation, but only a very slight decrease of IL-1, IL-6, IL-12 and

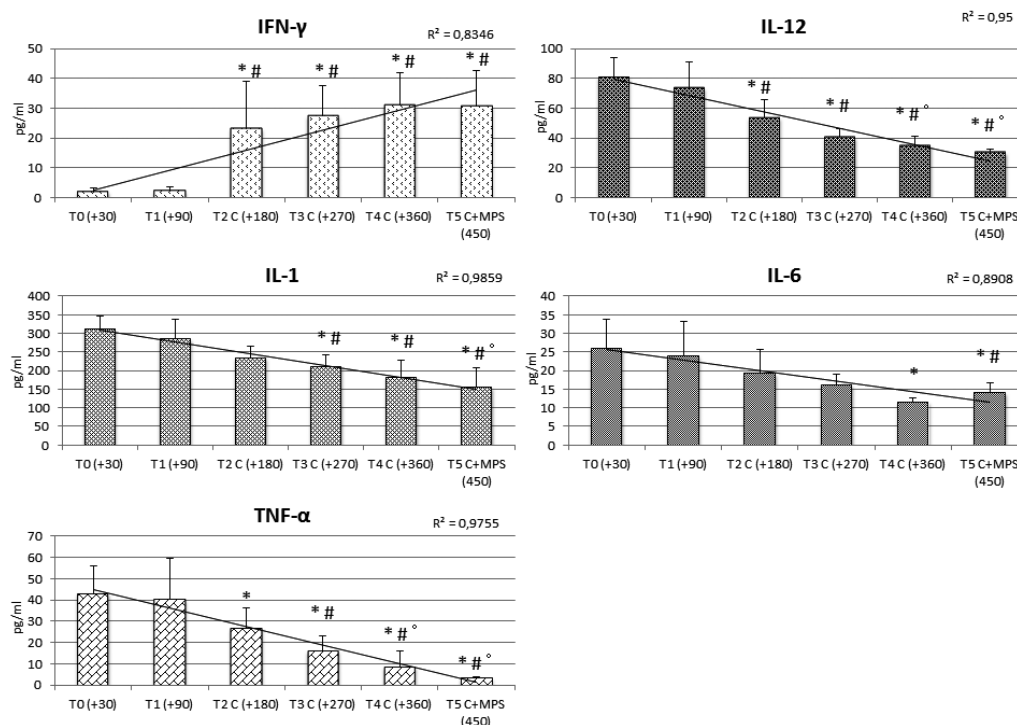


Fig. 5. Cytokine modulations during IPF treatment.

*: $p < 0.05$ vs T0; #: $p < 0.05$ vs T1 (+90); °: $p < 0.05$ vs T2 C (+180).

TNF- α when compared to the baseline. At last, IL-2, IL-4, IL-5, IL-8 and IL-12 did not show a significant modulation during IPF treatments (data not shown).

Rantes, MIP-1 α and MIP-1 β analysis on IPF treated patients

In order to further analyze chemokine ligands and proteins related to the first stage of macrophage inflammatory activation pathway, we performed an RNase protection assay where a set of coding sequences related to chemokine ligands and to the macrophage inflammatory proteins (MIP-1 α/β) were transcribed and hybridized *in vitro* to the RNase from PBL of target patients. As shown in Fig. 6, we determined a strong downregulation of Rantes/CCL5 protein in patients treated with IFN- γ 1b (T2) compared to untreated patients (T0) or patients treated with corticosteroids and azathioprine (T1) ($p < 0.05$). In addition, we showed that MIP-1 α and MIP-1 β were also dramatically decreased, particularly in regard to IFN- γ 1b-treated patients (Fig. 6).

DISCUSSION

IPF is an end-stage lung disease, clinically characterized by progressive dyspnea, worsening of pulmonary functions and respiratory failure (2). From a pathogenic point of view, IPF begins with an epithelial injury, which stimulates the release of inflammatory cytokines (6), followed by a later strong Th2 response that seems to lead to a failure of alveolar re-epithelialization, presence of fibroblasts/myofibroblasts, deposition of extracellular matrix and distortion of the lung architecture, which ultimately results in an uncontrolled fibro-proliferative response (7). Idiopathic pulmonary fibrosis seems also to be associated with circulating fibrocytes, which might cause an abnormal pulmonary vascular repair and remodeling (8, 9). For these reasons, corticosteroid and anti-inflammatory treatments were used in the first place, even though they have been demonstrated, by several studies, to be ineffective in order to improve survival and quality of life (11). Furthermore, several studies have indicated that biologic agents,

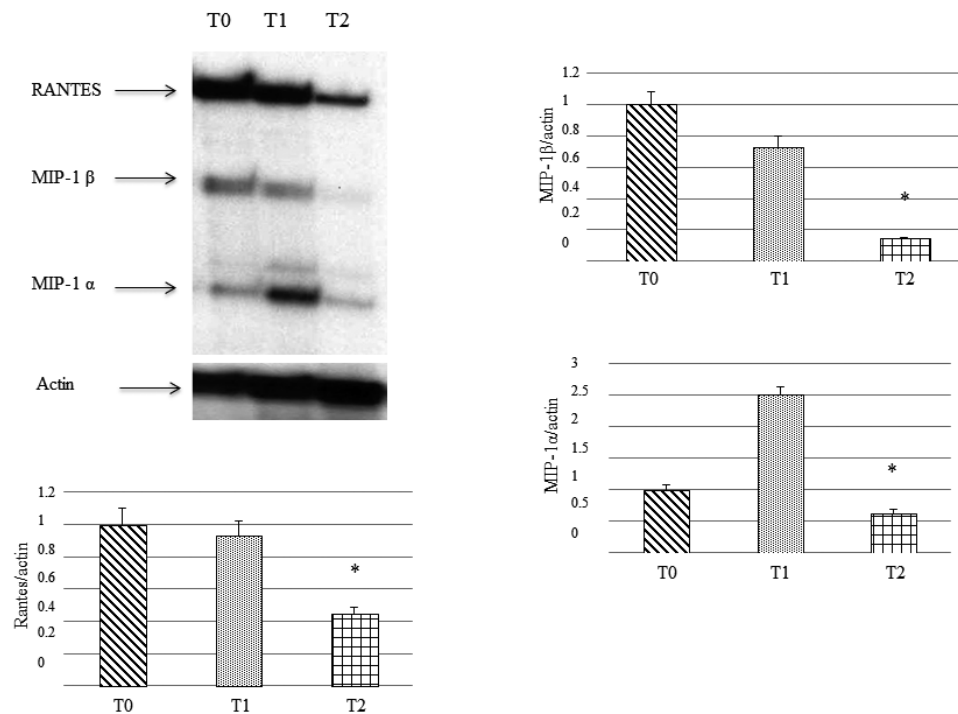


Fig. 6. Rantes, MIP-1α and MIP-1β modulation on IPF treated patients. Rantes, MIP-1α and MIP-1β protein analysis on untreated patients (T0) and on patients treated with corticosteroids and azathioprine (T1) or with IFN-γ 1b (T2). *: $p < 0.05$

including IFN-γ, able to modulate cytokine/chemokine interactions, could have some beneficial effect on patients with IPF. In fact, on the basis of studies on animal models, important advances have been made in identifying molecules that are able to block or induce lung fibrosis, including chemokines, cytokines, and their receptors. Specifically, an IFN-γ dependent down-regulation of transforming growth factor-β (TGF-β) transcription and, as a result, a dose-dependent inhibition of fibroblast proliferation, collagen-matrix synthesis and deposition has been demonstrated (13, 15). Clinical studies have led to conflicting findings: Ziesche et al. (16) reported that IFN-γ 1b was effective in patients with IPF resistant to three months of high doses of glucocorticoids. Instead, a Raghu et al. (17) large clinical trial in the USA failed to demonstrate an IFN-γ 1b effectiveness on progression-free survival, pulmonary function and quality of life in patients treated for 1 year. In this study, 11 newly diagnosed patients with IPF were enrolled (stage T00). All eligible patients received 600 mg X 3/die of N-acetyl cysteine for

30 days (stage T0). Subsequently, patients were randomly assigned (1:1) to receive 0.8 mg/Kg/die oral methylprednisolone for 1 month, after reducing the dosage to a maintenance dose of 12-16 mg/die and azathioprine 1-3 mg/Kg/die, increasing the dose every 2-4 weeks up to a maximum dose of 250 mg/die with or without 600 mg X 3/die of N-acetyl cysteine for other 90 days (stages T1A and T1B). IPF patients with a worsening in lung function, documented by (i) a decrease of TLC or CV $\geq 10\%$, of DLCO $\geq 15\%$, (ii) a reduction of SaO₂ during 6' WT $\geq 4\%$, or alternatively (iii) evidence of worsening disease on a chest radiograph, or (iv) of worsening dyspnea at rest or (v) on exertion despite treatment with glucocorticoids, azathioprine w/out NAC, were eligible for IFN-γ 1b treatment and they received 200 µg of IFN-γ 1b subcutaneously three times/week for a maximum total period of 360 days divided into four consecutive steps (T2, T3, T4 and T5). We demonstrated a consistent increase of oxidative stress during idiopathic pulmonary fibrosis, possibly correlated with the stage of the disease.

This is compatible with the pathogenic mechanisms characterized by an epithelial injury, which stimulates the release of inflammatory cytokines.

Furthermore, in the present study, an effective reduction of oxidative stress was documented only in patients treated with corticosteroids, azathioprine and NAC. Moreover, as previously described and in accordance with several previous studies, our results pointed out that corticosteroids and azathioprine w/out NAC are ineffective in improving biochemical endpoints. In addition, IFN- γ seems to improve these biochemical endpoints and the quality of life when patients are subjected to an extended therapy. In particular, our data demonstrated that patients treated with NAC w/out corticosteroids and azathioprine or a short-term therapy of IFN- γ showed a worsening of 6' WT and 6' WT dyspnea. Conversely, patients undergoing a prolonged treatment of IFN- γ have shown an improvement of 6' WT and 6' WT dyspnea in respect to a short therapy of the same drug and to the treatment with corticosteroids and azathioprine w/out NAC. These findings can be explained, at least partially, by an inflammatory cytokine modulation. In fact, patients treated with corticosteroids and azathioprine w/out NAC show a very slight decrease of IL-1, IL-6, IL-12 and TNF- α compared to the baseline. Instead, IFN- γ therapy evidenced, as expected, a significant increase of IFN- γ and a correlated strong reduction of IL-12, IL-1, IL-6 and TNF- α . Moreover, the modulation of these cytokines, in patients treated with IFN- γ , seems to be correlated in a time-dependent manner. All these results make it clear that corticosteroids and azathioprine w/out NAC, as opposed to IFN- γ , are ineffective in counterbalancing fibro-proliferative stimuli induced in idiopathic pulmonary fibrosis. As shown in Fig. 6, we also demonstrated that the Rantes/CCL5 protein, characterized as a chemotactic cytokine specific for recruitment of T cells, eosinophils and basophils, is downregulated at least at the transcriptional level in patients undergoing IFN- γ 1b treatment. Surprisingly, also the MIP-1 α and MIP-1 β proteins showed the same type of downregulation. Many studies (21) have determined a synergistic activity of Rantes and MIP proteins on the downregulation of T helper lymphocyte activity mostly related to HIV

infection. It is therefore plausible to hypothesize that IFN- γ may use such synergistic effects of Rantes/MIP transduction as a tool to attenuate T lymphocyte activity during the inflammatory-mediated biological response.

In conclusion, in this study we demonstrated the effectiveness of IFN- γ in a group of IPF patients in whom the treatment was prolonged for over a year. These data suggest a positive role of IFN- γ treatment in patients in the initial stage of the disease.

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LETTER TO THE EDITOR

SAFETY AND PHARMACODYNAMIC MECHANISM OF ROPIVACAINE LUMBAR ANESTHESIA IN CESAREAN SECTION

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Lumbar anesthesia is the preferred anesthetic approach for puerperae undergoing cesarean section in China. To observe the safety of administering different doses of ropivacaine for cesarean section and its pharmacodynamic mechanism, we randomly divided 180 pregnant women undergoing cesarean section into three groups: group A, 10 mg ropivacaine (0.50%); group B, 12 mg ropivacaine (0.50%); and group C, 14 mg ropivacaine (0.50%). Pharmacodynamic index, anesthesia quality and incidence of untoward reactions of each group were observed. Group A performed the poorest and group C the best in evaluation of sensory and motory block ($P<0.05$). With regard to evaluation of hemodynamic index, hemodynamic parameters of the three groups had significant differences after medication; mean arterial pressure (MAP) of patients in group B decreased at time points T_1 , T_2 and T_3 and heart rate (HR) became much higher at T_1 ($P<0.05$); MAP of the patients in group C decreased at T_1 , T_2 , T_3 and T_4 , but HR became higher at T_1 and T_2 ($P<0.05$); HR of group B was higher than that of group A at T_1 ($P<0.05$); MAP of the patients in group C had a significant decrease at T_1 , T_2 , T_3 and T_4 , but HR became higher at T_2 ($P<0.05$); MAP of patients in group C significantly decreased compared to group B at T_1 and T_2 , but HR became higher at T_2 ($P<0.05$). Fluctuation of oxyhemoglobin saturation (SpO_2) of all patients was between 95% and 99%. There was no occurrence of myocardial ischemia or arrhythmia. 1-min Apgar score of neonates of the three groups had no significant difference ($P>0.05$). The incidence of adverse reactions of the patients in group C was much higher than that of the patients in the other groups ($P<0.05$). Twelve mg ropivacaine (5%) is the most suitable dose for pregnant women undergoing cesarean section as it can achieve a sound anesthetic effect and high safety and, moreover, has little influence on respiratory and circulatory functions.

To the Editor,

Cesarean section, although lasting for a short time, requires a high dose of anesthesia during which the safety of the mother and child, as well as painless labor and good muscular relaxation, need to be ensured (1, 2). The most important point during anesthesia is to keep physiological indicators stable to avoid harming the fetus, therefore the selection of anesthetic drugs is important. With the constant

improvement of surgical methods, combined spinal epidural anesthesia as a novel anesthetic method has been extensively applied in cesarean section, featured by rapid onset, small anesthetic dose and definite effect (3).

Puerperea with reduced epidural cavity volume and cerebrospinal fluid induced by special pathophysiological changes usually have a higher anesthetic plane. Therefore, lumbar anesthesia has

Key words: cesarean section, ropivacaine, lumbar anesthesia, pharmacodynamic

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special requirements on the proportion, volume, dose, concentration and injection speed of local anesthetic drugs (4, 5).

Ropivacaine is a long-acting local anesthetic of amide derivatives. A clinical study (6) demonstrates that ropivacaine has low toxicity to central venous system and heart and blood vessels and can significantly block sensory and motor nerves. Additionally, it has also been found that ropivacaine, which has little effect on the blood flow of the uterus and placenta, is safe for mother and fetus (7). However, the anesthetic effect of different doses of ropivacaine in caesarean section is unclear and remains to be further studied. This study was designed to confirm the optimal dose of ropivacaine in lumbar anesthesia for pregnant women undergoing cesarean section by observing the pharmacodynamic performance and safety of ropivacaine at different doses.

MATERIALS AND METHODS

One hundred and eighty full-term and single-bearing primiparae who underwent cesarean section in the Binzhou Medical School Affiliated Hospital, Shandong, China, between February 2011 and December 2014 were selected as research subjects. The sample size of the study was determined using the formula $N = Z^2 \times (P \times (1 - P)) / E^2$ (N : sample size; Z : statistics; when confidence coefficient = 95%, $Z = 1.96$; when confidence coefficient = 90%, $Z = 1.64$; E = error value; P = probability value). The patients were aged 27.7 ± 7.6 years, weighed 74.5 ± 22.7 kg, had an average height of (160.2 ± 9.6) cm and had been pregnant for (39.9 ± 1.2) weeks. They were evaluated with cardiac function grade I or II using the American society of Anesthesiologists (ASA) physical status classification system. The subjects were randomly divided into three groups: group A, 10 mg ropivacaine (0.5%); group B, 12 mg ropivacaine (0.5%); and group C, 14 mg ropivacaine (0.5%). Age, height, weight and gestational weeks had no significant difference between the three groups ($P > 0.05$). The pregnant women included were those full-term and single-bearing primiparae who had cesarean section indication, were evaluated with cardiac function grade I or II using ASA

physical status classification system, had no allergic reaction to ropivacaine and signed informed consent. Those who had pregnancy complications including preeclampsia, placental abruption and placenta previa or contraindication to intraspinal anesthesia were excluded.

Anesthetic method

The puerperae fasted for 8 ~ 12 hours and did not drink for 4 hours before being admitted into the operation theatre (OT). Before admission to OT, they were injected with 0.5 mg atropine for 30 min. In the OT, oxygen supply, venous channel and infusion of compound sodium lactate were performed.

Each puerpera in all the three groups was injected with 3 mL of hyperbaric solution prepared by 0.5% ropivacaine (10 mg for group A, 12 mg for group B, 14 mg for group C) and 10% glucose solution under arachnoid in L_{2-3} space. After admission, blood pressure, electrocardiogram and oxyhemoglobin saturation were monitored; venous channel was established; 300 ~ 500 mL of Ringer's solution was injected in 0.5 h. Then the patients lay in a left lateral position and were punctured via L_{2-3} . When the cerebrospinal fluid was seen inside the needle, 3 mL of local anaesthesia solution was injected at a speed of 0.1 mL/s. After the lumbar anesthesia needle was retracted, an epidural tube was inserted for 2 ~ 3 cm. Then the puncture needle was removed. The patients were asked to take a horizontal position; the anesthetic plane was adjusted in 10 min. If the anesthetic plane was lower than T_{10} or the analgesic effect was poor, then 5 ~ 10 mL of lidocaine (2 %) was injected through epidural approach. When the systolic pressure was 40% lower than the basic blood pressure, the infusion was accelerated. When the systolic pressure was 70% lower than the basic blood pressure, 15 mg of ephedrine was injected. When the heart rate (HR) was lower than 55 time/min, 0.5 mg of atropine was intravenously injected.

Observation indexes

Hemodynamic parameters, including HR, mean arterial pressure (MAP) and oxygen saturation (SpO_2), were measured using a non-invasive hemodynamic monitoring system at the moment of

drug injection (T_0), 5 min after drug injection (T_1), 10 min after drug injection (T_2), 20 min after drug injection (T_3), 30 min after drug injection (T_4) and 60 min after drug injection (T_5). Onset time of sensory block, the highest plane of sensory block, sensory recovery time and the highest Bormage score of motor block (no motor block for one point, incapable of raising lower limbs for two points, incapable of bending the knees for three points, incapable of bending ankles for four points) and motor recovery time were recorded. The incidence of adverse reactions was observed. 1-min Apgar score of the newborns was evaluated.

Statistical analysis

SPSS ver. 19.0 was used for statistical analysis. Measurement data were expressed as mean $\pm$ SD. Comparison between groups was performed using Kolmogorov-Smirnov. Normality test and homogeneity test of variance were performed using the Levene method. Data conforming to normal distribution and variance homogeneity were processed by one-way analysis of variance, while data which did not conform to normal distribution and variance homogeneity were processed by rank sum test. Categorical data were processed by *Chi*-squared test. Differences were considered as having statistical significance if $P < 0.05$.

RESULTS

Comparison of pharmacodynamic index

Differences of onset time and perfection time of sensory block between the three groups had no statistical significance ($P > 0.05$). The highest motor block of groups B and C was much higher than that of group A ($P < 0.05$) and the highest block of group C was much higher than that of group B ($P < 0.05$). Duration of motor block of groups B and C was much longer than that of group A ($P < 0.05$). Compared to group A, onset time and perfection time of motor block of groups B and C was shorter ($P < 0.05$), duration of the block was much longer ($P < 0.05$) and cases of level of III Bormage score were much higher ($P < 0.05$) (Tables I and II).

Oxygen consumption increases in the gestation

period; decrease of oxygen saturation accelerates when respiration stops due to anesthesia induction or occurrence of eclampsia (7). In the gestation period, the growing uterus moves upward to compress the diaphragm, leading to weakened abdominal respiration, strengthened thoracic respiration and decreased expiratory reserve volume and residual volume. Hence, to avoid pulmonary ventilation insufficiency and severe hypoxemia, the control of plane is important when subarachnoid anesthesia is performed.

In this study, we selected the L_{2-3} intervertebral space as the puncture site. Generally, the intersection of ligature of bilateral crista iliacas and spinal cord is L_{3-4} spinous process interval or L_4 process. However, for puerpera, the pelvis expands and rotates under the effect of hormone and compression of fetus, therefore the ligation of the highest points of ilium will rise to L_{2-3} . Hence, it is necessary to refer to posterior superior iliac spine and search for a wide interspace of L_5S_1 . When a subject lies in the supine position, the highest point of the spinal cord is L_3 and the lowest point is T_6 . Hence puncture at the L_{2-3} interspace is beneficial for diffusion of the drug over the head. In this way, a lesser dose of the drug used for anesthesia is needed, and the toxicity to the spinal cord is therefore lower. To ensure the safety of mother and fetus, the sensory block plane of cesarean section needs to be T_{2-6} . Some scholars consider it should be T_4 , but considering the traction on the peritoneum and gastrointestinal tract, T_6 is more suitable.

Ropivacaine takes effect faster than bupivacaine, but it is weak in keeping the highest plane (8, 9). It is correlated to the slow onset and short half-life period of ropivacaine as well as its weak and slow penetration of thick myelin sheath. Tables I and II show that the sensory block plane gradually rose with the increase of drug dose; the sensory block plane of group B was the most suitable, group C was relatively high and group A was low. With the increase of drug dose, motor block onset and perfection time of groups B and C became shorter, duration of sensory and motor block prolonged gradually, motor block became deeper and intraoperative muscle effect was better.

Table I. Comparison of sensory block indexes between the three groups.

Group	No.	Block onset time (s)	Block perfection time (min)	The highest block plane (T)	Duration of block (min)
A	60	36.20±8.30	11.10±2.50	6.70±1.30	119±26
B	60	34.70±4.30	10.50±3.80	5.40±1.50*	144±27*
C	60	33.50±8.30	10.40±3.10	3.60±1.50*#	186±42*#

* $P < 0.05$ compared to group A; # $P < 0.05$, compared to group B

Table II. Comparison of motor block indexes between the three groups.

Group	No.	Block onset time (s)	Block perfection time (min)	Duration of block (min)	Bromage level III (n, %)
A	60	5.30±2.20	9.30±2.50	80.70±21.50	32(53%)
B	60	2.70±1.20*	6.30±3.00*	107.50±27.40*	60(100%)*
C	60	2.50±1.30*	5.60±2.70*	112.60±30.70*	60(100%)*

* $P < 0.05$ compared to group A. (mean±SD, N=60)

Changes of hemodynamic parameters

MAP and HR of the three groups had no significant differences before injection of the drug ($P > 0.05$). MAP and HR had no obvious variation at all time points after drug injection ($P > 0.05$). MAP of group B declined at T_1 , T_2 and T_3 , and HR became higher at T_1 ($P < 0.05$). MAP of group C had a significant decline at T_1 , T_2 , T_3 and T_4 , HR became higher at T_1 and T_2 ($P < 0.05$). Compared to group A, MAP of group B had no remarkable change after drug injection ($P > 0.05$), but the HR of group B became higher at T_1 ($P < 0.05$). MAP of group C significantly declined at T_1 , T_2 , T_3 and T_4 , but HR of group C was higher at T_1 and T_2 ($P < 0.05$). Compared to group B, MAP of group C had an obvious decrease at T_1 and T_2 , but HR of group C became higher at T_2 ($P < 0.05$). SpO_2 was always between 95% and 99% in all three groups. Furthermore, electrocardiograph manifestations of the three groups had no abnormal changes before or after injection of the drug (Table III).

Apgar scores of newborns in groups

The cases which had 10 points of Apgar score were 51, 57 and 54 in group A, B and C, respectively; the cases which had Apgar score equal to or greater

than 9 points and smaller than 10 points were 9, 3 and 6 in groups A, B and C, respectively. The difference of Apgar score between the three groups had no statistical significance ($P > 0.05$).

Incidence of untoward reaction

The incidence of adverse reactions of groups A and B was lower than that of group C ($P < 0.05$); the difference between groups A and B was not statistically significantly ($P > 0.05$) (Fig. 1).

DISCUSSION

With the development of society and the improvement of medicine in recent years, cesarean section has been applied more frequently in clinics. Due to the special physiological characteristics of puerperae, anesthesia is necessary in cesarean section (8, 9).

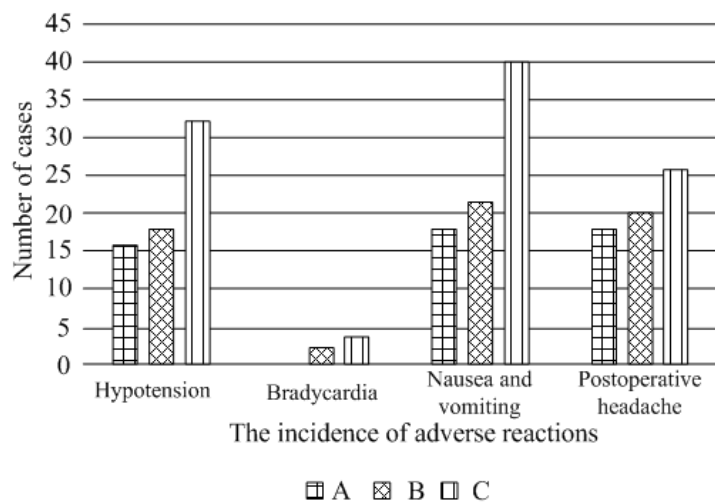
This study explored the application of ropivacaine in anesthesia for pregnant women undergoing cesarean section. The results demonstrated that subarachnoid anaesthesia using ropivacaine was feasible and safe; the different doses of ropivacaine had no influence on the Apgar score of the newborns,

Table III. Comparison of MAP, HR and SpO₂ before and after anesthesia.

Group	MAP(mmHg)	HR(time/min)	SpO ₂ (%)
A			
T ₀	77.20±4.40	81.80±4.30	98.50±1.20
T ₁	75.30±8.40	82.10±14.80	96.40±1.40
T ₂	77.10±5.80	80.10±12.70	97.50±1.20
T ₃	78.60±13.10	77.60±11.80	98.30±1.20
T ₄	79.70±4.60	80.90±5.10	98.20±1.30
T ₅	80.60±6.70	83.20±5.60	98.30±1.10
B			
T ₀	83.80±7.70	82.70±7.80	97.60±1.20
T ₁	74.80±10.20 ^Δ	91.50±13.10 ^{Δ*}	98.20±1.30
T ₂	73.60±7.60 ^Δ	80.60±9.20	98.50±1.40
T ₃	74.60±10.20 ^Δ	86.20±11.30	97.40±1.30
T ₄	85.90±9.30	85.10±7.60	98.30±1.10
T ₅	82.50±5.80	84.70±7.30	98.20±1.40
C			
T ₀	83.10±7.80	82.80±6.90	98.20±1.40
T ₁	62.60±11.70 ^{Δ*#}	91.60±11.20 ^{Δ*}	96.40±1.30
T ₂	65.20±4.80 ^{Δ*#}	94.40±12.30 ^{Δ*#}	96.80±1.30
T ₃	67.30±13.70 ^{Δ*}	86.70±9.80	97.70±1.10
T ₄	65.20±11.60 ^{Δ*#}	85.60±9.30	98.40±1.10
T ₅	81.80±7.20	84.20±4.80	98.30±1.30

^Δ $P < 0.05$ compared to before injection of drug; * $P < 0.05$ compared to group A;

[#] $P < 0.05$ compared to group B. (mean±SD, N=60)

**Fig. 1.** Comparison of adverse reactions between the three groups.

which might be correlated to the low plasma concentration of fetus and the timely correction after blood pressure reduction (10). Furthermore, the sensory block plane of group B was the most appropriate, the sensory block plane of group C was slightly high, and the sensory block plane of group A was insufficient. With the increase of block plane, the onset and perfection time of motor block of groups B and C significantly shortened, motor maintenance time extended, motor block deepened, and muscle relaxation during surgery was better; group C, had rapid onset, had a higher incidence of bradycardia, hypotension and emesis than groups A and C, which might be correlated to the strong block efficacy of ropivacaine under the effect of high concentration of A fibers and C fibers (11). The incidence of postoperative headache was in a correlation to the diameter of lumbar anesthetic puncture needle and the flow volume of cerebrospinal fluid (12).

To sum up, lumbar anesthesia using ropivacaine is effective and safe for cesarean section. 12 mg of ropivacaine (0.5%) is effective in lumbar anesthesia for women undergoing cesarean section, and is featured by little influence on mother and fetus, rapid onset, definite effect and little adverse reaction.

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LETTER TO THE EDITOR

CYCLOSPORINE-A AUGMENTS ENDOPLASMIC RETICULUM STRESS MARKERS AND EXPRESSION OF MATRIX PROTEIN mRNAS.R. RAO¹, R. SUBBARAYAN² and D.M. GIRIJA³

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Cyclosporine-A induces gingival overgrowth with disturbance in the homeostasis of cells and connective tissue proteins. Human gingival fibroblasts were cultured with cyclosporine A, and the expression of two vital endoplasmic stress markers and two prime matrix proteins (connective tissue growth factor (CTGF and periostin) were assessed by RT-PCR. We found that expression of Glucose-Regulated Protein 78 (GRP78/BIP) and CCAAT/enhancer binding protein, C/EBP homologous protein (CHOP) were significantly increased, along with CTGF and periostin, suggesting a role for these factors in gingival overgrowth.

To the Editor,

One of the major complications of cyclosporine treatment is gingival overgrowth, which is disfiguring and interferes with the masticatory function. Cyclosporine induces endoplasmic reticulum stress (ER stress) in renal tubular cells (1) which can lead to epithelial mesenchymal transition (EMT) and cell death, apoptosis, autophagy, and inflammation culminating in fibrosis (2). TGF- β , the key regulator of all fibrotic conditions, stimulates downstream a key mediator viz CTGF which induces matrix synthesis and accumulation (3). CTGF can also act independently from TGF- β stimulation. Hence, it is ideal to estimate the CTGF expression in exaggerated matrix synthesis. Sume et al. (4) demonstrated EMT markers in drug-induced gingival overgrowth. Periostin (also known as osteoblast specific factor 2), a matricellular protein, has been correlated with

induced EMT (5). Since EMT can lead to fibrosis, we included periostin as the second marker for fibrosis. Hence, in order to observe whether ER stress plays a role in this overgrowth, the expression of ER stress markers (GRP-78/BIP and CHOP) and CTGF and periostin gene expression were assessed.

MATERIALS AND METHODS

The study was approved by the Institutional Ethics Committee of Sri Ramachandra University. Informed consent was obtained from patients prior to the excision of gingival tissue. Gingival tissue samples were obtained from healthy individuals (25–35 years old) during crown-lengthening procedures at the Department of Periodontology, Faculty of Dental Sciences, Sri Ramachandra University, Chennai, India.

Key words: gingival fibroblast, cyclosporine-A, ER stress markers, matrix proteins

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Isolation and culture of human gingival fibroblasts

Human gingival fibroblasts (HGF) were cultured using the enzymatic digestion method and the optimum concentration of cyclosporine-A (CsA) of 25 μ M was determined by MTT assay as described in our previous publication (6).

Reverse transcriptase PCR analysis

Gene expression of GRP78/BIP, CHOP, CTGF and periostin was carried out by RT-PCR. RNA from CsA-treated and untreated human gingival fibroblasts isolated using TRIzol RNA isolation reagent (Invitrogen, Bangalore, India). Then, cDNA was synthesized using the Verso cDNA synthesis kit (ThermoFisher Scientific, Bangalore, India). The conversion and cycling program was performed as per the manufacturer's instructions. To confirm the homogeneity of RNA loading, GAPDH was used as a housekeeping gene.

RESULTS

Based on the gene expression results, cells treated with 25 μ M of CsA- showed a significantly increased expression of GRP78/BIP, CHOP, CTGF and periostin when compared to untreated cells ($p < 0.001$) (Fig. 1A). The histogram (Fig. 1B) represents the normalization of gene expression using GAPDH as a reference gene between the untreated and the CsA-treated gingival fibroblasts. Values are expressed as the mean and standard deviation of three independent experiments. Statistical analysis was performed by two-way ANOVA using Bonferroni's post-hoc test in the GraphpadPrism 6.0.

DISCUSSION

In the present study, the addition of cyclosporine

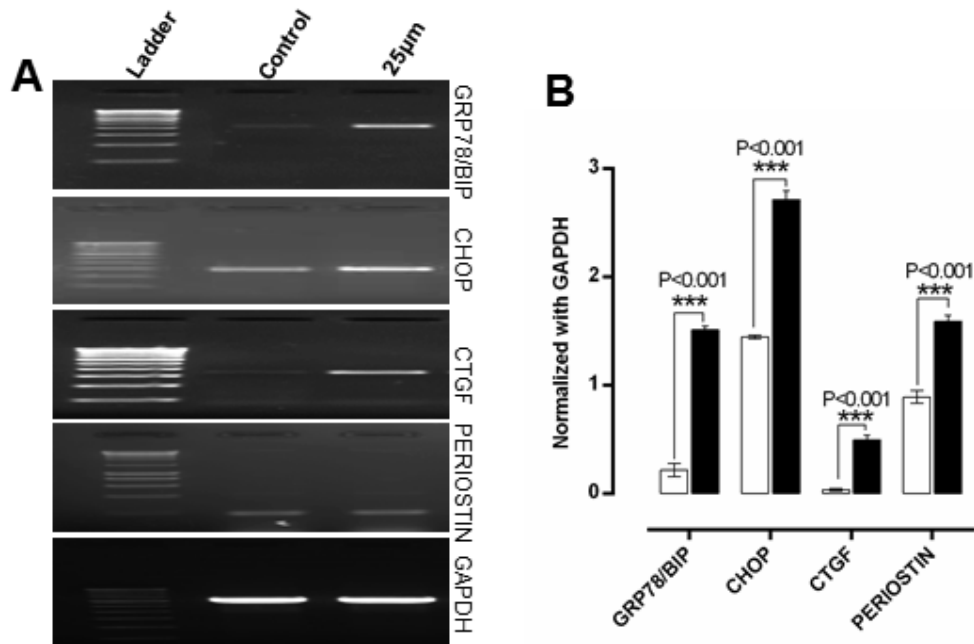


Fig. 1. Expression of markers GRP78/CHOP, CHOP, CTGF and periostin in human gingival fibroblast cells at 4th passage was analyzed by RT-PCR. **A)** Expression of Endoplasmic reticulum stress factors in Control and CsA induced gingival fibroblast. **B)** Histogram showing GAPDH internal housekeeping gene to normalize the relative expression of target gene based on the percentage of expression. Values are expressed as mean and standard deviation of three independent experiments. All genes were analyzed on a 1.5% agarose gel electrophoresis and visualized by gel documentation unit (Bio Rad, USA). 100 bp DNA ladder was used as a marker.

Table I. List of primers used in this study.

GENE	ACCESSION NUMBER	FORWARD PRIMER	REVERSE PRIMER
GAPDH	NM_001256799	AATGGGCAGCCGTTAGGAAA	GCGCCCAATACGACCAAATC
GRP78 /BIP	NM_005347	GAACGTCTGATTGGCGATGC	GAGTCGAGCCACCAACAAGA
CHOP	NM_001195053	TCCAACCTGCAGAGATGGCAG	TCCTCCTCTTCCTCCTGAGC
CTGF	NM_001901.2	CACCCGGGTTACCAATGACA	TCCGGGACAGTTGTAATGGC

A to human gingival fibroblasts induced a significant increase in the gene expression of ER stress markers (GRP78 and CHOP) and matrix proteins (periostin and CTGF). GRP78 is the first ER protein to delink from other silent proteins in ER stress, and therefore, it is considered a hallmark of ER stress. In human and murine pulmonary fibroblasts, GRP78 has been found to contribute to TGF- β -induced myo-fibroblast differentiation and collagen production. p-eIF2 α or CHOP however have not been found to act via these pathways (7). A concomitant increase in CTGF gene expression along with ER stress markers was observed in the present study. Since CTGF plays a central role in fibrosis (8), its overexpression may represent fibrosis. Shing-Hwa Liu et al. (9), in a rat unilateral ureteral obstruction (UUO) model, observed the attenuation of GRP78, CHOP and other ER stress markers along with α -Smooth Muscle Actin and CTGF following treatment with 4-phenylbutyrate.

Periostin, a matrix cellular protein, is a major inducer of epithelial mesenchymal transition (EMT) (5). Considering the diverse roles of Periostin in collagen metabolism, angiogenesis and EMT, we

can hypothesize that this matrix cellular protein may contribute significantly to the pathogenesis of fibrosis and angiogenesis observed in gingival overgrowth.

In conclusion, the present study provides preliminary evidence for the differential expression of ER stress markers and pro fibrotic mediator (CTGF and POSTN) in CsA treated human gingival fibroblasts, hence suggesting their potential role in cyclosporine-induced gingival overgrowth). Therapeutic intervention in the form of antioxidants and anti-ER stress agents such as 4-phenylbutyric acid can potentially spare patients from gingival surgery, which is currently the only option for patients with overgrowth.

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LETTER TO THE EDITOR

THE BIOMEDICAL ASPECTS OF ORAL MUCOSAL EPITHELIAL CELL CULTURE IN MAMMALS

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In recent years, there has been a growing interest in epithelial cell tissue culture, particularly oral mucosa and its application utilizing *in vitro* cell culture in medicine. This involves tests using animal models to better understand oral mucosa function, and the differences in its construction in various animal models. The use of buccal pouch mucosal cell culture provides insight into the processes of trans mucosal transport and regeneration of the oral epithelium. The processes associated with epithelium regeneration is the base for stem cell research and/or oral cancer investigation. These artificially cultured tissue equivalents are used in transplant surgery for the treatment of a variety of tissue dysfunctions, i.e. eye, esophagus, or urethra. In this review, the most recent results from studies carried out on in animal models, which may be applied in areas such as regenerative medicine and reconstructive surgery, were explored.

To the Editor,

Oral mucosa has received special attention due to its regenerative properties. Regeneration occurs faster in this tissue than in other epithelial tissues (1). Oral mucosa consists of epithelium and lamina propria. Buccal mucosa epithelium is the stratified, non-keratinized squamous epithelium without a stratum corneum or a granular layer (2). The basal layer is created by proliferating cells that can be assigned to three fractions: epithelial stem cells (ESCs), migratory cells with limited proliferative capacity (TACs – transit-amplifying cells) and post-

mitotic differentiating cells (DCs) (3).

Most research performed on mammalian oral mucosa is conducted on small animals. They are easier to maintain, and a larger population of animals can be kept and cared for, whereas breeding of large animals poses more problems and is more expensive (4). The main species used in experiments are typically mouse, rat, hamster, cavia, and rabbit. However, in clinical trials, the pig is often used as an animal model (5). The primary advantages of using pigs are anatomical and physiological similarities to the human circulatory, digestive, and nervous

Key words: buccal mucosa, animal model, primary culture, gene marker

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systems. The use of large animals is also linked to the demands of the European Medicines Agency (EMA) for conducting clinical studies on tissue engineering in large animals (5). Additionally, clinical trials on larger animals, such as pigs or dogs, are more convenient because of the ability to conduct research and endoscopic transplantation (6).

The great advantage of large animal breeding is the possibility of biological material collection for cell culture after slaughter. Primary cultures that arise from tissues and organs enable a larger amount of usable material. In this case, there is no need for experimental animal breeding and acquisition of all necessary permissions.

Primary epithelial cell culture – animal test model

Epithelial cell cultures derived from animal models aid in progressing medical research. They give insight into physiological and pathological processes, providing the basis for new treatment options for humans and animals.

Depending on the experiment, an appropriate animal model is chosen. Similarity to human epithelial tissue is a primary goal. The first criterion for choosing the animal model is keratosis of the superficial layers of the epithelium. In humans and experimental animals, the palate epithelium undergoes the process of keratosis. In contrast, the human buccal epithelium has no stratum corneum, which is present in rodents such as mouse, rat, cavia, and rabbit (4, 6) and in ruminants (sheep and cattle) (4). Keratosis derived from the buccal pouch is not detected in dogs and pigs, however, in rabbits, parakeratosis may be observed (4, 6). The second criterion is the thickness of the epithelium. In humans, the region of the palate is approximately 180 microns. A similar thickness of the epithelium is found in sheep and dogs, while the pig is characterized by a thinner layer of the epithelium (4). Other studies have also compared the thickness of the buccal epithelium. The thickest layer of buccal epithelium was observed in humans, pigs, and rabbits while in dogs, mice, and rats, the buccal mucosa epithelium was thinner (6).

Another histological criterion is the epithelia invagination in the lamina propria, which is similar within the palate as related to the number and length

of invaginations. Within the buccal pouch mucosa, invaginations vary between species (4). Analyzed experiments show pig and rabbit cells are less efficient in forming colonies and are characterized by a lower proliferation capability (6). According to Sa et al. (2016), oral epithelium isolated from dogs is histologically similar to human epithelial cells, with higher scores from porcine epithelium (4).

Buccal mucosa cells as an experimental model for in vitro culture

In *in vitro* culture of buccal pouch mucosa, epithelium obtained from humans, pigs, or rabbits have considerable thickness in relation to epithelium derived from mice or rats (6). However, the epithelial cells of humans, pigs, and rabbits show low colony forming efficiency, but this is a main problem of porcine cells (6). Buccal epithelial cells of rabbits and pigs proliferated only when associated with the feeder layer. Unlike mouse cells, rat and dog cells proliferated in the presence or absence of a feeder layer (6). In rabbit buccal cells, when cultured on a collagen medium, a high proliferation activity was obtained, which is well suited for long-term culture (7).

The most recently described experiment on the buccal mucosa analyzes three main issues. The first concerns the study of membrane permeability, which is associated with the mucosal administration of drugs. The second aspect is the conduct of research on oral cancer cells and related studies of the cell cycle and isolation of stem/progenitor cells. Another scope of analyzed research is cell engineering associated with transplantation.

In the studies on membrane permeability, an appropriate selection of conditions and material is required to eliminate additional factors that affect the diffusion potential of the epithelium. Keratinized epithelium of animals, which prevents diffusion, is not a suitable model, although a number of studies are carried out using rats and hamsters (8). For the porcine buccal mucosa model, it was found that the method of storage and freezing can influence the growth of epithelial permeability. In contrast, in the case of mucosa obtained from the palate, it can be used both fresh and frozen (9).

A popular animal model for oral cancer research

is the hamster. Induced carcinogenesis enables a better understanding of tumor development, providing favorable conditions for molecular tests that allow for signaling pathway tracing and the ability to obtain effective anti-cancer therapy (10). However, it is difficult to obtain a tumor model in animals, which would be a suitable source of knowledge, equivalent to human tumors in the oral cavity (11). In rats, carcinoma cells were isolated and cultured with a high grade metastasis for the purpose of understanding molecular mechanisms of tumor progression analysis (12). Additionally, hamster buccal mucosa stem cell/stroma were isolated and cultured in order to study tumor cells caused by dysregulated self-renewal multipotent cells (13).

Identification of marker genes of buccal pouch mucosa cells

Stem cells exhibit specific morphological and phenotypic features. These cells are of small diameter, having a high proliferative potential while being capable of self-renewal and tissue regeneration (14). However, there is a problem in distinguishing progenitor cells from stem cells and the ability to separate them. It is believed that small round cells having a high ratio of nuclear to nuclear cytoplasm area (>0.8) may be classified as progenitor stem cells. (7). Attempts have been made to isolate stem cells from rabbit mucosa, based on epithelial cell adhesion to collagen IV. The fraction of progenitor cells undergoing adhesion within 10 minutes is considered a putative stem cell fraction and these cells were characterized with the highest proliferative capacity (14).

There is substantial information regarding the wound healing process within the oral mucosa, which is characterized by an early occurring, dynamic proliferation and cell migration. The oral cavity retains its shape in contrast to epidermal cells where, in the course of cell migration, their size is reduced (1). The difference with the epidermis also relates to a method that damaged epithelium is reconstructed. In the buccal epithelium, keratinocytes migrate from the edge of the wound in the form of a wedge where wound surfaces combine to form the shape of the letter "V" (1).

In connection with mucosa wound healing, we analyzed the expression of connexins Cx26, Cx43,

and Cx30, and the results showed increased protein expression in the buccal epithelium as compared to the skin. Cx26 is not expressed in the basal layer but is found on the superficial layer at its bottom and middle region. Similar expression was observed for Cx30. Connexin 45 is common in the skin and is present in all layers of the buccal mucosa, but it exhibits lower expression in the basal layer. Epithelium reconstruction after injury is followed by decreased expression of connexins (1).

The keratins are common markers of buccal pouch mucosal cells. Most of the cells within the buccal mucosa are keratinocytes, which, in contrast to keratinizing epithelia, do not undergo a degeneration process during differentiation. In the basal layer of keratinocytes, genes encoding keratins K5, K14, and K19 are expressed. In the intermediate layer, which is characterized by differentiating cells, K4 and K13 are expressed (15).

Keratin K1 and K10 expression are characteristic for keratinized epithelium. K1 expression is observed in spinosus layer and K10 in basal layer. In oral non-keratinized epithelium both keratins may be characterized by very low expression levels. K10 expression strongly correlates with keratinization. During epithelial tissue regeneration following injury, the expression pattern of CK14 changes. Cells expressing the genes encoding CK14 are also identified in the suprabasal layer of the epithelial outgrowth (3, 16). There was also a decrease in CK14 and CK13 expression in cells growing at the wound edge (3). After healing, the expression of CK14 is present only in the basal layer (16). Other markers include transcription factor p63, responsible for differentiating endothelial progenitor cells, and PCNA, a marker of DNA replication and proliferation. Both genes are expressed in the basal layer in the normal tissue. (6). After injury, keratinocytes with PCNA and p63 expression are present at the site for tissue growth but double positive cells (p63/PCNA(+)) are not present. At 72 hours after injury, basal keratinocytes surrounding the wound margin show positive reaction for PCNA. At 120 hours, the majority of cells surrounding the wound margin are p63 positive without observed PCNA(+) (16). In another experiment, keratinocytes

PCNA(+) were observed in the basal layer only. The cells around the wound were PCNA(-) (3). p63(+) cells, occurring in the area of injury, may be undifferentiated cells exhibiting dynamic proliferation (16). Another marker used for detecting proliferating cells is bromodeoxyuridine (BrdU), a synthetic analogue of thymidine. BrdU(+) cells are detected in the basal layer of epithelial cells and over this layer (3). The expression of p75^{NGFR}, a nerve growth factor receptor, was also studied. It is a mediator in nervous tissue, guiding cell cycle survival or directing it to the apoptotic pathway. The activity of p75^{NGFR} was visualized in the cells of the basal layer keratinocytes and is a potential marker of stem cell keratinocytes (3). p75^{NGFR}(+) cells are located only in the basal layer, and their numbers are reduced in areas of injury, returning to baseline after rebuilding the damaged tissues (3). Ki67 is another marker of proliferative activity, detected in the basal layer as well as in several other layers of suprabasal location (4). Collagen IV is a marker of the basal membrane, which is substantially built by collagen protein and the laminae, forming a support structure for the epithelial cells of the basal layer (2).

The perspective of the mammal buccal mucous membrane application in medicine

Research on the buccal mucosa contributes to significant medical progress. This applies mainly to tissue engineering and the possibility of using cultured tissue for transplants in the reconstruction of damaged tissues in cases of mechanical trauma, malformations, and tumors. Clinical studies on porcine models were performed in order to obtain an equivalent buccal mucosa (5). Similar studies were carried out on rabbits using a co-culture of epithelial cells and fibroblasts, which contributed to the observation of the cytoskeleton and the expression of adhesive proteins without the presence of feeder cells (2).

A great number of experiments is conducted on the use of autologous transplants in ophthalmology. Autologous cells lining the mouth may be used in reconstruction of the ocular surface damaged by chemical burns, injuries, or eye infections. For the treatment of a cornea stem cell deficiency, cultured stem/progenitor cells are used for the reconstruction

of the cornea and conjunctiva surfaces (17). The results of the study, performed on rabbits, appear to be a promising form of treatment for ocular defects (6).

Autologous transplants are used in urology, but the procurement of tissues for transplants can lead to complications. With this in mind, it seems very convenient to use buccal tissue with respective morphological and mechanical properties (18). Research is aimed at the treatment of stenosis and developmental disorders of the urethra. A related example is the reconstruction of the urethra in dog using buccal mucosa and silk fibroin matrices (19). Tissue engineering can eliminate the problem of limited amounts of tissue available for transplantation. Scientists are exploring the problem of transplant failure due to necrosis caused by inadequate blood supply to the graft. The solution would be to carry out 3D culture (buccal epithelial cells, fibroblasts, and endothelial cells) (18).

The buccal mucosa is also used for transplantation after endoscopic resection of the mucosa and submucosa of the esophagus, which eliminates postoperative complications such as inflammation and esophageal stenosis (20).

All of these examples provide insight into the potential uses of primary cultures in medicine. Cell and tissue cultures, especially stem cell cultures, may have a crucial impact on reconstructive surgery development and become widely used in medicine.

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LETTER TO THE EDITOR

THE PIG AS A MODEL FOR PREMATURE INFANTS - THE IMPORTANCE OF IMMUNOGLOBULIN SUPPLEMENTATION FOR GROWTH AND DEVELOPMENT

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Preterm human neonates, contrary to preterm piglets, obtain immunoglobulins from their mothers via the placenta during intrauterine development. However, one should note that the majority of trans-placental transfer of immunoglobulins in humans takes place during the last trimester of pregnancy. It is also known that the feeding of limited amounts of colostrum or systemic infusion of small amounts of serum improves the survival of preterm and full-term piglets. Full-term piglets deprived of their mother's immunoglobulins exhibit strong apathy and develop watery diarrhoea, often resulting in death. The aim of the current study was to determine if provision of immunoglobulins using different approaches would be beneficial for survival outcomes. To reach the immunological sufficient level we infused immunoglobulins intravenously in amount mimicking the blood level in piglets fed with sow colostrum. Intravenous infusion of immunoglobulins in both preterm and full-term newborn piglets fully ensured their survival, growth and blood immunoglobulin G and protein levels similar to those observed in piglets fed colostrum. Piglets completely deprived of immunoglobulins exhibited significantly lower blood levels of immunoglobulins and protein compared to colostrum-fed animals. Piglets infused with only serum exhibited significantly lower blood immunoglobulin G level compared to those infused with immunoglobulins. In conclusion, based on the data obtained, we suggest that passive immune support provided by colostrum intake or early systemic infusion of Ig's in sufficient amounts is key to ensuring the general well-being of preterm and full-term new born piglets, used as an animal model for the human infant.

To the Editor,

Based on a WHO worldwide report, every year approximately 14.9 million infants are born before the 37th week of gestation, with a global prevalence of 11.1% of total births (1). The gestational age has an

effect not only on the neonates' organ developmental stage, but also on cellular and humoral immunity, and preterm infants have lower plasma immunoglobulin levels than full-term infants, which in turn contribute to the increased risk of infections (2, 3).

Key words: pig model, colostrum, immunoglobulin infusion, preterm infants

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A preterm pig model of about 90% gestational age, which mimics human neonates over 30 weeks of gestational age (75%), was used to study the growth and development of human preterm neonates with emphasis on sickness development and the effects of different infant formulae and nutritional recommendations (4, 5). In contrast to preterm or full-term new born infants, that are provided with maternal passive immunity *in utero*, new born piglets totally lack passive immunity transfer during the foetal period and are thus born without the protection of the circulating maternal immunoglobulins (Ig) (6).

The main aim of the current study was to investigate and prove the importance of sufficient immunoglobulin supplementation for the survival, growth and behaviour of full-term and preterm piglets. Two separate experiments were performed. In the first experiment, full-term piglets were intravenously (*i.v.*) infused with purified porcine Ig or crude porcine serum, and the growth and survival of these piglets were compared to those of piglets fed porcine colostrum containing Ig. In the second experiment, preterm piglets received purified Ig immediately after birth via injection into the umbilical vein. These piglets were fitted with an intra-gastric port which was used to feed the piglets a milk formula during a 2-week period.

MATERIALS AND METHODS

Animals and animal holding

The present study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. Efforts were made to minimize animal suffering.

The study was performed on 39 piglets. Preterm piglets (n=15) were delivered by caesarean section from 2 sows, 7 days before full gestation (115 days), corresponding to human neonates of approximately 32 weeks of gestational age. Full-term piglets (n=24), from 6 litters, born naturally, were isolated from their sows before suckling and randomly distributed into 4 experimental groups (n=6 pigs in each group).

The animals were maintained under a 12 h day-night cycle, with lights on from 08.00 am to 8.00 pm. The piglets were housed in pairs in special cages for preterm/full-term

piglets. The cages were equipped with dry bedding and heating pads to maintain a temperature of between 35–37°C and humidity around 56%. The pigs were allowed to move freely within the cages and had visual and tactile contact with each other.

Treatment and feeding procedures

Following arrival of the piglets to the laboratory, vascular catheters (Silastic Laboratory Tubing 508-001, Dow Corning, Auburn MI, USA) were inserted into the umbilical vein in all pigs for immunoglobulin/swine serum infusion as well as for primary (0h) blood sampling.

Full-term pigs (Experiment 1)

At the start of experiment 1, two groups of full-term pigs were infused, via the umbilical vein (23 ml/kg b.wt), with either a sterile filtered swine serum immunoglobulin preparation (306.75 mg IgG/ml) (Group 1) or normal sterile (16 ml/pig) swine serum (SS) (25.22 mg IgG/ml) (Group 2). A third group of piglets were fed with swine colostrum (10 ml/kg b.wt.) every 2 h for the first 12 h of life. The fourth group was deprived from immunoglobulins. All piglets were fed with the same infant formula (10 ml/kg b.wt; Similac Special care 24, Abbott Nutrition, Columbus, Ohio, USA) via an orogastric tube every 2 h for up to 48 h.

The swine serum Ig preparation was obtained from a pool of blood plasma from three multiparous sows, by ammonium sulphate precipitation (7). The Ig preparation was sterile filtrated and stored at -20°C until use. The normal swine serum was prepared by centrifugation of the blood obtained from three sows, which was then pooled, sterile filtrated and stored at -20°C until use.

Preterm pigs (Experiment 2)

At the start of experiment 2, 15 preterm piglets (Group 5) were used. All preterm pigs were infused (23 ml/kg b.wt) with the swine serum Ig solution (306.75 mg IgG/ml), in the same way in which the full-term pigs were infused. During the first 2 days after delivery, the preterm piglets were fed every hour with warm (37°C) infant formula (5 mL/kg b. wt.) via the orogastric tube.

At 48 hours, due to the increasing activity of the piglets, specially designed gastric port catheters for feeding were implanted. Briefly, the pigs were anesthetized using 0.5–1.5% air mixture of Fluothane (Zeneca, Gothenburg,

Sweden) and O₂ as a carrier gas, at approximately 0.5-1 L/min in a close-circuit respiratory system (Komesaroff Medical Developments, Melbourne, Australia). The surgery was performed under aseptic conditions. A 5-cm long incision was made posterior to the sternum, along the *linea alba*. The stomach was isolated and the specially designed gastric port catheter (Silastic, Laboratory Tubing 508-002, Dow Corning, Auburn MI, USA) was implanted via the *curvatura major* and fixed with purse string catgut sutures (KRUUSE Chromic Catgut, USP 4-0; Kruuse Svenska AB, Uppsala, Sweden). The abdomen was then stitched up using 3 layers of sutures, absorbable sutures for the muscle layers and non-absorbable sutures for the skin (Silon Monofil 2/0; CHIRANA, Prague, Czech Republic). Ampicillin (250-500 mg, Doktacillin, Astra Läkemedel, Södertälje, Sweden) was administered at the incision site. A jugular vein catheter (Silastic, Laboratory Tubing 508-001, Dow Corning, Auburn MI, USA) for blood sampling and infusions was also implanted during the same operation.

After surgery, the preterm piglets were infused i.v. for 6 h with 5 ml/kg/h of 0.9 % NaCl containing 10% glucose, after which they were switched to the gastric port feeding with formula, at a dose of 5 ml/kg every 1 h (24 per day) or 10ml/kg every 2 h (12 per day). Throughout the experiments, the piglets received increasing amounts of formula (about 5% increase per day) to ensure liquid, energy and nutrient supply. According to the neonatology guidelines, we aimed to reach enteral feeding with 135-180 ml/kg/day of formula, however the good tolerance to feeding and the welfare of animals were paramount.

Animal observations

The animals were thoroughly monitored during the experiments. The piglets' general health status, activity/ responsiveness and gastrointestinal clinical symptoms such as vomiting, chewing, constipation, diarrhoea and abdominal distension were evaluated. Before each feeding, food retention in the stomach was examined and if reflux of formula in gastric catheters was observed, the amount of formula fed was decreased by 1-2 ml (by the end of the study the amount of formula administered was around 15 ml/kg every 2 h). Prior to feeding, piglets were examined to confirm the absence of abdominal distension and respiratory distress. The piglets wore diapers for 24-h stool and urine collection. To assess bowel function, the

faecal consistency and amount were noted. The amount and intensity of the urine colour was also noted. The piglets were weighed once daily (prior to feeding at 10 a.m.) and the daily body weight gain, expressed as grams per kilogram, per day (g/kg/day), was estimated. The observation lasted 48 h for full-term piglets and 14 days for preterm piglets.

Blood sampling and analyses

Blood was collected at the start and end of the study into ice-chilled tubes coated with lithium heparin [BD Vacutainer®, 367884, Becton, Dickinson and Company (BD Medical), Franklin Lakes, New Jersey, USA] as an anticoagulant and then centrifuged at 3000 x g for 15 min. After centrifugation, plasma was removed and frozen at -20°C until analysis.

Total protein concentration (mg/ml) in plasma samples, swine serum and swine colostrum was estimated according to the method described by Lowry et al. (8), using bovine serum albumin (BSA, A5470, Sigma-Aldrich, St. Louis, MO, USA) as the standard.

The level of immunoglobulin G (IgG) (mg/ml) in plasma samples, swine serum and colostrum was analysed by single radial immunodiffusion (9), using anti-Pig IgG produced in rabbits (Sigma P0916, Sigma-Aldrich, St. Louis, MO, USA) and purified porcine IgG as the standard (Sigma 14381, Sigma-Aldrich, St. Louis, MO, USA).

Statistical analyses

All data are expressed as mean $\pm$ SD (standard deviation). ANOVA followed by the Tukey post-hoc test or Kruskal-Wallis test was used to indicate the statistical differences between the groups. All analyses were carried out using Statistica, version 7 (StatSoft, USA). In all statistical analyses $p < 0.05$ was considered significant.

RESULTS

Weight and clinical observations

In experiment 1, only 48 h following birth, the daily body weight gain was significantly ($p < 0.05$) increased in the piglets infused with Igs (Group 1) and in the colostrum-fed piglets (Group 3) (Table I). A body weight loss was observed in piglets i.v. injected with swine serum (Group 2) from 1.43 ± 0.28 kg to 1.33 ± 0.52 and in the control piglets without

any supplementation (Group 4) (1.27 ± 0.52 kg to 1.15 ± 0.30 kg) (Table I). It is worth mentioning that all piglets from Group 4 lost weight. Following i.v. Ig supplementation, at the end of the study on the 14th day following birth, a calculated daily body weight gain of 11.9 g/kg/day was observed in the preterm piglets (Group 5) (Experiment 2). During the first postnatal week, the preterm piglets displayed weight loss.

Upon physical examination, the preterm piglets showed no symptoms of having any gastrointestinal disorders or systemic infections, such as fever,

abdominal distension, bloody stools, feed retention in the stomach or vomiting. The piglets did not require respiratory or circulatory support. All piglets passed urine a few times a day and anuria was not noted. Both the preterm and full-term piglets from all treatment groups were alert and active between the feeding intervals, except the group of control pigs, fed only the infant formula (Group 4) without any supplement, which were observed to be lethargic and apathetic. The control group of piglets also had watery diarrhoea, while the piglets from the other groups passed normal stools.

Table I. Mean body weight, daily body weight gain, plasma total protein and immunoglobulin G content during the study period.

Pig Treatment groups	Time	Group 1 (n=6)	Group 2 (n=6)	Group 3 (n=6)	Group 4 (n=6)	Group 5 (n=15)
Body weight (kg)	0 h	1.40 ± 0.45	1.43 ± 0.28	1.15 ± 0.18	1.27 ± 0.52	1.14 ± 0.20
	48 h	1.55 ± 0.52	1.33 ± 0.52	1.27 ± 0.28	1.15 ± 0.30	1.15 ± 0.24
	14 d	-	-	-	-	1.33 ± 0.22
DBWG (g/kg/day)		$53.6 \pm 0.78a$	$-34.9 \pm 0.47b$	$50.4 \pm 0.35c$	$-45.8 \pm 0.54d$	$11.9 \pm 0.32e$
TPC (mg/ml)	0 h	$18.2 \pm 2.8a$	$15.8 \pm 1.9a$	$15.9 \pm 1.3a$	$21.3 \pm 2.0a$	$17.0 \pm 2.2a$
	48 h	$25.0 \pm 1.9b$	$24.4 \pm 0.1b$	$36.4 \pm 3.6c$	$19.7 \pm 3.1a$	$24.5 \pm 1.2b$
	14 d	-	-	-	-	$23.9 \pm 2.2ab$
IgGC (mg/ml)	0 h	$0.13 \pm 0.03a$	$0.08 \pm 0.02a$	$0.07 \pm 0.01ab$	$0.08 \pm 0.02ab$	$0.05 \pm 0.02b$
	48 h	$6.60 \pm 1.30c$	$1.80 \pm 0.08d$	$9.8 \pm 0.01e$	$0.06 \pm 0.01ab$	$5.70 \pm 1.60c$
	14 d	-	-	-	-	$4.40 \pm 1.40c$

BW, body weight; DBWG, daily body weight gain; TPC, total protein content; IgGC, immunoglobulin G content. Data expressed as mean $\pm$ SD values of BW, DBWG, TPC and IgGC at the beginning and at the end of experiment for all study groups (Group 1, full-term piglets which received an intravenous infusion of swine immunoglobulins, n=6; Group 2, full-term piglets which received an intravenous infusion of swine serum, n=6; Group 3, full-term piglets which received swine colostrum, n=6; Group 4, Similac-fed full-term piglets, n=6; Group 5, preterm piglets which received an intravenous infusion of swine immunoglobulins, n=15). Different letters given with particular results in column and in row describe significantly different between the groups and consecutive time points when $p < 0.05$.

Total protein (TP) and IgG plasma levels

The plasma protein levels at the time of birth were not significantly different between the full-term and preterm piglets (Table I). Forty-eight hours following the birth of the piglets, significantly increased total plasma protein levels were observed in all treatment groups, except for that of the control full-term piglets, fed infant formula without any supplement (Group 4).

Both preterm and full-term piglets displayed trace levels of IgG in the plasma samples obtained at the time of the birth (Table I). Forty-eight hours following the birth or caesarean of the piglets, the piglets infused with Ig or fed colostrum exhibited a significant ($p < 0.05$) increase in plasma IgG concentration. The infusion of swine serum (Group 2) also resulted in significantly increased plasma IgG levels, however, these increases were significantly lower than those observed in the Ig-infused or colostrum-reared piglets. The Ig deficiency was sustained in the control full-term piglets from Group 4, fed infant formula without any supplement. In the preterm piglets (Group 5), post-infusion plasma IgG levels were maintained throughout the study period (14 days).

DISCUSSION

In the current study we proved that both a preterm and full-term porcine model of human neonates need to be supported with the porcine Ig in sufficient amounts (either *via i.v.* infusion or colostrum feeding) to ensure adequate growth of the animals, and specific for piglets' alertness and vitality. In addition, the use of gastric ports simplified the feeding and allowed us to perform prolonged observations. We demonstrated that *i.v.* infusion of porcine serum Ig immediately after birth results in increased serum IgG levels in both full-term and preterm piglets, which mimics the benefits of natural colostrum intake. In a previously published study, preterm piglets were infused with sow serum to create passive immunity (11). However, the amount of infused Ig was unable to protect the animals from the development of necrotizing enterocolitis. Our results demonstrated that full-term piglets, which received porcine serum in amounts, mentioned by Shen et al. (11), displayed

significantly increased plasma levels of IgG and TP in comparison to the piglets fed infant formula without any supplementation. However, the plasma IgG amounts still remained significantly lower than those observed in piglets infused with purified Ig (Group 1) and those fed colostrum (Group 3). The full-term piglets that were not provided with any form of immune support displayed low plasma levels of IgG and TP; in fact, TP levels seemed to decrease during the 48 hours following birth. This appeared to have a serious negative effect and led to the deterioration of the general health status of the piglets, including the development of apathy and watery diarrhoea. In conclusion, based on our data, we suggest that passive immune support provided by colostrum intake or early systemic infusion of Ig in sufficient amounts is key to ensuring the general well-being of preterm and full-term newborn piglets. However, more detailed research is needed to confirm the positive influence of enteral feeding with passive immunity support.

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LETTER TO THE EDITOR

THE EFFICACY OF PROANTHOCYANIDINS AND SECNIDAZOLE IN THE TREATMENT OF CHRONIC PERIODONTITIS AFTER SCALING AND ROOT PLANING THERAPY

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The aim of this study is to evaluate the clinical and microbiological effect of the systemic antibiotic therapy of proanthocyanidins and secnidazole on periodontitis. Seventy-five subjects with chronic periodontitis were randomly divided into two treatment groups (secnidazole or proanthocyanidins) and one placebo control group (25 cases each). Plaque index (PI), gingival index (GI), gingival bleeding index (BI), probing pocket depth (PPD), and clinical attachment level (CAL) were carried out at baseline, post-treatment and 3 months after treatment. Microbial analysis was performed at baseline and post-treatment. The results show that the two treatment groups had greater mean reduction in BI, GI, and PPD evaluated at both post-treatment and 3 months after treatment compared to the control group ($p < 0.05$), but there were no significant differences in those of PI and CAL (except CAL evaluated at post-treatment, $p > 0.05$). After treatment, culturable bacteria counts significantly decreased. In conclusion, the adjunctive use of proanthocyanidins or secnidazole in combination with scaling and root planing in adults with periodontitis is effective in reducing the pathogenic flora and achieves significantly better clinical results to a certain degree.

To the Editor,

Metronidazole (1), tinidazole (2), and ornidazole (3) have been used to treat periodontitis. Secnidazole, which is a 5-nitroimidazole derivative with properties similar to metronidazole, with the exception of a more prolonged blood concentration (4), has not been used to treat periodontitis. Numerous recent studies have demonstrated the antibacterial activity of proanthocyanidins (5). The aim of the present clinical trial was to evaluate the clinical and microbiologic efficiency of proanthocyanidins or secnidazole in

conjunction with scaling and root planing (SRP) therapy in patients with chronic periodontitis.

MATERIALS AND METHODS

Patients

The study was approved by the Ethics Committee of the 9th People's Hospital, Medical College, Shanghai Jiao Tong University (Approval No. 2013-41). Chronic periodontitis was diagnosed based on the criteria of the American Academy of Periodontology (1999) (6) by one

Key words: inhibitory, secnidazole, proanthocyanidins, bacteria, chronic periodontitis

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experienced dentist. Seventy-five patients with chronic periodontitis were included. All of the subjects signed informed written consent before the study.

Inclusion criteria

The age of the patients, who were non-smokers, ranged from 18 to 75 years; mean age 52. Patients had at least six permanent teeth including incisors and/or first molars with at least one site each with PPD and clinical attachment level (CAL) ≥ 5 mm and at least six teeth other than first molars and incisors with at least one site each with PPD and CAL ≥ 5 mm.

Exclusion criteria

Patients having allergies or hyper-sensitive skin reactions against secnidazole, systemic diseases, were undergoing or required extensive dental or orthodontic treatment, were pregnant or breastfeeding, had extensive caries, had had periodontal treatment within the past 6 months, or had undergone antibiotic therapy in the previous 3 months were excluded from the study.

Study design and group methods

This clinical trial was a randomized, double blind, placebo controlled study (Trial registration: chictr.org, Identifier ChiCTR-TRC-13003576). Patients received full-mouth periodontal SRP, and then secnidazole/proanthocyanidins (Zhejiang Nanyang Pharmaceutical Co., Ltd. / Shenyang Zhongke Pharmaceutical Co. Ltd.) were administered within 48 hours. The subjects were randomly divided into 3 groups: secnidazole, proanthocyanidins and placebo group ($n=25$ per group). A one-month course of secnidazole 1g per day and proanthocyanidins 1.75 g per day were given orally. Clinical parameters were recorded at baseline, post-treatment and 3 months after treatment with secnidazole and proanthocyanidins. Laboratory parameters were assessed at baseline and post-treatment.

Clinical parameters

Clinical parameters for all of the teeth were measured at baseline, post-treatment and 3 months after treatment. The patients' complaints included: bleeding gums, sore gums, occlusal tooth pain, loose teeth. The following indexes were used for patient evaluation: i) L  e-Silness gingival index, GI (0-absence of inflammation; 1-mild

inflammation with slight change in colour and little change in texture; 2-moderate inflammation with moderate glazing, redness, edema, hypertrophy and bleeding on pressure; 3-severe inflammation with marked redness, hypertrophy, tendency for spontaneous bleeding and ulceration). ii) Gingival bleeding index, BI: 0 = gingival of normal texture and color, no bleeding; 1 = gingival normal, bleeds on probing; 2 = bleeding on probing, change in color, no oedema; 3 = bleeding on probing, change in color, slight oedema; 4 = bleeding on probing, change in color, obvious oedema; 5=bleeding on probing, spontaneous bleeding, change in color, marked oedema. iii) Turesky modified Quigley-Hein Plaque Index, PI: 0 = No plaque, 1 = Slight flecks at the cervical margin, 2 = thin continuous band (≤ 1 mm) at cervical margin, 3 A band of plaque > 1 mm but $< 1/3$ of the area, 4 covering $1/3$ $2/3$ of the area, 5 covering $\geq 2/3$ of the area. All clinical evaluations were performed by the same periodontist. iv) PPD was measured from the bottom of the periodontal pocket to the gingival line using a periodontal probe, with a controlled pressure at mesial, distal, buccal, and lingual sites. For each tooth, we considered the greatest PPD value measured among the four as the representative value. v) Clinical attachment level, CAL, defined as the depth (in mm) was measured from the cemento-enamel junction (CEJ) to the bottom of the gingival sulcus for each site corresponding to a tooth, and six pre-specified sites per tooth (excluding the third molars) were measured via a periodontal probe. For each tooth, we considered the largest CAL value measured among the six as the representative value.

Isolation and identification of suspicious pathogenic bacteria (expressed by colony forming unit, CFU), WBC count, and platelet count were also carried out.

Microbe samples collection and culture procedures

The samples were collected from the facial surface of selected teeth at baseline and post-treatment. A standard size (No. 15) paper point was inserted into the periodontal pocket for 2 min, then was withdrawn and placed in a sterile Eppendroff vial containing 1 ml of prerduced thioglycollate medium (Difco, Detroit, Mich., USA). The samples were vortexed for 10 s. After 10^5 times dilution, 50 μ l of each sample were inoculated onto 9 special agar plates in triplicate and cultured at 37  C. Plates including Anaerobic CDC blood agar plate (CDC),

Table I. Clinical results at baseline, post-treatment (PT) and 3 months after treatment (3 M after) with secnidazole or proanthocyanidins treatment.

Groups	period	PI	BI	GI	PPD	CAL
secnidazole	baseline	1.4 ± 0.5	1.9 ± 0.8	2.0 ± 0.9	6.4 ± 1.6	5.2 ± 1.8
	PT	1.2 ± 0.6	0.9 ± 0.6*	0.9 ± 0.7*	5.0 ± 1.1*	4.2 ± 1.0*
	3 M after	1.3 ± 0.6	0.9 ± 0.7*	1.2 ± 0.9*	5.4 ± 1.2*	4.4 ± 0.9
proanthocyanidins	baseline	1.5 ± 0.6	2.0 ± 0.8	1.9 ± 1.0	6.3 ± 1.2	5.2 ± 1.0
	PT	1.1 ± 0.5	1.0 ± 0.9*	1.2 ± 0.8*	5.1 ± 1.3*	4.3 ± 1.1*
	3 M after	1.3 ± 0.6	1.2 ± 0.9*	1.3 ± 0.9	5.3 ± 1.2*	4.5 ± 0.9
control group	baseline	1.3 ± 0.9	1.9 ± 0.9	1.8 ± 0.9	6.1 ± 1.2	5.0 ± 1.7
	PT	1.1 ± 0.8	1.3 ± 0.8	1.3 ± 1.0	5.2 ± 1.1*	4.3 ± 0.9
	3 M after	1.2 ± 0.8	1.3 ± 0.9	1.3 ± 0.8	5.3 ± 0.8	4.4 ± 0.9

* Differences of the post-treatment and 3 months after treatment as compared with baseline, ($p < 0.05$).

Table II. Changes in values between baseline and post-treatment of the percentages of bacterial frequency (BF) and bacteria colony-forming units (CFU, lgXG mean ± SD) in each treatment (n=25).

Suspected pathogen	secnidazole		proanthocyanidins		control group	
	BF	CFU	BF	CFU	BF	CFU
staphylococcus	15.2	3.0 ± 1.5	13.4	2.6 ± 1.5	5.8	0.4 ± 0.2
peptostreptococcus	9.5	1.3 ± 0.6	10.3	1.3 ± 0.8	3.1	0.3 ± 0.2
<i>P. asaccharolyticus</i>	26.2	3.9 ± 1.8	21.4	3.6 ± 1.3	6.0	0.8 ± 0.4
<i>P. melaninogenicus</i>	19.2	3.0 ± 1.7	23.4	3.1 ± 2.0	4.4	0.5 ± 0.3
<i>P. gingivalis</i>	18.6	3.3 ± 1.8	22.6	2.9 ± 1.4	4.2	0.6 ± 0.3
Fusobacterium	19.0	1.6 ± 1.0	20.3	2.2 ± 0.9	3.1	0.6 ± 0.4
<i>T. forsythia</i>	20.8	2.1 ± 1.7	16.9	2.6 ± 1.9	2.8	0.7 ± 0.4
<i>A. actinomycetemcomitans</i>	6.9	0.9 ± 0.6	5.0	0.9 ± 0.1	2.5	0.3 ± 0.1
Capnocytophagas	16.2	2.2 ± 1.0	12.5	2.0 ± 0.8	1.8	0.7 ± 0.4
other gram negative anaerobic bacillus	4.4	1.4 ± 0.4	5.7	1.3 ± 0.7	2.1	0.3 ± 0.1
other gram positive anaerobic bacillus	5.1	0.7 ± 0.1	3.8	1.1 ± 0.5	1.5	0.2 ± 0.1

kanamycin-vancomycin laked blood plate (KVLB), V-Rogosa selective blood agar plate (V-Rogosa), FS blood agar plate (FS), and PS blood agar plate (PS) for anaerobic bacteria were incubated in an anaerobic work station (BUG BOX DUAL, Fuskinn, UK) in 80% N₂, 10% H₂ and 10% CO₂ at 37°C for at least 4 days. Plates including Tryptic Soy Agar plate (TSA) for aerobic bacteria were incubated in an anaerobic work station in 90% N₂ and 10% CO₂ at 37°C for at least 2 days. After incubation, colonies formed on the plates were initially identified with the morphology using a stereomicroscope, and the colony forming unit (CFU) counts were recorded accordingly. The isolated bacteria were followed by 16S rRNA sequencing analysis. The measured 16S rRNA gene sequences of isolated strains were checked for their sequence homology by comparing with GenBank files. ATCC reference species were used as control.

Statistical analysis

Data points using SPSS11.5 analysis. Statistical analyses of the clinical results were carried out by one-way ANOVA ($\alpha=0.05$). The viable counts of bacterial frequencies were compared using *chi*-squared test. Multiple group means of CFU value were compared with single factor analysis of variance, and the comparison between any two means was performed with the least significant difference (LSD) method.

RESULTS

The two treatment groups showed greater mean reduction in BI, GI, and PPD evaluated at both post-treatment and 3 months after treatment compared to the control group ($p < 0.05$), but there were no significant differences in those of PI and CAL (except CAL evaluated at post-treatment, $P > 0.05$. Table I). There were no significant differences in the detection rate of each bacterium among the three groups ($P > 0.05$), but the detection rate had statistical differences within each group ($P < 0.05$) as shown in Table II. The composition of bacteria had no statistical difference among the three groups ($P > 0.05$), however, significant differences were found within each group ($P < 0.05$) as shown in Table II. Secnidazole and proanthocyanidins showed similar biological effects in this study.

DISCUSSION

These results showed that there were significant differences in the BI, GI and PPD between baseline and after secnidazole or proanthocyanidins treatment. The CAL reduction for secnidazole and proanthocyanidins in post-treatment were 1.07 ± 0.45 mm and 0.91 ± 0.42 mm. There were no significant statistical differences between the two treatment groups. Previous studies demonstrated that SRP plus systemic antimicrobial had better results in CAL and PPD change than SRP alone in the treatment of periodontitis (7). This clinical study showed that the adjunctive use of secnidazole in combination with scaling and root planing in adults with periodontitis is effective in reducing the pathogenic flora, and achieves significantly better clinical results than SRP therapy alone to a certain degree. Proanthocyanidins have shown inhibitory effects on the formation of oral biofilms *in vitro* (8). The inhibitory effect of proanthocyanidins on biofilm seems to be an explanation for its antibacterial effect in this study. Antibiotics given immediately after SRP provided an opportunity to control the bacterial biofilm formation again. The use of the systemic anti-microbials in combination with SRP achieved better clinical and microbial results to those of SRP alone (9, 10). Most cases of chronic periodontitis can be treated successfully by SRP, but certain defective sites continue to show periodontal tissue destruction despite conventional periodontal therapy. To solve this problem, antibiotics may be administered as an adjuvant treatment (3, 11). In conclusion, the adjunctive use of secnidazole or proanthocyanidins in combination with scaling and root planing in adults with periodontitis is effective in reducing the pathogenic flora, and achieves significantly better clinical results to a certain degree.

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LETTER TO THE EDITOR

CORRELATION BETWEEN CYSTATIN C AND RETINOPATHY OF TYPE-TWO DIABETES MELLITUS PATIENTS

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Diabetic retinopathy is one of most common diabetic microvascular complications. In recent years the incidence of the disease has increased, hence early diagnosis and treatment are of great importance. In order to find reliable biological indexes to diagnose and treat type-two diabetes mellitus promptly, this study focused on the correlation between Cystatin C (Cys C) and retinopathy of type-two diabetes mellitus patients. One hundred and eighty type-two diabetes mellitus patients and one hundred healthy controls (the control group) were chosen in this study. Of the patients ninety-eight patients had type-two diabetes mellitus without retinopathy (non-diabetic retinopathy group) and eighty-two had type-two diabetes mellitus with retinopathy (diabetic retinopathy group). Correlation of Cys C and type-two diabetic retinopathy was analyzed by examining the waist-hip ratio, fasting blood glucose (FBG), total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), glycosylated hemoglobin (HbA_{1c}), and Cys C of both groups. The results showed that FBG, TC, TG, LDL-C, HbA_{1c}, Cys C in the type-two diabetes mellitus patients group were higher than those of the control group ($P<0.05$). Age, course of diabetes, FBG, HbA_{1c}, and Cys C levels were statistically significant in both the DR group and NDR group ($P<0.05$). The result of logistic regression analysis indicates that there was a positive correlation between type-two diabetic retinopathy development and age, course of diabetes, and Cys C level ($P<0.05$). Thus, it can be seen that changes of Cys C levels can assist early diagnosis and treatment of diabetic retinopathy to some extent. The patients with high Cys C level, long course of diabetes, and old age are more likely to have diabetic retinopathy.

To the Editor,

Diabetic retinopathy (DR), a common diabetic eye complication, if not timely treated, may lead to lifelong blindness and has become the major cause of blindness in diabetic patients (1, 2). In recent years, as diabetic patients increase, the development of DR also shows an unprecedented growing tendency. Especially among adult type-two diabetic patients, 20-40% of patients have retinopathy, and

8% of patients lose their sight (3, 4). Therefore, early diagnosis and treatment of DR is of great significance.

Cys C, a cysteine proteinase inhibitor made of one hundred and twenty-two amino acids, exists extensively in karyocytes and body fluid in various tissues (5). Research has found that Cys C levels could predict early nephropathy of type-two diabetes mellitus patients (6). Reports also claimed that age-

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related macular degeneration (AMD) was potentially caused by growing levels of Cys C (7). The trace of Cys C was also found at vitreum and various layers of retinal cells (8). However, these studies were insufficient to prove a necessary correlation between Cys C and type-two diabetic retinopathy, and no specific data suggested that Cys C was a dangerous factor for type-two diabetes mellitus patients with retinopathy. The present study therefore investigated the correlation between Cys C level and the development of retinopathy by observing related index of Cys C level and retinopathy. The study also aims to increase the cognition level of DR, find the possible effective biological index used in early prevention, discovery, diagnosis and treatment, and to improve the patients' quality of life.

MATERIALS AND METHODS

One hundred and eighty type-two diabetes mellitus patients who were treated in the the First Affiliated Hospital of Zhengzhou University, Henan, China, between May 2013 and May 2015 were chosen for this study. All patients met the diabetic diagnosis and classification system released by World Health Organization in 1999 (9). Of these 180 patients, 106 were males and 74 were females; mean age: 55.2 ± 5.3 years; mean body mass index: 21.52 ± 2.26 kg/m²; the mean course of diabetes was 9.24 ± 7.31 years. Of 100 healthy controls 54 were males and 46 were females; mean age: 51.2 ± 3.2 years; mean body mass index: 22.71 ± 2.39 kg/m². The patients with type-one diabetes mellitus, serious cardiovascular and cerebrovascular diseases, severe hepatic and renal dysfunction, haematological diseases, malignant tumor and acute diabetes were excluded from the study. This study was reviewed and approved by the ethics committee of the First Affiliated Hospital of Zhengzhou University and written informed consent was obtained from all patients.

Research methods

Measurements of waistlines and hiplines of all patients were recorded to calculate the waist-to-hip ratios (WHR), and the calculation formula of WHR was: $\text{WHR} = \text{length of waistline (cm)} / \text{length of hipline (cm)}$. Fasting venous blood was collected, and Cys C, FBG, TC, TG, HDL-C and LDL-C concentration was

measured by latex-enhanced immutoturbidimetry using Hitach 7600 autobiochemistry, while HbA_{1c} was measured by BioRad D10 high performance liquid analysis. Fundus radiography examination was performed on all patients by TRC-NW300, TOPCON. One or two 45° posterior pole photochromes were taken for each eye and were analyzed by an ophthalmologist to make the diagnosis. DR can be divided into six stages: I-VI: DR at stages I to III is non-proliferative retinopathy, and DR at stage IV or VI is proliferative retinopathy. The patients were divided into two groups according to radiography results, i.e., NPR group (n= 98) and DR group (n=82).

Statistical analysis

Enumeration data were expressed as percentage (%), and measurement data were expressed as mean $\pm$ SD. Comparison of normal distribution test between the two groups was performed by analysis of variance, and comparison of rates was processed by *chi*-squared test. Correlation analysis was performed by Pearson correlation analysis, and Cys C and other variables were analyzed by logistic regression analysis. All data were analyzed by SPSS 20.0. $P < 0.05$ meant that difference was statistically significant.

RESULTS

Comparison of general materials among three groups

FBG, TC, TG, LDL-C, HbA_{1c} and Cys C levels in the DR and the NDR groups were higher than those of the control group ($P < 0.05$). By comparing the patients in the DR and NDR groups, we found that differences in age, course of diabetes, FBG, HbA_{1c} and Cys C were statistically significant and the other indicators had no statistical significance (Table I).

Correlations between diabetic retinopathy and various indexes

Diabetic retinopathy was positively correlated to Cys C, FBG, HbA_{1c}, course of diabetes and age (Table II).

Logistic multiple regression analysis of dangerous factors concerning type-two diabetic retinopathy

Regression analysis was performed by taking whether or not there was retinopathy as a dependent

Table I. Comparison between the three groups.

Indexes	The control group (n=100)	DR group (n=82)	NDR group (n=98)	Statistics	P
Age (years)	51.2±3.2	57.4±4.4* [#]	51.2±4.1	F=23.11	<0.05
Course of diabetes (years)	/	9.73±6.28* [#]	6.28±4.58*	t=0.619	<0.05
Weight (kg/m ²)	22.71±2.39	21.12±2.05	21.52±2.26	F=1.682	>0.05
Waist-to-hip ratios (%)	0.88±0.42	0.96±0.31	0.93±0.46	F=1.742	>0.05
FBG (mmol/L)	5.20±2.49	9.19±2.20* [#]	7.66±3.41*	F=83.764	<0.05
Cys C (mg/L)	0.59±0.49	1.15±0.26* [#]	0.82±0.75*	F=44.016	<0.05
HbA _{1c} (%)	5.51±0.46	11.14±1.27* [#]	9.08±1.68*	F=765.091	<0.05
TC (mmol/L)	4.74±0.51	5.14±0.79*	5.10±0.94*	F=13.106	<0.05
TG (mmol/L)	2.12±0.97	2.61±0.60*	2.44±0.67*	F=17.942	<0.05
HDL-C (mmol/L)	1.40±0.61	1.18±0.43*	1.26±0.43*	F=7.922	<0.05
LDL-C (mmol/L)	2.33±0.63	2.89±0.88*	2.56±0.58*	F=25.743	<0.05

Note: *means $P<0.05$, compared to the control group; [#]means $P<0.05$, compared to the NDR group. (mean±SD)

variable, and the course of diabetes, age, Cys C, FBG and HbA_{1c} as independent variables. The results suggested that Cys C (odd ratio (OR)=0.104, 95% confidence interval (CI)=0.028-0.386, $P<0.05$), course of diabetes (OR=0.892 95% CI 0.840-0.946, $P<0.05$), and age (OR=0.639, 95% CI=0.512-0.798, $P<0.05$) were risk factors leading to type-two diabetic retinopathy (Table III).

DISCUSSION

Diabetic retinopathy is one of the most common diabetic microvascular complications. In most cases, it coexists with diabetic nephropathy and the incidence of the disease increases with age. It has been proved that a total of 35.7% of diabetic patients in China had concurrent retinopathy. Therefore, to discover DR timely and offer effective treatment has

become a major issue for the prevention and control of the disease (10, 11).

The present study found that DR was in a positive correlation with age, the course of diabetes, Cys C, FBG and HbA_{1c}. The logistic multiple regression analysis suggested that the course of diabetes, age and Cys C were risk factors leading to retinopathy. This study also found that, HbA_{1c}, blood lipid and blood glucose were not risk factors influencing retinopathy, the reason for which might be that the treated patients had these factors under control or that the sample size was too small. Cys C being a key independent risk factor for age and course of diabetes was not easy to control. Owing to special metabolic process and expression sites, Cys C often connects with renal function changes and age-related eye disease (12, 13). Studies in recent years have shown that Cys C is also involved in the physiological and

Table II. Analysis of related factors leading to type-two diabetic retinopathy.

Variables	R	P
Age	0.327	<0.05
Course of diabetes	0.491	<0.05
FBG	0.357	<0.05
HbA _{1c}	0.221	<0.05
Cys C	0.615	<0.05

Table III. Logistic multiple regression analysis of risk factors concerning type-two diabetic retinopathy.

Factors	B	SE	P	95%CI
Age	0.448	0.113	0.000	0.512-0.798
Course of diabetes	0.112	0.029	0.000	0.840-0.946
Cys C	2.261	0.668	0.001	0.028-0.386

pathological process of vessel wall remodeling, angiogenesis, inflammation and neurodegeneration (14). Moreover, pathophysiological changes were often connected with precipitates of macula lutea, macular oedema, optic neuropathy, retinal neovascularization, inflammatory process and over expression of glial cells (15, 16). Therefore, the common pathway of Cys C and DR may be the reason they can be closely related and the theoretical basis of using Cys C level to predict severe DR.

In conclusion, Cys C level is an independent risk factor leading to diabetic retinopathy and the measurement of Cys C has great clinical significance in evaluating the development of chronic diabetic complications.

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LETTER TO THE EDITOR

SALIVARY CORTISOL, ALPHA-AMYLASE AND IMMUNOGLOBULIN A RESPONSES TO A MORNING SESSION OF BASKETBALL OR VOLLEYBALL TRAINING IN BOYS AGED 14–18 YEARS

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This study investigates whether a single session of routine morning basketball or volleyball training affects saliva levels of cortisol, alpha-amylase (sAA) and secretory immunoglobulin A (sIgA) in boys aged 14–18 years. Twenty-nine boys who participate in basketball or volleyball training, recruited from the Marcin Gortat's Athletic Championship School in Lodz, were enrolled in the study. The 90-minute routine exercise program included 15 minutes of warm-up followed by basketball or volleyball practice. Unstimulated saliva samples were collected prior to and immediately after the exercise, and were analysed using ELISA. One training session resulted in a significant increase of sAA concentration in all participants, as well as in the volleyball and basketball subgroups ($p=0.00022$; $p=0.0029$; $p=0.0011$; respectively). Post-exercise cortisol levels were significantly lower than pre-exercise levels ($p=0.00002$) throughout the group, as well as in the volleyball and basketball subgroups ($p=0.0048$; $p=0.0019$; $p=0.0048$; respectively). The exercise protocol did not significantly affect sIgA level, either in the whole examined group or the volleyball subgroup, however a weak significant increase of sIgA was observed in the basketball subgroup ($p=0.046$). The routine morning training session comprising a warm-up followed by basketball or volleyball practice seems to activate the sympatho-adrenal-medullary system, with a subsequent increase of alpha-amylase, but does not affect oral immunity in 14-18-year-old boys.

To the Editor,

Heavy exercise and training are considered physical stressors which may affect the function of the sympathetic nervous system and influence the immunological status. During intensive physical activity, the sympathoadrenal medullary (SAM) system and hypothalamic-pituitary-adrenal axis (HPA) are activated in a way that resembles stress reaction (1). Sympathetic stimulation seems to also

increase salivary protein secretion (2). Salivary alpha-amylase (sAA), synthesized in the acinar cells of the salivary glands is a digestive calcium-containing metalloenzyme, which is important also for mucosal immunity (3). As there is direct evidence for the sensitivity of sAA to changes in adrenergic activation (3), and measurements of catecholamines in saliva have been problematic, sAA was adopted to be a valid marker of catecholaminic activity and the

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simple indicator of the SAM response to stress (2). A number of studies report increased level of alpha-amylase in response to exercise (4-6). However, another study reports decreases in sAA (7).

Activation of the HPA is associated with release of corticotrophin-releasing hormone, and adrenocorticotrophic hormone which, in turn, stimulates the secretion of glucocorticoid hormones, mainly cortisol (1). As the levels of cortisol in its free form in blood correlate with those in saliva, and salivary measurements of steroids are unaffected by variations in flow rate, a non-invasive saliva sampling has become the method of choice for assessing the HPA activation, also in response to exercise (3, 8). It has been suggested, that training early in the morning may have a negative influence on immunity status due to the possibility of a synergic suppressive effect by the circadian rhythm of cortisol secretion, which peaks in early morning, and exercise (9).

It has also been suggested that exercise can influence the mucosal immune system, and increase the risk of recurrent upper respiratory tract infections, via decreases in salivary immunoglobulin class A (sIgA) levels (3, 10). Although there are many data on hormonal and immunological response to exercise in adults, such data are rather rare in adolescents. Furthermore, although basketball is the most popular team sport among adolescents, and volleyball is also popular, to the best of our knowledge no studies have been published on boys' hormone responses to those sports activities. Additionally, there is only one study that examined immunological response to basketball practice, and pointed out that basketball exercise can increase sIgA levels (11). The objective of the present study was therefore to examine whether a single session of routine morning basketball or volleyball training affects saliva levels of cortisol, sAA and sIgA in boys aged 14–18 years.

MATERIALS AND METHODS

Subjects

Twenty-nine boys aged from 14 to 18 years (mean age 16.76 ± 1.6 years), who do basketball or volleyball training, participated in the study. All subjects were recruited from the Marcin Gortat's Athletic Championship School in

Lodz, which provides sport education in lower and upper secondary school levels. Twelve of the participants who agreed to participate in the study were attending classes with basketball profile (mean age 16.07 ± 1.7 years), and 17 in the volleyball program (mean age 17.03 ± 1.01 years). All participants were in regular training 7.5 hours a week (5 sessions of 90 minutes) of the chosen sport discipline. All subjects were generally healthy, were non-smokers and were not using any medication or supplements that could affect the immune system function. All subjects 18 years of age gave written informed consent, while consent by parents or guardian was given for subjects under 18. The study protocol was approved by the Medical University of Lodz Bioethics Committee, Resolution Number RNN/311/13/KE, and conformed to the principles of the Helsinki Declaration.

Study projects

The routine 90-minute session of training of all participants took place in the hall of the City Sports and Recreation Centre. The subjects exercised from 7:30 to 9:00 am. The exercise program included 15 min of warm-up followed by basketball or volleyball practice. All study participants were familiar with the training protocol as it was a part of the regular training procedure for basketball or volleyball school players. Unstimulated saliva samples were collected prior to and immediately after the exercise session.

Saliva collection

Unstimulated whole saliva (UWS) was collected by spitting into a glass tube with a screw top, for 3 min. Subjects were seated in an upright position and asked to relax during spitting. The participants were instructed to avoid swallowing the saliva during sample collection to allow the saliva to accumulate in the floor of the mouth and to spit into test tubes every 30 s. In our study passive drooling of unstimulated saliva was used, as the literature indicates that stimulated saliva secretion increases the concentration of s-IgA secreted from the parotid saliva gland, probably due to increased epithelial cell transcytosis of IgA (3, 10). All saliva samples were kept in shipping boxes with dry ice and transported to the laboratory within 20 min from collection of post-training saliva samples. The samples were stored at -80°C until laboratory analysis. Saliva samples were centrifuged and

sAA, sIgA and cortisol were assayed using commercial diagnostic kits.

Saliva analysis

The saliva concentration of cortisol was measured by competitive enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D System, Abingdon, UK). Results were expressed in ng/ml. The sensitivity of the immunoassays was 0.071 ng/ml. No significant interference was observed. Cross-reactivity for: prednisolone was 4.4%; Reichstein's substance S 3.4%; progesterone 1.7%; cortisone 0.2%; other compounds <0.1%. Secretory IgA in saliva was measured using specific ELISA (Immunodiagnostik AG, Bensheim, Germany) in accordance with the test procedure. Results were expressed in ng/ml. The detection limit was 13.4 ng/ml. No cross reactivity was noted with other proteins in saliva. Quantitative assessment of alpha-amylase in saliva was performed using competitive ELISA (EUROIMMUN, Lübeck, Germany) in agreement with the test instructions. Results were expressed in U/ml. The detection limit of sAA was 3.6 U/ml. Cross reactions with other amylases were virtually excluded, as the specificity

for the assay was above 99.7%. The EUROIMMUN ELISA reported correspondence with salimetrics alpha amylase enzyme assay of $R=0.98$.

Statistical analysis

All laboratory data was tested for normality using the Shapiro-Wilk test. As cortisol, IgA and alpha-amylase concentrations were found to have non-normal distribution, the differences between their median values before and after the exercise session were tested with non-parametric test: the Wilcoxon signed-rank test. To analyze differences between subgroups (volleyball vs basketball subgroup) the Mann-Whitney test was used. Correlations were assessed by the Spearman's rank correlation coefficient. Differences were considered significant if p was less than 0.05. Statistical analysis was performed using Statistica for Windows software (version 10.0).

RESULTS

None of the pre-training values were found to differ between the volleyball and basketball groups. Similarly, no statistically significant differences were

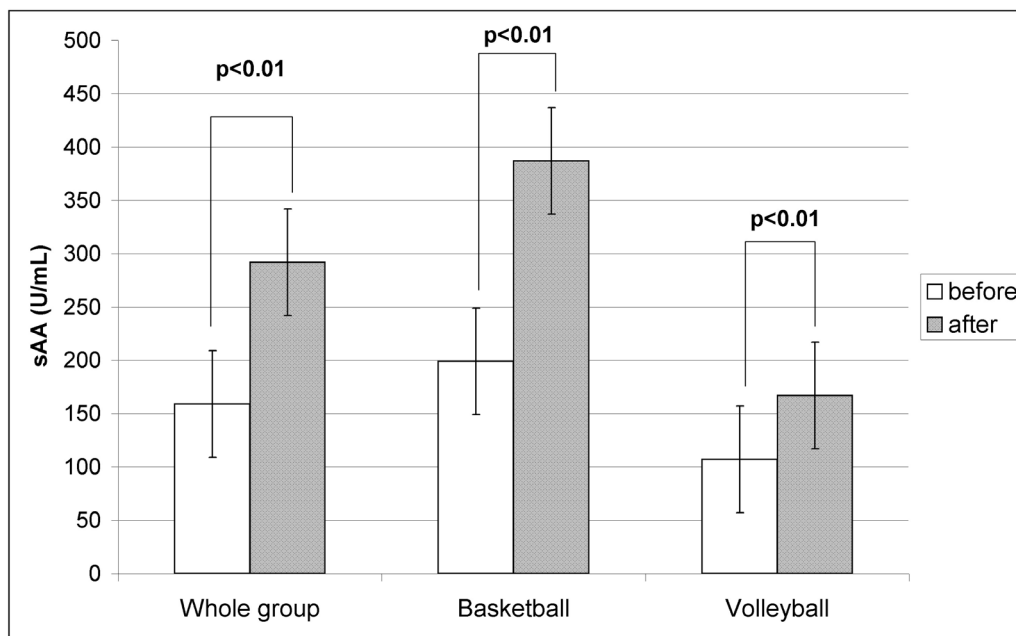


Fig. 1. Pre- and post-exercise sAA concentrations in whole group and in the basketball and volleyball subgroups. The bars show medians with interquartile ranges.

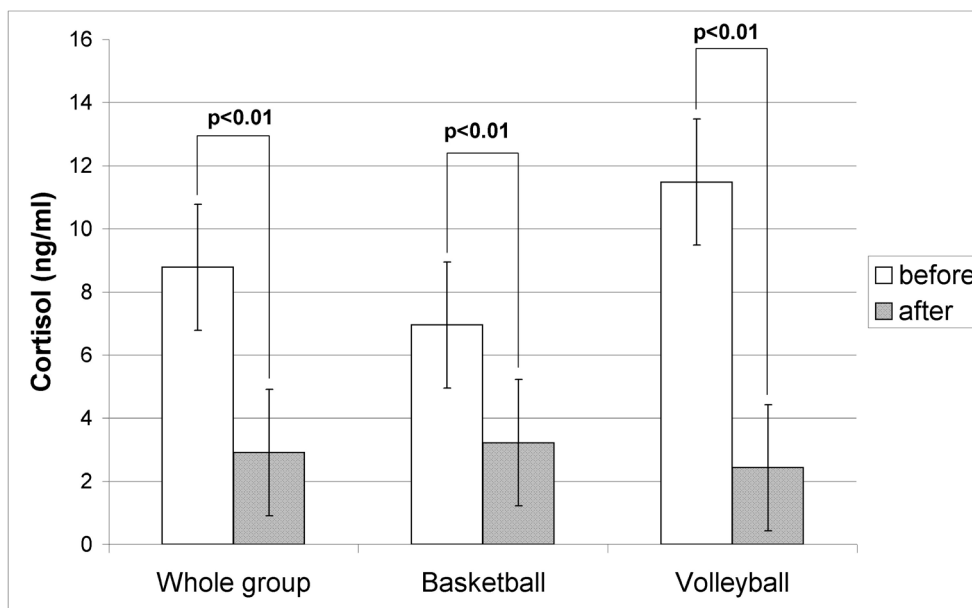


Fig. 2. Pre- and post-exercise cortisol concentrations in whole group and in the basketball and volleyball subgroups. The bars show medians with interquartile ranges.

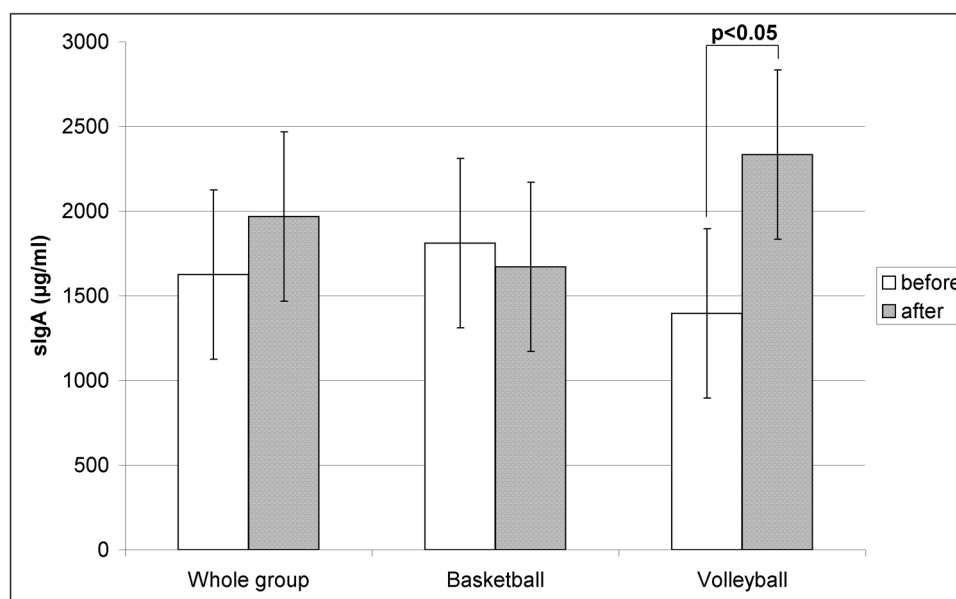


Fig. 3. Pre- and post-exercise sIgA concentrations in whole group and in the basketball and volleyball subgroups. The bars show medians with interquartile ranges.

found between those two subgroups with regard to the post-training saliva parameters: cortisol, sAA or sIgA. The results of the present study showed that one session of school training results in a significant increase of sAA concentration in all participants, as well as in the volleyball and basketball subgroups ($Z=3.69$, $p=0.00022$; $Z=2.97$, $p=0.0029$; $Z=2.53$, $p=0.0011$, respectively) (Fig. 1). Salivary cortisol was significantly lower post-exercise than pre-exercise ($Z=4.26$, $p=0.00002$) in all participants, as well as in the volleyball and basketball subgroups ($Z=2.81$, $p=0.0048$; $Z=3.11$, $p=0.0019$; $Z=2.81$, $p=0.0048$, respectively) (Fig. 2). Although no significant changes were observed in sIgA level, either in the whole group or the basketball subgroup ($p>0.05$), a weak significant increase of sIgA was observed in the volleyball group ($Z=1.99$; $p=0.046$) (Fig. 3). No correlation was found between pre-exercise values of cortisol and sAA or IgA. However, post-training cortisol concentration positively correlated with post-training alpha-amylase concentration ($r = 0.367$, $p < 0.05$). No correlation was found between age of participants and any of the saliva parameters.

DISCUSSION

The findings of the present study indicate a decrease of cortisol level after volleyball or basketball training session, which is in accordance with previous studies concerning the response to rugby training exercise protocols (12, 13). It was shown that neither soccer practice, soccer match competition nor short bouts of sprint cycling have any significant effect on saliva cortisol concentration in boys (14, 15). In contrast to our results, some studies have found cortisol levels to increase during exercise in a competition environment (4, 6). It has been suggested that when components of real competitions are removed, the stress related to exercise itself is not sufficient to induce a significant impact on cortisol in saliva (8). As the time of exercise ranged in the present study from 7.30 to 9.00 am, it is possible that the training-related stress was insufficient to affect the diurnal pattern of cortisol secretion, with a significant burst of concentration 30 min after

awakening, followed by a rapid decrease until 10 am (16). Also, Smilios et al. noted that in exercise protocols performed at submaximum velocities cortisol concentration decreased in accordance with the circadian rhythm (17).

The results of our study indicate that sAA concentration increases in response to exercise. This is in line with previous studies, as post-exercise increases of sAA were observed in both non-competition (5), and in competition environments (4, 6). Salivary alpha-amylase has been reported to be a more sensitive marker of exercise-induced stimulation of adrenomedullary nervous system than cortisol, as it is produced locally in the salivary glands (3). Also, Kivlighan and Granger reported larger increases of sAA compared with cortisol in response to an ergometer competition (6). The positive correlation of post-exercise values of sAA and cortisol may indicate that if the physical stressor caused greater activity of SAM, the HPA was also activated during the exercise session, but the reaction of SAM was stronger or faster. In contrast to our data, others found no correlation of those two parameters in males (11, 13).

Some findings are in accordance with ours, as they show sIgA concentration to be unaffected immediately after submaximal swimming (9) as well as sprint cycling (15). However, strenuous and prolonged (>2h) bouts of acute exercise or a competitive marathon race have been reported to cause a decrease in sIgA concentration in trained athletes (3, 10). In contrast, moderate exercise can be associated even with increased sIgA levels (3). The present study also reveals an increase of sIgA when training included a volleyball game, which is regarded by Centers for Disease Control and Prevention and the American College of Sports Medicine as moderate activity.

In conclusion, a routine morning training session (from 7.30 am to 9.00 am) consisting of warm-up followed by a basketball or volleyball match appears to cause the sympatho-adrenal-medullary system activation, with a subsequent increase of alpha-amylase immediately after exercise. However, it does not appear to affect oral immunity in boys who do basketball or volleyball training.

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LETTER TO THE EDITOR

EFFECTS OF HUMAN PARATHYROID HORMONE ON BONE MORPHOGENETIC PROTEIN SIGNAL PATHWAY FOLLOWING SPINAL FUSION IN DIABETIC RATS

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Osteoporosis is a major complication in patients with diabetes mellitus. Thus, it is crucial to study the signal mechanisms responsible for enhancement of bone mass in diabetes. Administration of human parathyroid hormone (hPTH) has been reported to prevent osteoblast apoptosis and have anabolic effects on bone in animals and humans. In the present study, we examined the effects of hPTH on expression of bone morphogenetic protein type 2 (BMP-2) and its receptor BMPR2 in diabetic rats following spinal fusion. Our data show that hPTH amplified BMP-2 and BMPR2 in bone tissues of non-diabetic rats, but not in diabetic rats. Our data further demonstrate that hPTH plays a role in regulating BMP-2 and BMPR2 via mTOR-PI3K signal pathway. We suggest specific signaling pathways by which hPTH regulates BMP-2 via mTOR-PI3K mechanism in bone formation following spinal fusion. Notably, our data indicate under diabetic conditions this signal pathway is impaired, thereby likely affecting bone formation after spinal fusion. The subsequent induction of BMP-2 and BMPR2 are likely a part of the protective effects aimed at attenuating pathological bone damage as a result of diabetes.

To the Editor,

Osteoporosis is a major complication in patients with diabetes mellitus, especially in those with insulin dependency (1-3). The major causes of bone loss in these patients and animal models include primary disturbances in calcium or vitamin D metabolism, chronic malnutrition, chronic vascular disease of bone as well as a negative bone balance resulting from decreased bone formation (1-3). Without doubt, it is crucial to maintain or enhance bone mass in diabetic patients with worsening bone formation, and provide treatment with drugs that have anabolic effects by increasing bone formation.

Intermittent administration of human parathyroid hormone (hPTH) has been reported to prevent

osteoblast apoptosis and have anabolic effects on bone in animals and humans (4-7). Thus, hPTH is one of the candidates for the treatment of diabetes-induced osteoporosis. Furthermore, recent studies have reported that bone morphogenetic proteins (BMPs) enhance bone formation (8). Numerous animal studies have established that BMPs (i.e., BMP-2) can play a beneficial role in regulating spinal fusion (8). However, there is a lack of data on the effect of BMP-2 on spinal fusion in the osteoporotic spine, in particular, on spinal fusion in diabetes.

Accordingly, the purpose of this study was to examine the effects of hPTH on BMP-2 levels and expression of its receptor, namely BMPR2 in bone tissues of control rats and diabetic rats following

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spinal fusion. We hypothesized that systemic administration of hPTH increases expression of BMP-2 and BMPR2, and the effects of hPTH are lessened in diabetic animals.

In addition, mammalian target of rapamycin (mTOR) is a serine threonine protein kinase. Activation of mTOR, in particular, mTOR complex 1 (mTORC1) that is more sensitive to rapamycin, leads to promotion of the phosphorylation of downstream effectors, such as p70 ribosomal S6 protein kinase (p70S6K) and this further governs mRNA translation (9). The mTORC1 is well known for its critical roles in the regulation of protein synthesis and growth, furthermore the compelling evidence supports the widespread dysregulation of mTOR in development of diabetes (10). Thus, we further hypothesized that mTOR-phosphatidylinositol 3-kinase (p-PI3K) pathways are engaged in regulation of hPTH in expression of BMP-2 signal pathway.

MATERIALS AND METHODS

Animals

All the animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Shandong University, which were in compliance with the Guidelines of the International Association for the Study of Pain. Ninety-two male Sprague-Dawley rats (9 weeks old) weighing 200–250 g were used in this study.

Induction of diabetes

Streptozotocin (STZ) was freshly dissolved in 0.9% sterile saline and diabetes was induced by a single injection of STZ (65 mg/kg i.p., Sigma Co.) as described previously. Diabetes was confirmed by measurements of blood glucose concentrations in samples obtained from the tail vein 4 weeks after injection of STZ. Rats with blood glucose concentration > 350 mg/dl were included in the study (n=48). Age- and body weight-matched rats injected with saline were used as controls of non-diabetes (n=44).

Spinal surgical procedures

The STZ rats and non-STZ rats were anesthetized with an isoflurane-oxygen mixture (2–5% isoflurane in 100% oxygen). An endotracheal tube was first inserted and attached to a ventilator during the surgery. After shaving

and prepping the surgical site, the rats were placed prone on the operating table. Under aseptic conditions, a dorsal skin incision at the L1–L3 level was made. The dorsal muscles were shifted laterally and the spinous processes were exposed. Soft tissues including periosteum were removed from the spinous processes and dorsal part of bony vertebral arches. Titanium microplates with thickness 0.5 mm and titanium screws were used for spinal fusion. Spinous processes of two adjacent vertebrae were fixed. The space between bony vertebral arches and spinous processes was filled with augmentation material and covered with a HyproSorb resorbable collagen membrane. The soft tissues and skin were sutured with resorbable thread. For pain relief and infection prevention those rats immediately received buprenorphine (0.02 mg/kg, daily) and gentamicine (0.5 mg/kg, daily) subcutaneously for 5 days.

Administration of drugs

Subcutaneous injections of hPTH (4.0 µg/kg body weight, daily) was initiated just after spinal surgery and continued until the rats were sacrificed for collection of samples. Accordingly, STZ rats and non-STZ rats were randomized into four groups: STZ rats were given subcutaneous injections of 0.9% saline (n=16); STZ rats were given subcutaneous injections of hPTH (n=32); non-STZ rats that received saline (n=16) and hPTH (n=28). In a subset of experiments, rapamycin (mTOR inhibitor, obtained from Selleckchem; 1 mg/kg daily) and LY294002 (PI3K inhibitor, obtained from Sigma-Aldrich; 0.5 mg/kg daily) were given in STZ rats and non-STZ rats (n=14 in each group) until the rats were sacrificed 8 weeks after the spinal surgery. With overdose of isoflurane autologous bone tissues were harvested from the iliac crests bilaterally through the fascial incisions for biochemical analysis.

ELISA

Total protein was extracted by homogenizing sample in ice-cold radioimmunoprecipitation assay buffer with protease inhibitor cocktail kit. The lysates were centrifuged and the supernatants were collected for measurements of protein concentrations using a bicinchoninic acid assay reagent kit. The levels of BMP-2 were determined using an ELISA assay kit (Abcam Co.) according to the provided description and modification. Briefly, polystyrene 96-well microtiter immunoplates were coated with affinity-purified polyclonal rabbit anti-BMP-2 antibody. Parallel

wells were coated with purified rabbit IgG for evaluation of nonspecific signal. After overnight incubation at room temperature and 2 h of incubation with the coating buffer containing 50 mM carbonate buffer (pH 9.5) in 2% BSA, plates were washed with 50 mM Tris-HCl. After extensive washing, the diluted samples and the BMP-2 standard solutions were distributed in each plate and left at room temperature overnight. The plates were then washed and incubated with anti-BMP-2 galactosidase per well. Then, the plates were washed and incubated with substrate solution. After an incubation of 2 h at 37°C, the optical density was measured using an ELISA reader.

Western blot analysis

The protein expression of BMPR2, p-mTOR and p-PI3K was determined by using a standard Western blot analysis. After being denatured by heating at 95°C in an SDS sample buffer, the supernatant samples were loaded onto 4-20% Mini-Protean TGX Precast gels and then electrically transferred to polyvinylidene fluoride membranes. Membranes were incubated with rabbit anti-BMPR2, anti-p-mTOR and anti-p-PI3K primary antibodies (1:500, obtained from Abcam Co.). After being fully washed, the membrane was incubated with horseradish peroxidase-linked anti-rabbit secondary antibody (1:250, obtained from Santa Cruz Co.) and visualized for immunoreactivity. The membrane was also processed to detect β -actin for equal loading. The bands recognized by the primary antibody were visualized by exposure of the membrane onto an X-ray film. The film was then scanned and the optical densities of protein bands were analyzed using the Scion image software (obtained from the NIH).

Statistical analysis

All data were analyzed using a two-way repeated-measures analysis of variance. Values were presented as means $\pm$ standard error of mean (SEM). For all analyses, differences were considered significant at $P < 0.05$. All statistical analyses were performed by using SPSS for Windows version 13.0 (SPSS Inc., Chicago, USA).

RESULTS

Measurements of blood glucose

Previous studies have reported that the levels of blood glucose begin to increase 1 day after systemic

injection of STZ and remain at a higher level for >10 weeks (11, 12). Consistent with the previous studies, the results of our current study show that the animals (n=48) had developed hyperglycemia by 4 weeks after STZ ($P < 0.05$ vs. non-STZ rats, n=44) as compared with non-STZ rats of saline control. At 12 weeks after STZ the samples were obtained and hyperglycemia was still observed ($P < 0.05$ vs non-STZ rats) and injection of hPTH did not alter the levels of blood glucose per se.

Effects of hPTH on the levels of BMP-2 and expression of BMPR2

Fig. 1A shows that administration of hPTH significantly increased the levels of BMP-2 in non-STZ rats ($P < 0.05$ vs saline control rats, n=16 in each group) as compared with saline control group. Fig. 1B further shows that hPTH significantly increased protein expression of BMPR2 in non-STZ rats ($P < 0.05$ vs saline control rats, n=8 in control; n=10 with injection of hPTH) as compared with saline control group. Notably, Fig. 1, A and B demonstrate that the effects of hPTH on BMP-2 and BMPR2 were blunted in STZ rats ($P > 0.05$, saline control vs hPTH).

Effects of hPTH on mTOR-PI3K signal pathway

Likewise, Fig. 1C shows that administration of hPTH significantly amplified expression of p-mTOR and p-PI3K in non-STZ rats ($P < 0.05$ vs saline control rats, n=8 in each group) as compared with saline control group. Nonetheless, hPTH failed to increase both p-mTOR and p-PI3K in STZ rats ($P > 0.05$, saline control vs hPTH).

Effects of blocking mTOR and PI3K on amplified BMP-2 and BMPR2

In order to examine the role played by mTOR-PI3K pathways in regulating the amplified BMP-2 and BMPR2 evoked by hPTH, rapamycin and LY294002 were used to inhibit mTOR and PI3K signals, respectively. Fig. 2, A and B demonstrate that blocking mTOR by using rapamycin significantly attenuated elevation in the levels of BMP-2 and expression of BMPR2 in non-STZ rats ($P < 0.05$ vs saline control and hPTH injection with rapamycin).

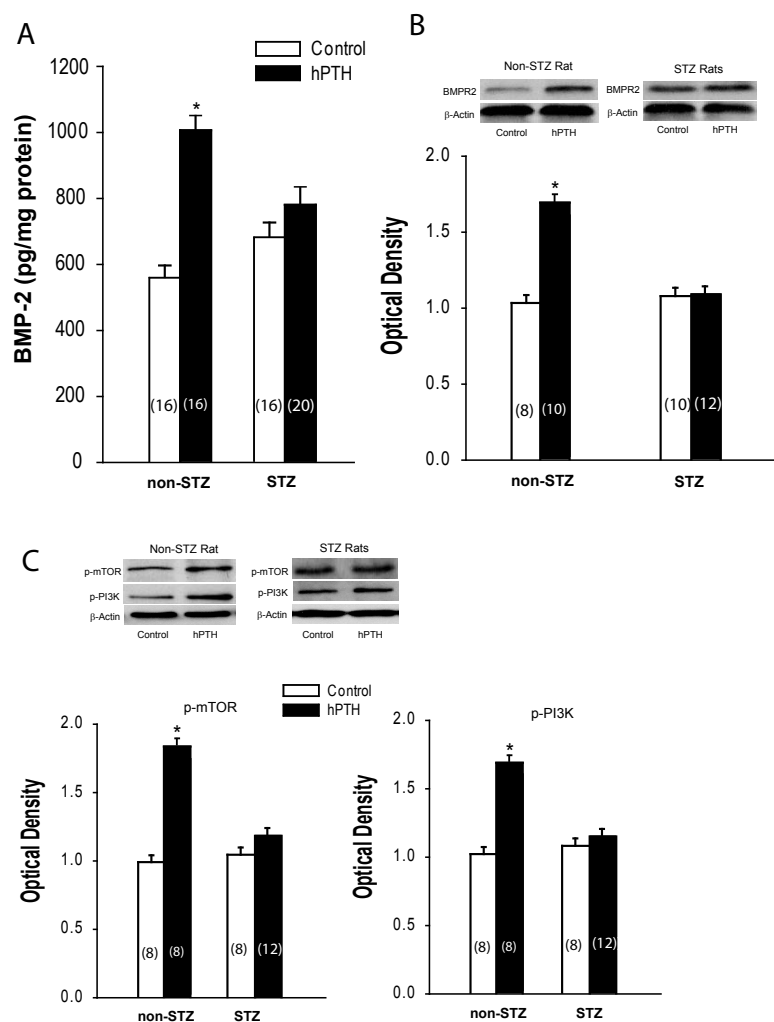


Fig. 1. Effects of hPTH on BMP-2/BMPR2 and mTOR/PI3K signal pathways. A,B) hPTH significantly increased both BMP-2 and BMPR2 in non-STZ rats. C) Effects of hPTH on expression of mTOR-PI3K. mTOR and PI3K were amplified by hPTH in non-STZ rats. * $P < 0.05$ vs saline control rats. The number of animals is indicated in each bar. In B and C, representative bands are shown in the top panels.

Similarly, Fig. 2, C and D show that LY294002 attenuated the amplified BMP-2 and BMPR2 evoked by hPTH in non-STZ rats ($P < 0.05$ vs saline control and hPTH injection with LY294002).

DISCUSSION

The results of the current study demonstrate that i) hPTH amplifies BMP-2 and BMPR2 in bone tissues of non-diabetic rats, but not in diabetic rats; and ii) the effects of hPTH are via mTOR-PI3K signal pathway.

PTH is secreted by the parathyroid glands in

response to low serum calcium and vitamin D levels (13). PTH stimulates calcium reabsorption, phosphate excretion, and the production of active vitamin D in the kidney (13). Both PTH and PTH-related peptide (PTHrP) act through the G protein coupled parathyroid hormone 1 receptor (PTH1R), which is most highly expressed in kidney and bone and affects signaling in osteoblasts and osteocytes (13-15).

Interestingly, administration of hPTH decreases the period of time required for graft bone healing and produces a more solid fusion mass in the rat posterolateral spinal arthrodesis model (16). The

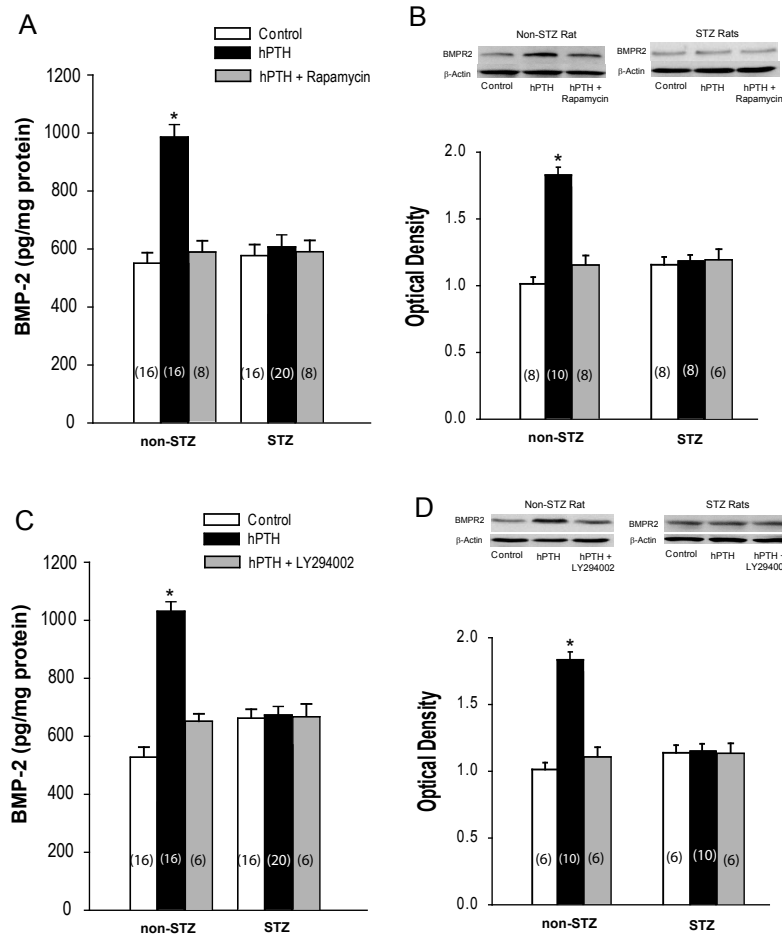


Fig. 2. Effects of blocking mTOR and PI3K on BMP-2 and BMPR2 signals. A,B) Inhibition of mTOR by rapamycin blunted the effects of hPTH on BMP-2 and BMPR2. C,D) Inhibition of PI3K pathway by LY294002 diminished the increases of BMP-2 and BMPR2 evoked by hPTH. * $P < 0.05$ vs saline control and hPTH plus respective inhibitors, rapamycin and LY294002. The number of animals is indicated in each bar. The top panels (B and D) show typical bands that are representative of expression of BMPR2.

accelerating effects of PTH on bone graft healing are considered to be beneficial for spinal fusion surgery and other skeletal diseases requiring bone grafts (16). Additional study suggests that hPTH given alone or with insulin has an anabolic effect on STZ-induced osteopenia by increasing the bone formation rate significantly (17). Results of the present study show that systemic administration of hPTH amplifies the levels of BMP-2 and expression of its receptor BMPR2 in bone tissues following spinal fusion.

BMPs are originally identified to induce the formation of bone and cartilage when they are

implanted at ectopic sites in rats (8, 18). Nevertheless, BMP-like molecules have been found in vertebrates as well as in invertebrates, and BMPs are known to exhibit a wide range of biological effects on various cell types (8, 18). In addition to induction of bone and cartilage tissues, BMPs regulate tooth, kidney, skin, hair, muscle, haematopoietic and neuronal development, and maintain the iron metabolism and vascular homeostasis *in vivo* (8). Notably, among BMPs BMP-2 acts as a disulfide-linked homodimer and induces bone and cartilage formation and plays a key role in osteoblast differentiation (8). To the best of

our knowledge, the results of our current study show for the first time the role played by hPTH in regulating BMP-2 signal pathway. In particular, our data suggest that the effects of hPTH are impaired in STZ animals after spinal fusion, which is consistent with the notion that diabetes has the worse bone formation.

The mTOR signaling pathways are important mechanisms responsible for osteoclast survival in bone tissues (19, 20). Rapamycin is a classical inhibitor of mTOR activity. Prior studies have demonstrated that rapamycin plays a role in regulating osteolysis and survival in animal models of bone cancer (21). Also, administration of rapamycin significantly attenuates PI3K expression, suggesting that mTOR-PI3K signal pathway is involved in bone formation. In the present study, our data show that blocking mTOR or PI3K significantly attenuates the promoting effects of hPTH on BMP-2. Thus, mTOR-PI3K signal pathway is necessary to be involved in the role played by hPTH in amplifying BMP-2 and BMPR2 in bone formation following spinal fusion.

In conclusion, our data suggest specific signal pathways by which hPTH regulates BMP-2 pathway during bone formation after spinal fusion. Also, mTOR-PI3K signals are engaged in the effects of hPTH on BMP-2. Notably, our data indicate that under diabetic conditions these signal pathways are lessened, thereby likely affecting bone formation after spinal fusion. The subsequent induction of BMP-2 and its target signal pathways are likely a part of the protective effects aimed at attenuating pathological bone damage as a result of diabetes. Thus, targeting one or more of these signaling molecules has clinical implications for management of spinal fusion in diabetic patients.

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LETTER TO THE EDITOR

EFFECTS OF ANESTHESIA USING PROPOFOL AND ETOMIDATE ON T LYMPHOCYTE SUBPOPULATION OF INFECTIOUS SHOCK PATIENTS IN PERIOPERATIVE PERIOD

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Infectious or septic shock is induced when a toxic microorganism invades blood circulation in the human body. Emergency operation is an effective method for treating infectious shock in the early stages although the use of anesthesia is more complex due to the internal disorders caused by the disease. This study explored the effects of propofol and etomidate anesthesia on the cellular immune function (T lymphocyte subpopulation) of infectious shock patients, aiming to provide a basis for the selection of the proper anesthetic method. One hundred and twenty patients with infectious shock were selected and randomly divided into an observation and a control group. The control group were narcotized using propofol, while the observation group were narcotized using etomidate. The effects on the immune functions of patients and drug-related adverse reactions were compared between the two groups. Results demonstrated that the levels of CD_3^+ and CD_4^+ of the two groups were similar before anesthesia and the differences had no statistical significance ($P>0.05$). After anesthesia, the levels of both groups showed a tendency to decrease and the levels of CD_3^+ and CD_4^+ of the observation group were much higher than those of the control group in the different periods. The differences were statistically significant ($P<0.05$); the differences of CD_8^+ level and CD_4^+/CD_8^+ between the two groups had no statistical significance before anesthesia ($P>0.05$); after anesthesia, CD_8^+ level and CD_4^+/CD_8^+ of the observation group were all much higher than those of the control group in the different periods and the differences had statistical significance ($P<0.05$). Therefore, the conclusion is that etomidate anesthesia has little influence on the immune functions of infectious shock patients in perioperative period and the incidence of adverse reaction is low, hence, worth clinical promotion.

To the Editor,

Infectious shock refers to septic syndrome accompanied by shock induced by pathogenic microorganism, toxicity or its metabolites. Immune dysfunction of the human body is a major cause for infectious shock. The occurrence of infectious shock

is the result of the immune disorder of inflammatory reaction and anti-inflammatory reaction (1, 2). Manifestations are pale complexion, anxiety, nausea and rapid heart rate. With its progression, patients gradually demonstrate loss of consciousness, slow breathing, decreased blood pressure and often,

Key words: propofol, anesthesia, etomidate, T lymphocyte subgroups

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important organ failure (3, 4). Clinically, systemic anesthesia is required for patients with severe systemic shock before surgery, but anesthesia can produce direct or indirect influence on the immune system of the human body. Therefore, the selection of a safe anesthetic drug is important as it can ensure a successful surgical outcome (5).

Propofol, a frequently used anesthetic drug, can promote the increase of hypothalamo-pituitary-adrenal axis, excite sympathetic nervous system, induce the secretion of more catecholamine and glucocorticoids into the blood and effectively inhibit immune function (6). Moreover, propofol retains sodium, potassium and calcium ion channel, directly regulating cellular immunity (7). Etomidate, also a common anesthetic drug, rapidly enters the brain, organs that present rich blood perfusion, muscle and adipose tissue which are normally slow in intake. It also has a short half-life period and loses activity after being hydrolyzed by esterase in liver and plasma (8). This study discussed the effects of different anesthetic drugs on human immune functions by analyzing the effects of propofol and etomidate on T lymphocyte subgroups of infectious shock patients in perioperative period.

MATERIALS AND METHODS

Research object

One-hundred-and-twenty patients who were diagnosed with infectious shock in our hospital from May 2013 to May 2015 and were to undergo focal clearance surgery, were selected. All patients showed apathy and slow response and had $>38^{\circ}\text{C}$ or $<36^{\circ}\text{C}$ body temperature, 90 times/min higher heart rate, 20 times/min higher respiratory frequency and 90 mmHg (1 mmHg=0.133 Kpa) higher systolic pressure. Patients with high blood pressure, diabetes, coronary heart disease, cerebral infarction or hepatic renal dysfunction were excluded. All patients were randomly divided into an observation group and a control group. In the control group, comprising 31 males and 28 females (mean age 42.5 ± 2.3), anesthesia using propofol was administered; there were 23 cases of biliary tract disease, 12 cases of diffused purulent peritonitis and 5 cases of acute necrotizing pancreatitis. In the observation group, comprising 35 males and 25 females (mean age 41.4 ± 2.2), anesthesia using etomidate

was administered; there were 21 cases of biliary tract disease, 11 cases of diffuse purulent peritonitis, 11 cases of gastrointestinal operation-induced infection, 9 cases of gastric ulcer and 8 cases of acute necrotizing pancreatitis. The Medical Ethics Committee of the hospital approved this study and all subjects signed prior informed consent.

Anesthetic methods

Patients in the control group were narcotized by propofol and were given 1~2 mg/kg propofol, 0.01~0.02 mg/kg midazolam, 2~4 g/kg fentanyl and 0.1 mg/kg vecuronium bromide for anesthesia induction; the dose depended on the clinical symptoms and disease history of patients. Tracheal intubation was then performed immediately for mechanical ventilation. During surgery, propofol [4~6mg/(kg·h)] and remifentanyl [10~12 $\mu\text{g}/(\text{kg}\cdot\text{h})$] were continuously pumped and vecuronium bromide was discontinuously injected through veins for anesthesia maintenance. The dose of drugs was adjusted during surgery according to the vital signs of patients. Patients in the observation group were narcotized by etomidate. Etomidate [0.2~0.3 mg/(kg·h)] or 4~6 mg/(kg·h) were used for whole-course anesthesia; the dose depended on the clinical symptoms and disease history of patients. Conventional anti-infection treatment was carried out in both groups using relevant anti-bacterial drugs. Moreover, fluid infusion and dilatation of blood volume were performed according to the vital signs of the patients.

Observation indexes

Before anesthesia, at the end of surgery and at day 1 and day 3 after surgery, 2 mL of peripheral venous blood was collected from each subject and transferred into an anticoagulant tube. T lymphocyte subgroups (CD_3^+ , CD_4^+ , CD_8^+ and $\text{CD}_4^+/\text{CD}_8^+$) were detected using a flow cytometer. The incidence of drug-related adverse reactions in the two groups was observed.

Statistical analysis

SPSS ver. 20.0 was used for data processing. Measurement data were expressed as mean $\pm$ standard deviation (SD). Comparison between the two groups was performed using *t*-test. Analysis of variance was adopted for multi-group comparison. Categorical data were processed by *chi*-squared test. Difference was considered as statistically significant if $P<0.05$.

RESULTS

Comparison of CD_3^+ and CD_4^+

Before anesthesia, the levels of CD_3^+ and CD_4^+ between the two groups had no statistical significance ($P>0.05$). After anesthesia, the levels of CD_3^+ and CD_4^+ in the two groups showed different degrees of decrease, which was statistically significant compared to those before anesthesia ($P<0.05$). The levels of CD_3^+ and CD_4^+ of the observation group were much higher than those of the control group at the end of surgery, at day 0 after surgery and at day 3 after surgery; the difference had statistical significance ($P<0.05$) (Table I).

Comparison of CD_3^+ and CD_4^+/CD_8^+

Before anesthesia, the differences of CD_8^+ level and CD_4^+/CD_8^+ between the two groups had no statistical significance ($P>0.05$). After anesthesia, the level of CD_8^+ decreased in both groups, but the difference had no statistical significance ($P>0.05$). CD_4^+/CD_8^+ of the control group showed a remarkable decrease after anesthesia compared to before anesthesia and the difference was statistically significant ($P<0.05$). CD_4^+/CD_8^+ of the observation group demonstrated no remarkable change after anesthesia compared to before anesthesia ($P>0.05$) (Table II).

Table I. Comparison of the levels of $CD3^+$ and $CD4^+$ between two groups before and after anesthesia (mean $\pm$ SD).

Group		Before anesthesia	At the end of surgery	Day 1 after surgery	Day 3 after surgery
Control group	CD_3^+	70.6 $\pm$ 6.2	60.8 $\pm$ 9.3 [#]	65.3 $\pm$ 8.2 [#]	69.0 $\pm$ 9.0 [#]
	CD_4^+	38.8 $\pm$ 3.2	23.4 $\pm$ 2.4 [#]	26.2 $\pm$ 3.1 [#]	32.3 $\pm$ 3.6 [#]
Observation group	CD_3^+	70.5 $\pm$ 5.9	66.4 $\pm$ 7.3 ^{*#}	68.7 $\pm$ 8.3 ^{*#}	71.8 $\pm$ 7.4 [*]
	CD_4^+	38.5 $\pm$ 5.3	33.1 $\pm$ 3.0 ^{*#}	38.1 $\pm$ 3.5 [*]	38.2 $\pm$ 5.4 [*]

[#]: $P<0.05$, compared to before anesthesia; ^{*}: $P<0.05$ compared to the control group.

Table II. Comparison of CD_8^+ level and CD_4^+/CD_8^+ between two groups before and after anesthesia (mean $\pm$ SD).

Group		Before anesthesia	After surgery	Day 1 after surgery	Day 3 after surgery
Control group	CD_8^+	31.5 $\pm$ 3.2	30.0 $\pm$ 5.4	30.6 $\pm$ 4.5	30.6 $\pm$ 3.9
	CD_4^+/CD_8^+	1.7 $\pm$ 0.7	1.2 $\pm$ 0.6 [#]	1.3 $\pm$ 0.8 [#]	1.4 $\pm$ 0.3 [#]
Observation group	CD_8^+	31.8 $\pm$ 2.2	31.7 $\pm$ 6.3	31.6 $\pm$ 6.2	31.8 $\pm$ 5.4
	CD_4^+/CD_8^+	1.7 $\pm$ 0.6	1.6 $\pm$ 0.4 [*]	1.7 $\pm$ 1.0 [*]	1.7 $\pm$ 0.4 [*]

[#]: means $P<0.05$, compared to before anesthesia; ^{*}: $P<0.05$, compared to control group.

Table III. Comparison of the incidence of drug-related adverse reaction.

	Control group		Observation group	
	N	Incidence	N	Incidence
Dizziness	4	6.7%	2	3.3%
Stomach ache	3	5.0%	1	1.7%
Nausea and vomiting	6	10.0%	2	3.3%
Total	13	21.7%	5	8.3%*

*: $P < 0.05$, compared to the control group.

Comparison of fluorescence staining results of corneal epithelium

The incidence of drug-related adverse reaction of patients in the observation group was 8.3%, much lower than that of the control group (21.7%); the difference had statistical significance ($P < 0.05$) (Table III).

DISCUSSION

Recent studies demonstrated that T cells mediate specific cellular immunity, and play an important role in anti-infection in the perioperative period (9). CD_3^+ , the co-marker on the surface of all T cells can form T cell antigen receptor (TCR)– CD_3 compound by binding with TCR. It is also involved in the signal transduction of major histocompatibility complex (MHC)-I and MHC-II restrictive T cells and is the major unit of T cells in antigen recognition and signal transduction. Decreased CD_3^+ level can weaken the immune functions of the human body. CD_4^+ can assist B-cell differentiation in producing antibodies and activate other cells to produce immune reaction. CD_8^+ can inhibit immune functions as well as the secretion, synthesis and proliferation of antibody (10, 11, 12). The relative balance of CD_4^+/CD_8^+ is of important significance to the steady state of T-lymphocyte subgroups (13-15).

Currently, specific cellular immunity can be

reflected through detecting CD_3^+ , CD_4^+ , CD_8^+ and CD_4^+/CD_8^+ . Research results demonstrated that the levels of CD_3^+ and CD_4^+ of two groups showed a remarkable decrease one day after surgery compared to before anesthesia, but there was no obvious change in the level of CD_8^+ . The indexes gradually increased and all the indexes recovered to the levels of pre-anesthesia, indicating that immunosuppression induced by narcotics in the perioperative period was temporary and reversible. This type of temporary immunosuppression may increase the risks of postoperative infection, therefore, the reasonable selection of narcotics is of great significance to the prognosis of patients.

This study preliminarily explored the application of propofol and etomidate in the anesthesia of patients with infectious shock in the perioperative period and found that both drugs could induce the decline of immune functions of patients, although the decline caused by propofol was more remarkable and patients showed better tolerance to etomidate. The conclusion obtained in this study provides an important reference for the selection of anesthetic drugs clinically.

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LETTER TO THE EDITOR

INDOLEAMINE 2,3-DIOXYGENASE AND REGULATORY T CELLS IN INTESTINAL MUCOSA IN CHILDREN WITH INFLAMMATORY BOWEL DISEASE

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Impaired immune regulation has been suggested as an underlying mechanism of inflammatory bowel disease. Indoleamine 2,3-dioxygenase (IDO) and regulatory T cells expressing FOXP3 are crucial elements of immune regulation. Conversion of FOXP3⁺ lymphocytes to Tregs is one of the functions of IDO. The aim of this study was to evaluate the number of cells expressing FOXP3 and IDO in the lamina propria of intestinal mucosa and to evaluate correlations between these parameters and disease activity. Sixty-six children newly diagnosed with inflammatory bowel disease (41 patients with ulcerative colitis and 25 patients with Crohn's disease) were included in the study. Clinical activity of the disease was assessed by the Pediatric Ulcerative Colitis Activity Index and the Pediatric Crohn's Disease Activity Index. Histopathological activity was scored according to the system described by Geboes. The infiltration of FOXP3⁺ and IDO⁺ cells was evaluated by immunohistochemistry. Sixteen patients with a diagnosis of irritable bowel syndrome (IBS) served as a control group. Lamina propria demonstrated a significantly higher infiltration of FOXP3⁺ and IDO⁺ cells in inflammatory bowel disease compared to the control group ($p=0.001$, $p=0.004$, respectively). The number of IDO⁺ and FOXP3⁺ cells correlated with clinical and histopathologic activity of Crohn's disease. A positive correlation between the number of IDO⁺ and FOXP3⁺ cells was found in both types of inflammatory disease but not in patients with IBS. We conclude that indoleamine dioxygenase and FOXP3⁺ cells are upregulated in the intestinal mucosa of children with inflammatory bowel disease. IDO mediated conversion of FOXP3⁺ T cells to Tregs predominantly occurs in inflammation.

To the Editor,

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), is a chronic condition of complex pathogenesis

involving numerous mechanisms of innate and adaptive immunity. It constitutes an emerging problem in pediatrics due to an increasing incidence and ineffective treatment. Accumulating evidence

Key words: regulatory T cells, indoleamine dioxygenase, IDO, inflammatory bowel disease, children

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suggests that inflammation is caused by an excessive immune response to commensal intestinal flora in the presence of environmental triggers and genetic predispositions. Impaired immune regulation has been hypothesized as a potential mechanism for the abnormal inflammatory response to enteric bacterial antigens.

Indoleamine 2,3-dioxygenase (IDO) catalyzes the initial step in tryptophan degradation along the kynurenine pathway (1). Tryptophan starvation by IDO consumption inhibits T-cell activation, whereas products of tryptophan catabolism, such as kynurenine derivatives, downregulate T-cell proliferation and survival (1). Thus, IDO is considered an immunosuppressive enzyme that plays a crucial role in local immune regulation.

IDO is constitutively expressed in some immune cells, including dendritic cells (DC) and macrophages naturally present in normal tissues. IDO also favors the emergence of Tregs with a CD4⁺CD25^{high}FOXP3⁺ phenotype via conversion from CD4⁺CD25⁻FOXP3⁻ cells (1). Tregs have been shown to be crucial in the maintenance of self-tolerance and the prevention of autoimmune disease. FOXP3, a member of the forkhead-winged helix family of transcription factors, is a functional marker of Treg activity (2). The basic biological activity of Tregs lays in their ability to inhibit the activation and proliferation of effector cells (B,T,NK), by cell contact dependent mechanisms and through the release of anti-inflammatory cytokines, including IL-10 and TGFβ (2). Although some studies have demonstrated the expression of FOXP3 in other groups of lymphocytes with regulatory functions, this transcription factor has been widely used for identifying and monitoring Treg populations (3).

IDO and FOXP3 expressing cells have been subjects of interest in studies of inflammatory bowel disease, which is expected to be a condition exhibiting inappropriate immune regulation. Previous results reveal an overexpression of IDO and an increased number of FOXP3⁺ cells in inflamed intestinal mucosa in adults. IDO and FOXP3 have not been evaluated simultaneously in any study and the correlation between the two biomarkers in pediatric inflammatory bowel disease has yet to be clarified.

The aim of this study was to evaluate the number of IDO⁺ and FOXP3⁺ cells in the intestinal mucosa of children with inflammatory bowel disease and to explore the correlations between these parameters and disease activity.

MATERIALS AND METHODS

Patients and controls

Sixty-six pediatric patients (aged 2-18 years, 22 females, 44 males) from the Department of Pediatrics, Pediatric Gastroenterology, Hepatology and Nutrition of the Medical University of Gdansk, who were newly diagnosed with IBD, were included in the study. Informed consent was obtained from parents and patients over the age of 15 years.

Patients were divided into two subgroups: patients with CD (n=25) and those with UC (n=41). Diagnosis was based on colonoscopy and confirmed by histopathology (biopsy specimens) according to the current consensus (4). The disease activity was determined by the Pediatric Ulcerative Colitis Activity Index (PUCAI) for UC and Pediatric Crohn's Disease Activity Index (PCDAI) for CD (5, 6). Disease severity was staged as mild, moderate or severe, based on PUCAI and PCDAI indices (5, 6).

Histopathological activity was scored according to the system described by Geboes et al. (7)

Sixteen patients (aged 1-16, 7 females, 9 males), with irritable bowel syndrome (IBS) served as the control group. Abdominal pain was an indication for colonoscopy, which revealed no endoscopic or histological abnormalities. These patients were subsequently diagnosed as having IBS.

Antibodies and immunohistochemistry

Mouse anti-human monoclonal antibody against FOXP3 (Abcam, ab20034) and rabbit polyclonal antibodies against IDO (Santa Cruz, sc 25809) were used.

Immunohistochemistry for FOXP3 and IDO was performed according to the following protocol: 4 μm-thick serial sections were cut, deparaffinized and subjected to a heat-induced epitope retrieval step before being incubated with the primary antibodies. Sections were immersed in Target Retrieval Solution (Dako Cytomation, Denmark) and heated in a pressure cooker.

The slides were incubated for 1.5 h with the antibody against FOXP3 at a concentration of 1:800, and with the antibody against IDO at a concentration of 1:50.

Detection of the reaction was carried out by means of the Novolink polymer detection system (Novocastra Laboratories) according to the manufacturer's instruction with 3,3-diaminobenzidine tetrahydrochloride as a chromogen. Slides were counterstained with hematoxylin and mounted. Appropriate positive control (normal tonsil) and negative control (the primary antibody was replaced with normal mouse IgG at an appropriate dilution) were included for each case.

FOXP3⁺ cells, representing Tregs and IDO⁺ cells were evaluated in lamina propria of intestinal mucosa in random areas in IBS and in IBD within areas of active disease features. Lymphoid follicles, focal lymphadenoplasia, and exulcerations were excluded from analysis.

FOXP3⁺ and IDO⁺ cells were counted in eight high power (x400) microscopic fields in areas of positive cells for each tissue sample (microscope Olympus BX40). The

measurements from three high power fields (HPFs) with a maximum number of cells were subjected to further analysis.

Statistical analysis

Differences between variables were compared using U Mann –Whitney and Kruskal-Wallis tests. Relationships between variables were tested with Spearman's rank correlation coefficient. The value of $p < 0.05$ was considered significant. All analyses were performed with Statistica 10 software (Stat Soft. Inc. USA).

The protocol of the study was approved by the Independent Bioethical Committee of the Medical University of Gdansk.

RESULTS

Study group

The median patient age was 14 years (range 2-18 years) in the IBD group, and 14 years (range 1-16 years) in controls with IBS.

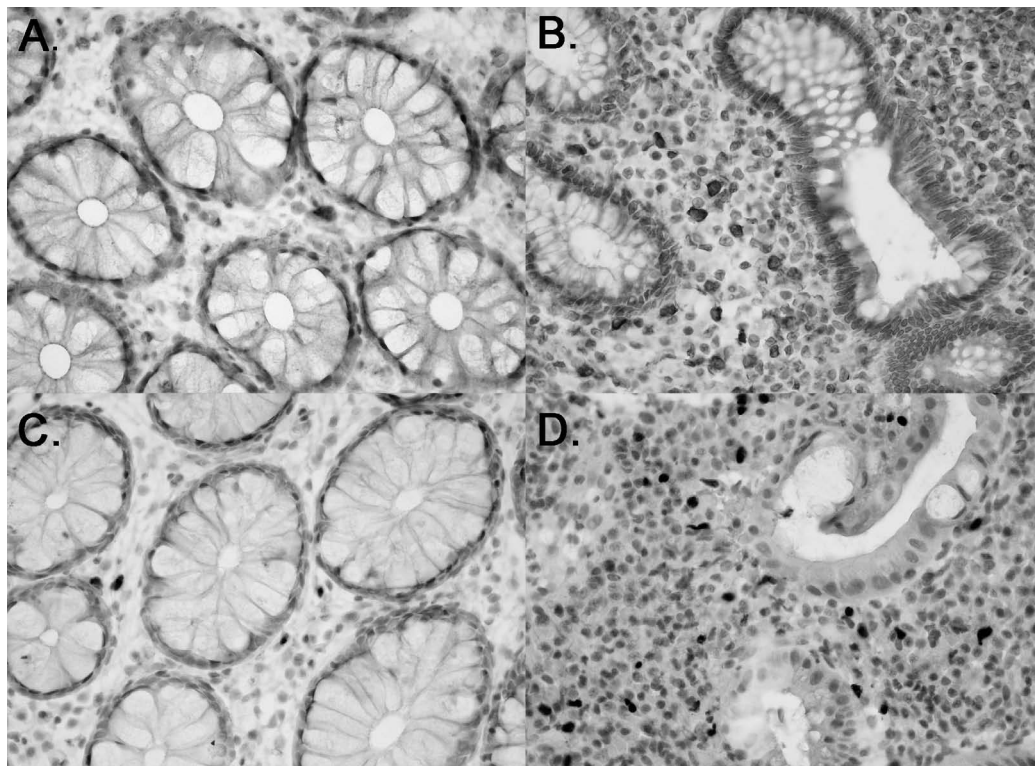


Fig. 1. Microphotographs of IHC staining indicating IDO⁺ and FOXP3⁺ cells in intestinal lamina propria (400x). **A)** IDO expression in non-inflamed intestinal mucosa. **B)** IDO expression in IBD (ulcerative colitis case). **C)** FOXP3 expression in non-inflamed intestinal mucosa. **D)** FOXP3 expression in IBD (Crohn's disease case).

UC

The median patient age was 14 years (range 6-17 years). Median PUCAI was 30 (range 10-75). The distribution of disease severity in patients with UC is shown in Table I.

CD

The median patient age was 15 years (range 2-18 years). Median PCDAI amounted to 35 (range 15-75). The distribution of the disease severity in patients with CD is shown in Table I.

Intestinal IDO⁺ cells in lamina propria

IDO immunoreactivity was localized in the cytoplasm of a small subpopulation of mononuclear cells within intestinal mucosa, without epithelial infiltration. IHC staining of IDO⁺ cells is shown on the microphotograph (Fig. 1). Detailed data on number of IDO⁺ cells in relation to clinical characteristics are summarized in Table I.

IBD vs control

The median number of IDO⁺ cells in the IBD

and control group amounted to 5.67 cells/HPF (range 0-32.67) and 0.67 cell/HPF (range 0-2.67), respectively. Infiltration of IDO⁺ cells in lamina propria was significantly higher in patients with IBD than in controls ($p=0.004$) (Table II).

UC vs control

The median number of IDO⁺ cells in UC amounted to 4.0 cells/HPF (0-30.67) and was significantly higher than in controls ($p=0.014$) (Table II).

CD vs control

The median number of IDO⁺ cells in CD amounted to 9.0 cells/HPF (0-32.67) and was significantly higher than in controls ($p=0.004$) (Table II). Children with CD and UC did not differ significantly in terms of the number of IDO⁺ cells infiltrating the lamina propria ($p=0.513$).

A significant correlation was found between the number of intestinal IDO⁺ cells and clinical activity of the disease in patients with CD ($r=0.68$, $p=0.0002$ $p<0.001$), but not in patients with UC ($r=0.034$ $p=0.834$).

The median number of IDO⁺ cells in mild, moderate and severe CD was 0 (range 0-9.67), 10.33

Table I. Clinical characteristics of the study group and the distribution of IDO⁺ and FOXP3⁺ cells (Tregs) numbers.

	Number of patients	Median number of IDO ⁺ cells	Median number of FOXP3 ⁺ cells (Tregs)
IBD	66	5.67 (range 0.0-32.67)	80.5 (range 2.0-375.67)
UC	41	4.0 (range 0.0-30.67)	43.0 (range 2.0-336.33)
CD	25	9.0 (range 0.0-32.67)	126.0 (range 3.0-375.67)
Controls- IBS	16	0.0 (range 0.0-2.67)	10.17 (range 5.0-30.0)
PUCAI for CD			
Mild	24	3.67 (range 0-30.33)	62.0 (range 3.0-336.33)
Moderate	15	5 (range 0-30.67)	39.3 (range 2.0-212.33)
Severe	2	5.17 (range – 10.33)	50.92 (range 15.5-86.33)
PCDAI for UC			
Mild	8	0.0 (range 0.0-9.67)	58.17 (range 18.0-214.33)
Moderate	14	10.33 (range 0.0-18.0)	151.17 (range 3.0-375.67)
Severe	3	15.5 (range 0.0-32.67)	134.33 (range 37.33-210.0)

IBD: patients with Inflammatory Bowel Disease, UC: patients with Ulcerative Colitis, CD: patients with Crohn's Disease, IBS: patients with Irritable Bowel Syndrome.

Table II. Numbers of IDO⁺ and FOXP3⁺ cells (Tregs) in IBD patients compared to control group.

	IBD	IBS	IBD vs IBS p-value	UC	UC vs IBS p-value	CD	CD vs IBS p-value
IDO ⁺ cells/HPF median/range	5.67 0-32.67	0.0 0-2.67	0.004	4.0 0-30.67	0.014	9.0 0-32.67	0.004
FOXP3 ⁺ cells (Tregs)/HPF median/range	80.5 2.0-375.67	10.17 5.0-30.0	<0.001	43.0 2.0-336.33	<0.001	126.0 3.0-375.67	<0.001

IBD: patients with inflammatory bowel disease, IBS: patients with irritable bowel syndrome, control group, UC: patients with Ulcerative Colitis, CD: patients with Crohn's Disease

(range 0-18.0), 15.5 (range 0-32.67), respectively (Table I). The differences between disease subtypes were significant ($p=0.02$). The median number of IDO⁺ cells in mild, moderate and severe UC was 3.67 (range 0-30.33), 5.0 (range 0-30.67), 5.17 (range 0-10.33), respectively (Table I). Differences between disease subtypes were not significant ($p=0.8$). No correlation between the number of IDO⁺ cells and histopathologic activity of the disease was found in CD ($r=0.24$, $p=0.26$) and UC ($r=0.08$, $p=0.63$).

No correlation between the number of IDO⁺ cells and age was found in CD ($r=0.060$, $p=0.780$), UC ($r=0.23$, $p=0.15$) or controls ($r=-0.18$, $p=0.52$). No statistically significant difference in IDO⁺ cell number was found between male and female subjects ($p=0.72$).

Intestinal FOXP3⁺ cells in lamina propria

FOXP3 was expressed in the cytoplasm of a subpopulation of mononuclear cells in intestinal lamina propria. No epithelial involvement of these cells was observed. IHC staining of FOXP3⁺ cells within intestinal mucosa is shown on the microphotograph (Fig. 1). Detailed data on the number of FOXP3⁺ cells in relation to clinical characteristics is summarized in Table I.

IBD vs control

The median number of FOXP3⁺ cells was 80.5 cells/HPF (range 2.0-375.67) and 10.17 (range 5.0-

30.0) in patients with IBD and in the control group, respectively. The number of FOXP3⁺ cells was significantly higher in patients with IBD than in control group patients ($p<0.001$) (Table II).

UC vs control

The median number of FOXP3⁺ cells in UC amounted to 43.0 cells/HPF (2.0-336.33) and was significantly higher than in controls ($p<0.001$) (Table II).

CD vs control

The median number of FOXP3⁺ cells in CD amounted to 126.0 cells/HPF (3.0-375.67) and was significantly higher than in controls ($p<0.001$) (Table II).

CD vs UC

Patients with CD presented with a significantly higher number of FOXP3⁺ cells compared to UC patients ($p=0.016$). No significant correlation was found between the number of intestinal FOXP3⁺ cells and clinical activity in UC ($r=0.08$, $p=0.63$) or CD patients ($r=0.378$, $p=0.062$). The median number of FOXP3⁺ cells in mild, moderate and severe CD was 58.17 (range 18-214.33), 151.17 (range 3-375.67), and 134.33 (range 37.33-210), respectively. Differences between the disease subtypes were not significant ($p=0.251$). The median number of FOXP3⁺ cells in mild, moderate and severe UC was 62.0 (range 3.0-336.33), 39.33 (range 2-216.33), and

50.92 (range 15.5-86.33), respectively. Differences between the disease subtypes was not significant ($p=0.875$).

FOXP3⁺ cell number correlated with age in the UC group ($r=0.38$, $p=0.01$), but not in the CD group ($r=-0.073$, $p=0.727$) or in IBS patients ($r=-0.24$, $p=0.36$). FOXP3⁺ cell number correlated with histopathologic activity in CD ($r=0.42$, $p=0.04$), but not in UC patients ($r=0.09$, $p=0.58$). No statistically significant difference in FOXP3⁺ infiltrates was found between males and females ($p=0.877$).

A correlation between the number of IDO⁺ cells and FOXP3⁺ cells was found in patients with IBD ($r=0.79$, $p<0.001$) as well as in patients with UC ($r=0.85$, $p<0.001$) and patients with CD ($r=0.56$, $p=0.004$). This correlation was not observed in the control group ($r=0.29$, $p=0.27$).

DISCUSSION

The results of our study demonstrated a higher number of IDO⁺ cells in the intestinal mucosa of children with inflammatory bowel disease compared to non-inflamed mucosa of patients with irritable bowel syndrome, providing further evidence that this immunosuppressive enzyme is upregulated in IBD. These findings are consistent with the results of other studies (8-10). Overexpression of IDO in the gut reflects its role in intestinal immune regulation. The first study addressing this issue was presented by Wolf et al. and revealed an elevated expression of IDO mRNA and an increased infiltration of IDO cells in the gut mucosa of patients with IBD by immunochemistry (8). The role of IDO in IBD was then confirmed with experimental studies (9). In another paper, Zhou et al. also demonstrated an increased number of IDO⁺ cells in lesional biopsies of 51 adult IBD patients compared to healthy controls who underwent routine screening colonoscopy (10). In our study, patients with irritable bowel syndrome-non-inflammatory, functional condition constituted the control group. For pediatric patients, colonoscopy is regarded as an invasive procedure that requires special indications and is not recommended in asymptomatic patients. Contrary to Zhou, who found a correlation between the severity of the disease and

the number of IDO⁺ cells in both UC and CD patients, we observed this correlation only in children with CD. We would like to emphasize that the criteria used for the assessment of inflammatory activity in pediatric patients with bowel disease are different from those used in adult IBD. Furthermore, the severity of pediatric UC and CD is determined using different scoring systems with fewer, exclusively clinical, criteria used in PUCAI (7).

The second studied parameter was the number of FOXP3⁺ cells in the intestinal lamina propria of children with IBD. FOXP3⁺ cell number has been shown to represent mainly regulatory T cells. We have demonstrated an accumulation of FOXP3⁺ cells in inflamed mucosa, which is consistent with the results of other studies (11-13).

Some authors, however, claim that the expansion of these cells is not numerically adequate for the situation. Maul et al. have demonstrated lower numbers of intestinal FOXP3⁺ cells in IBD than in acute diverticulitis, a condition expected to exhibit undisturbed immunoregulatory function (11). In the present study we also demonstrated significantly higher numbers of FOXP3⁺ cells in patients with CD versus patients with UC. This finding, reported previously by other authors, needs to be examined in a larger cohort of patients (13). Moreover, we found a correlation between the number of FOXP3⁺ cells and histopathologic activity of CD. This suggests that immune regulation in Crohn's disease may be adjusted to the intensity of inflammation. The differences in the local regulatory response observed in CD and UC may point to the presence of another immunological feature discriminating these two entities.

Our final discussion point concerns the correlation between the number of IDO⁺ cells and FOXP3⁺ cells. This positive correlation between two studied parameters was found in the experimental studied group, but not in the IBS group. These results may reflect the conversion of FOXP3⁻ cells to FOXP3⁺ regulatory cells, which is one of the activities of IDO. The lack of correlation in the control group suggests that this phenomenon does not play an important role in a non-inflammatory situation.

Consistent with previous results, our study

provides further data that two crucial elements of the immunosuppressive network are upregulated in human inflammatory bowel disease. These facts do not support the theory of deficient immune regulation. Nevertheless, these mechanisms, although numerically increased, are still supposed not to be sufficient. Experimental studies show that therapies using regulatory cells and IDO agonists bring improvement in IBD, which means that immune regulation is still a target for future treatment strategies (14, 15).

It is worth noting that the present study is the first concerning the number of intestinal IDO⁺ cells and FOXP3⁺ cells in pediatric IBD patients. Contrary to the majority of publications, the analysis was performed at onset of the disease, before treatment introduction. It reflects better immunological background and enables to eliminate the influence of different medications on the course of the disease. A relatively small cohort is one weakness of our investigation.

In conclusion, indoleamine 2,3-dioxygenase and FOXP3⁺ cells are upregulated in the intestinal mucosa of children with inflammatory bowel disease. The positive correlation between the number of regulatory cells and IDO⁺ cells in IBD but not in controls suggests that IDO-mediated conversion of Tregs from FOXP⁺ T cells predominantly occurs in inflammation.

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LETTER TO THE EDITOR

CORRELATION BETWEEN BLOOD ASYMMETRIC DIMETHYLARGININE LEVEL AND THE COMPLICATIONS OF PATIENTS WITH CARDIOVASCULAR DISEASES

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This study aimed to investigate the correlation between blood asymmetric dimethylarginine (ADMA) and the complications of patients with cardiovascular diseases through studying the level changes of ADMA, endothelial nitric oxide synthase (eNOS) and NO. Two hundred research subjects with small differences in gender ratio and age, including 50 patients with hypertension combined with myocardial infarction, 50 patients with hypertension, 50 patients with myocardial infarction and 50 healthy normal controls, were enrolled. Relevant basic indexes were measured and recorded; the blood ADMA levels of all the research subjects were detected using high pressure liquid chromatography (HPLC) within the required time. Furthermore, the levels of eNOS and NO were detected using enzyme-linked immunosorbent assay and the relevant information, such as blood pressure, was recorded. The comparison and analysis results of data obtained through detection demonstrated that the subjects in the four groups were well comparable. It was found that the myocardial infarction combined with hypertension group had a much higher serum ADMA level and relatively low levels of eNOS and NO compared to those of the other three groups; the myocardial infarction group and the hypertension group had a much higher serum ADMA level compared to that of the healthy control group and the two groups had much lower levels of eNOS and NO. Moreover, the serum ADMA level was in a positive correlation with the severity of cardiovascular diseases and it showed a significant difference in patients with different severity of hypertension. The change of blood ADMA level can induce acute myocardial infarction as well as the occurrence of cardiovascular disease-associated complications.

To the Editor,

Hypertension, a commonly seen cardiovascular disease in modern society (1, 2), is featured by increased arterial pressure and may damage the functions of multiple organs such as heart, brain, blood vessels and kidney; heart is one of the most vulnerable organs in hypertension. Hypertension can also induce a series of cardiovascular complications, one of which

is myocardial infarction (3). Myocardial infarction refers to local necrosis of myocardium caused by severe persistent ischemia because of coronary arterial occlusion and interrupted blood flow, hence greatly damages the human body. The recent clinical studies suggest that the impairment of vascular endothelial function can induce relevant cardiovascular diseases such as acute myocardial infarction.

Key words: blood ADMA level, complications of cardiovascular diseases, hypertension, myocardial infarction, nitric oxide

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The latest research in both China and abroad has verified that vascular endothelial function and blood pressure are in a close correlation with the generation, synthesis and metabolism of nitric oxide (NO) (4, 5) and they could be regarded as the important factors for determining the occurrence of major cardiovascular diseases such as arteriosclerosis and hypertension and the relevant complications. Asymmetric dimethylarginine (ADMA) is an L-arginine analogue which is synthesized in the human body, and the most ADMA metabolism is realized through dimethyl arginine dimethyl amion acid hydrolase (DDAH2) (6, 7). ADMA can compete with L-arginine to inhibit the activity of nitric oxide synthase (NOS); therefore, it can reduce the generation of NO which is a cardiovascular protective substance, cause the disorder of endothelial function, and induce the occurrence of cardiovascular diseases and relevant complications. Thus, studying the level of blood ADMA has been a new direction for the prevention and treatment of cardiovascular diseases and its complications.

This study investigated the correlation of the levels of serum ADMA, NO and endothelial nitric oxide synthase (eNOS) with the complications of patients with cardiovascular diseases through analyzing the levels of those indexes of patients with hypertension combined with myocardial infarction, hypertension and myocardial infarction and healthy controls (8), aiming to provide a theoretical basis for treatment.

MATERIALS AND METHODS

Study population

The research subjects included in the experiment were 50 patients with hypertension combined with myocardial infarction (group 1), 50 patients with hypertension grades 1, 2 and 3 (group 2), 50 with myocardial infarction (group 3) who were confirmed in the cardiovascular department of the First People's Hospital of Nanjing, Jiangsu, China and 50 control subjects who were examined as healthy by physical examination. Of the 200 subjects, there were 105 males and 95 females, with an average age of 60.32 ± 9.16 years. In group 1 there were 25 females and 25 males and there were 12 cases of diabetes, 16

cases of hyperlipidemia, and 22 cases of smoking and drinking (9, 10). In group 2 there were 26 males and 24 females, and there were 15 cases of diabetes, 10 cases of hyperlipidemia and 25 cases of smoking and drinking. In the group 3, there were 29 males and 21 females and there were 11 cases of diabetes, 12 cases of hyperlipidemia and 27 cases of smoking and drinking. In the healthy control group, there were 25 males and 25 females, with normal body indicators and without cardiovascular diseases or other relevant diseases. All the study subjects gave informed consent to participate in the study. The clinical trials were carried out in the First People's Hospital of Nanjing, Jiangsu, China.

Main instruments and reagents

The main instruments included spectrophotometer, high performance liquid chromatograph, fluorescence detector, column oven, PH measuring instrument, centrifugation instrument, medical cryogenic refrigerator, water bath, drying oven, electronic scale, fully automatic biochemical analyser, anastar chromatographic work station. The main reagents included ADMA standard substance, NO detection reagent, eNOS enzyme-linked immuno sorbent assay (ELISA) reagent, distilled water, ethyl alcohol, potassium chloride, pure water filter, acetonitrile, NDA stock solution.

Basic indicators of experimental subjects in the four groups

Prior to the detection of levels of ADMA, NO and eNOS, the basic information was recorded and relevant biochemical indicators were detected, after which the data were timely recorded and analyzed.

ADMA level

The level of ADMA was detected using high pressure liquid chromatography (HPLC) (11, 12). Within 5 min, 30 ml of potassium chloride was fully mixed with serum sample solution which had been prepared previously and shaken evenly. After 15 min of standing, 40 ml of NDA solution was added and shaken evenly for 15 min. Then the solution was left until fluorescent products produced. Then it was put in a warm water bath. A moderate amount of the sample was taken out and analyzed by HPLC. Information, such as the level of ADMA and blood pressure, was recorded.

eNOS and NO levels

The levels of eNOS and NO were detected using ELISA. The levels were reflected by the metabolic product of NO. In the detection of NO, NO₃⁻ was restored to NO₂⁻ and then the level of NO₂⁻ was detected. The prepared solid phase carrier was added with the sample containing eNOS antibody (13). The carrier was washed using substrate. After catalysis, peroxidase and specified acid preparation were added. Finally, the absorbance was detected using a spectrophotometer; the level of eNOS was detected.

Correlation between ADMA and the severity of disease

To investigate the correlation between the serum ADMA level and the severity of disease, the serum NO, eNOS and ADMA concentrations were compared between the patients with grade 1 ~ 3 hypertension.

Statistical analysis

All the data obtained from the experiments were statistically processed by SPSS ver. 17.0 and expressed as mean $\pm$ standard deviation (SD). Difference was considered with statistical significance if $p < 0.05$ and with remarkable statistical significance if $p < 0.01$.

RESULTS

Comparison of relevant data between subjects

After the examination of all research subjects, the gender, age, blood pressure, diseases and blood biochemical indexes were recorded and compared. The results demonstrated that, the total cholesterol, diastolic blood pressure and systolic pressure of the 200 subjects had significant difference ($p < 0.05$) and the other indexes had no remarkable

Table I. *The comparison of relevant indexes between the four groups.*

Group	Hypertension +myocardial infarction	Hypertension	Myocardial infarction	Healthy control
Gender (male/female)	25/25	26/24	29/21	25/25
Age (year)	65.30 $\pm$ 10.05	65.14 $\pm$ 9.25	62.35 $\pm$ 12.55	64.12 $\pm$ 11.24
Diabetes	12	15	11	0
Hyperlipidemia	16	10	12	0
Smoking and drinking	22	25	27	26
Systolic pressure (mmHg)	150.02 $\pm$ 10.35	164.21 $\pm$ 17.68	128.47 $\pm$ 15.03	132.21 $\pm$ 12.11
Diastolic blood pressure (mmHg)	91.58 $\pm$ 7.22	95.05 $\pm$ 9.54	85.33 $\pm$ 9.88	76.87 $\pm$ 8.68
Total cholesterol (mmol/L)	8.66 $\pm$ 1.02	8.55 $\pm$ 1.56	7.03 $\pm$ 1.65	5.45 $\pm$ 1.66
Low density lipoprotein(mmol/L)	3.58 $\pm$ 1.22	3.33 $\pm$ 1.29	3.10 $\pm$ 1.87	3.28 $\pm$ 1.77

difference, suggesting that the different groups were comparable. The differences of specific indexes are shown in Table I.

ADMA, eNOS and NO levels in groups

The ADMA, eNOS and NO levels of the four groups were recorded and compared. The comparison results demonstrated that the ADMA level of group 1 was much higher than that of the other three groups, and the eNOS and NO levels of group were much lower than those of the other three groups ($p < 0.01$); the ADMA level of group 2 and group 3 was significantly higher than that of the control

group and the eNOS and NO levels of group 2 and group 3 were much lower than those of the control group ($p < 0.01$); but the differences of the indexes between group 2 and group 3 were not statistically significant. The results indicated that ADMA could inhibit the activity of eNOS, reduce the generation of NO, induce endothelial function damage, and promote the occurrence of hypertension and its relevant complications. The differences of specific indexes are shown in Table II.

The levels of ADMA, eNOS and NO of the males and females in the four groups are shown in Table III. No obvious differences were found in the values

Table II. *The levels of ADMA, eNOS and NO in the four groups.*

Group	n	ADMA($\mu\text{mol/L}$)	eNOS(U/ml)	NO($\mu\text{mol/L}$)
Hypertension combined with myocardial infarction group	50	3.96 $\pm$ 0.85	43.21 $\pm$ 15.35	54.26 $\pm$ 11.25
Hypertension group	50	1.42 $\pm$ 0.26	55.36 $\pm$ 12.88	62.54 $\pm$ 10.84
Myocardial infarction group	50	1.75 $\pm$ 0.33	58.23 $\pm$ 16.24	65.71 $\pm$ 15.36
Healthy control group	50	0.56 $\pm$ 0.17	69.12 $\pm$ 25.64	78.86 $\pm$ 8.94

Table III. *The levels of ADMA, eNOS and NO of the males and females in the four groups.*

Group	Hypertension +myocardial infarction		Hypertension		Myocardial infarction		Healthy control	
	Male	Female	Male	Female	Male	Female	Male	Female
ADMA ($\mu\text{mol/L}$)	3.93 $\pm$ 0.89	3.89 $\pm$ 0.78	1.45 $\pm$ 0.23	1.41 $\pm$ 0.25	1.76 $\pm$ 0.32	1.75 $\pm$ 0.33	0.55 $\pm$ 0.18	0.55 $\pm$ 0.16
eNOS (U/ml)	44.16 $\pm$ 14.40	42.36 $\pm$ 14.50	51.45 $\pm$ 16.78	54.47 $\pm$ 11.99	58.29 $\pm$ 16.18	58.19 $\pm$ 16.20	68.98 $\pm$ 25.78	68.49 $\pm$ 25.00
NO ($\mu\text{mol/L}$)	55.21 $\pm$ 10.30	54.19 $\pm$ 11.18	62.24 $\pm$ 11.14	62.35 $\pm$ 10.65	64.96 $\pm$ 16.11	63.67 $\pm$ 13.32	79.05 $\pm$ 8.75	78.41 $\pm$ 8.49

between the males and females in different groups ($p > 0.05$).

Correlation between ADMA and the severity of hypertension

The comparison of serum NO, eNOS and ADMA levels between the patients with grade 1 ~ 3 hypertension suggested that the patients with grade 3 hypertension had the highest level of serum ADMA, followed by the patients with grade 2 and 1 hypertension, and the patients with grade 1 hypertension had the highest levels of serum NO and eNOS, followed by the patients with grade 2 and 3 of hypertension. It indicated that the serum ADMA level was in a positive correlation with the severity of hypertension, and the ADMA level of the patients with different grades of hypertension had remarkable differences. The results are shown in Fig. 1.

DISCUSSION

The incidence of hypertension has shown an increasing tendency in recent years. Hypertension,

a cardiovascular disease featured by increased arterial pressure, is accompanied with dysfunction of multiple organs such as heart, brain and blood vessels; other cardiovascular complications, such as acute myocardial infarction which has a high death rate, may also occur with hypertension. Recent studies found that vascular endothelial dysfunction could induce the occurrence of some cardiovascular diseases and relevant complications. Vascular endothelium, a psychological barrier between circulating blood and vascular smooth muscle (14), plays an important physiological function. When vascular endothelial dysfunction occurs, for example, endothelial-dependent vasodilation functions weaken and inflammatory reactions occur, and atherosclerosis (15) will form to induce cardiovascular disease. The cause for vascular endothelial dysfunction is complex, and the significantly reduced generation of NO is one of the major causes.

NO, a strong and effective endogenous vasorelaxation factor, can relax blood vessels and involve multiple physiological and pathological processes. Therefore, the abnormal change of the

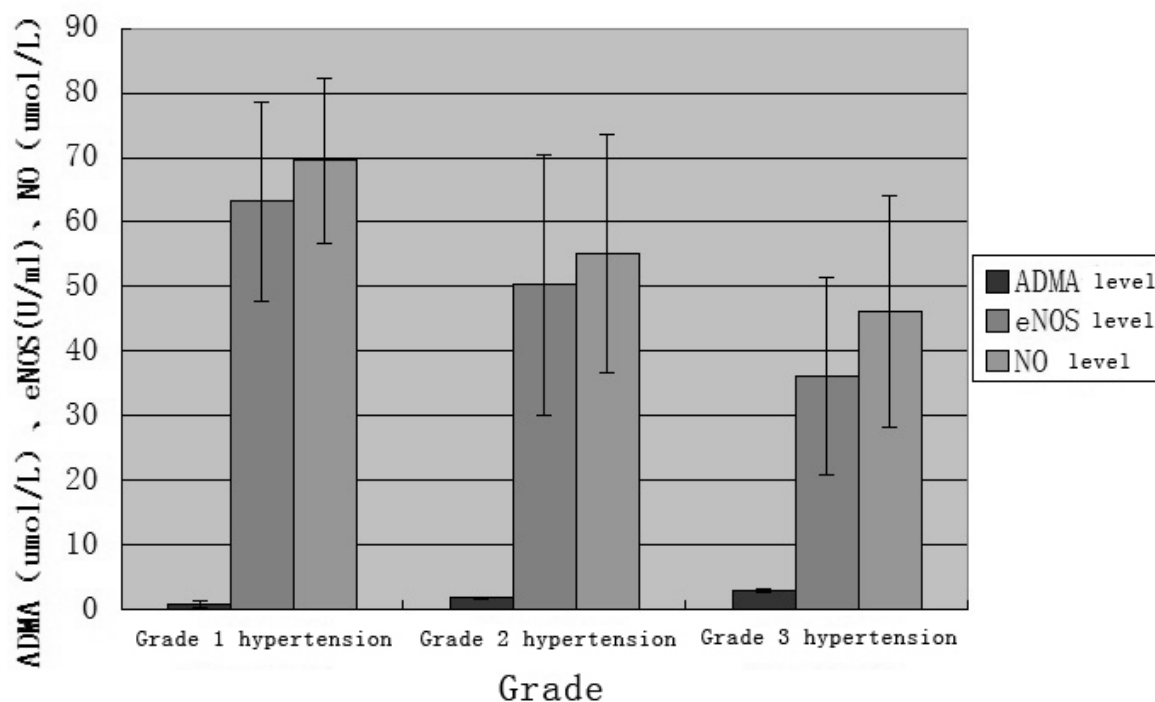


Fig. 1. Comparison of serum ADMA, NO and eNOS levels between patients with different grades of hypertension.

release volume of NO may induce the disorder of endothelial function, cause endothelial cell dysfunction, and finally induce cardiovascular disease and a series of complications. NO is generated based on L-arginine under the effect of NOS. eNOS, one type of NOS, is mainly expressed in vascular endothelial cells and is involved in the occurrence and development of many cardiovascular diseases. Thus, once the activity of eNOS is inhibited, the generation of NO will be severely reduced, resulting in endothelial dysfunction; ADMA is the main substance inhibiting the activity of eNOS.

ADMA can reduce the generation of NO by inhibiting the activity of eNOS through competing with L-arginine. Through detecting the levels of NO, eNOS and ADMA of the patients with hypertension in combination with myocardial infarction, those with only hypertension, or only myocardial infarction as well as healthy controls, this study found that the patients with hypertension in combination with myocardial infarction had much lower levels of NO and eNOS but a much higher ADMA level compared to those with only hypertension or myocardial infarction. Moreover, the patients with hypertension and those with myocardial infarction had much lower levels of NO and eNOS and a much higher ADMA level compared to the healthy controls. The results indicated that ADMA could inhibit the activity of eNOS and reduce the generation of NO, i.e., the higher the level of ADMA was, the less the output of NO and the more serious the vascular endothelial dysfunction would be; ADMA had an important role in the development of cardiovascular diseases and relevant complications. This study also compared the concentrations of serum NO, eNOS and ADMA of the patients with different grades of hypertension and found that the patients with a higher grade of hypertension had lower levels of NO and eNOS but a higher level of ADMA, indicating the serum ADMA level was in a positive correlation with the severity of hypertension.

This study analyzed the correlation between blood ADMA level and the complications of patients with cardiovascular diseases through detecting the levels of ADMA and NO in patients and eventually found that ADMA could block vascular endothelial

function by inhibiting the activity of eNOS and the generation of NO. This work leads to further understanding the role of ADMA in cardiovascular diseases and the relevant complications and, in addition, it is beneficial to motivate the further study of ADMA and the prevention and treatment of complications of patients with cardiovascular diseases in the future.

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LETTER TO THE EDITOR

EVALUATION OF TOLERANCE AND SAFETY OF CONVERSION FROM MYCOPHENOLATE MOFETIL TO ENTERIC-COATED MYCOPHENOLIC ACID IN RENAL TRANSPLANT RECIPIENTS

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This study was designed to evaluate the curative effect of conversion from mycophenolate mofetil (MMF) to enteric-coated mycophenolate sodium tablets (EC-MPS) and its safety. One hundred and twenty renal transplant recipients who developed MMF-associated chronic diarrhea were selected as research subjects and treated with EC-MPS. The patients were followed up for 12 months to compare the improvement of gastrointestinal symptoms and the indexes such as carbon dioxide combining power (CO_2CP), serum sodium, serum potassium, serum creatinine (Scr) and 24-h urine protein before and after conversion treatment. One hundred and ten of the 120 patients tolerated the conversion treatment and the dose increment of EC-MPS at week 28. After initiating the conversion treatment, the improvement rate of diarrhea within 2 weeks was 95% (114/120). Indexes, such as CO_2CP , serum sodium, serum potassium, after conversion treatment were higher than those before treatment ($P < 0.05$). No acute rejection reactions were observed in the 12-month follow-up. Indexes of Scr and 24-h urine protein had significant improvement after conversion treatment compared to before conversion treatment ($P < 0.05$). Compared to before treatment, the average values of indexes in gastrointestinal symptom rating scale in the 12th month remained stable, except for the increase of dose. For renal transplant recipients who received suboptimal EC-MPS treatment due to gastrointestinal symptoms, conversion from MMF to EC-MPS can significantly lower gastrointestinal symptom load, improve quality of life, relieve electrolyte disturbance and improve the injured functions of transplanted kidney, without increasing the risks of acute rejection reactions.

To the Editor,

Chronic renal failure can be divided into renal function compensatory period, renal function decompensatory period, renal function failure period and uraemia period. In the uraemia period, i.e., the end period of nephropathy, patients should undergo dialysis or renal transplant urgently (1, 2). Although the survival rate of patients who receive

renal transplant becomes increasingly higher with the advancement of medical technologies, patients may have severe postoperative complications due to surgical stress and the application of high-dose hormone (3). Diaz et al. (4) found that 10.9% of patients who underwent heart transplant stopped taking mycophenolate mofetil (MMF) and 38.70% of patients reduced the dose of MMD due to

Key words: mycophenolate mofetil, enteric-coated mycophenolate sodium tablets, diarrhea, tolerance

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gastrointestinal complications. On this account, scholars who are specialized in transplants try to find the perfect immunosuppressor.

The classical triple immunosuppression scheme which combines MMF with steroid and calcineurin inhibitor has been extensively recognized and applied and by clinicians for its definite immunosuppression effect; however, MMF may induce diarrhea after renal transplantation. Enteric-coated mycophenolate sodium tablets (EC-MPS) are a novel immunosuppressor which has the same source and base as MMF, and the conversion from MMF treatment to ES-MPS treatment can increase the exposure of MPA in patients experiencing intolerable MMF-induced gastrointestinal reactions (5). This study selected 120 renal transplant recipients who had undergone MMF treatment in the People's Hospital of Zhengzhou, Zhengzhou, Henan, China and treated them with EC-MPS, aiming to observe the effect of the conversion treatment.

MATERIALS AND METHODS

General data

One hundred and twenty patients with uremia who were transplanted with kidneys from cadaveric donors in the People's Hospital of Zhengzhou, Zhengzhou, Henan, China and previously treated with MMF from June 2012 to June 2014 were randomly selected. The average age was 52 ± 13.6 years; 7.2 ± 4.4 years had passed since transplantation; 64% of them were males. For the application of calcineurin inhibitor, 80% (96/120) applied tacrolimus and 20% (24/120) applied cyclosporine. All patients developed gastrointestinal reactions, diarrhea mostly, after surgery. The stools of all patients were watery or pasty; the frequency of defecation was 3 times/day; and the daily amount of defecation exceeded 0.2 kg. Patients with mild diarrhea had suffered from diarrhea for two weeks and patients with severe symptoms had suffered for one month. The patients included conformed to the following criteria: renal transplant at least three months previously; the interval between treatment with 250 mg/d~1500 mg/d MMF and enrolment in the study was at least four weeks; being treated with reduced dose of MMF due to intolerant gastrointestinal reactions; current immunosuppressive therapy remained for at least four

weeks; aged 18 years or over. Patients who developed gastrointestinal reactions not regarding MMF, developed acute rejection reaction within four weeks prior to the study, suffered from mental diseases, pregnant or nursing females and patients who received trial drug treatment within thirty days before enrolment were excluded. All patients signed informed consent before conversion treatment and the study was approved by the medical ethics committee of the People's Hospital of Zhengzhou, Zhengzhou, Henan, China.

Immunosuppression

All patients were converted from MMF to equimolar dose of EC-MPS (720 mg of EC-MPS is equivalent to 1000 mg of MMF). A minimal dose increase of EC-MPS (180 mg/d) was administered at week 7 ± 1 after baseline. The night dose of EC-MPS was the first dose modified. If the new increased dose of EC-MPS was well tolerated, the dose of EC-MPS was increased by 180 mg/d every 7 ± 1 weeks. The same methodology and criteria for EC-MPS increase were used until follow-up visit 5 or when a maximal dose of EC-MPS of 720 mg twice a day on cyclosporine or 540 mg twice a day on tacrolimus was achieved. The doses of concomitant immunosuppressants, such as corticosteroids or CNI, were not modified unless indicated by the physician.

Observation index

Safety evaluation. Patients were self-evaluated with gastrointestinal symptom rating scale (GSRS) before conversion treatment and every 7 ± 1 weeks after conversion treatment (day 0, weeks 7, 14, 21, 28, 35, month 12). The severity and frequency of diarrhea were monitored. The indexes, such as carbon dioxide combining power (CO_2CP), serum sodium, serum potassium, serum creatinine (Scr) and 24-h urine protein, were recorded. Evaluation items of GSRS included abdominal pain, reflex symptom, dyspepsia, diarrhea, constipation. Those items were scaled for 0~3 points (0 point for no symptoms; 1 point for mild symptoms; 2 points for medium symptoms; 3 points for severe symptoms) (10).

Evaluation of curative effect: From the beginning of the follow-up and at week 28, Scr and 24-h urine protein were detected. Renal function was evaluated based on the levels of serum Scr and urea. If the level of Scr increased

from 15% to 20%, then renal biopsy was collected. The incidence of acute rejection could be confirmed if the level of serum Scr increased for at least 15% compared to that at baseline.

Statistical analysis

SPSS19.0 statistics software was used for data analysis. Measurement data were expressed as Mean $\pm$ SD. Multi-group comparison was performed using single-factor analysis of variance. Pairwise comparison between groups adopted SNK-q method. Enumeration data was expressed by percentage. Comparison between groups was performed using *chi*-squared test. Difference was considered to have statistical significance if $P < 0.05$.

RESULTS

The conditions of conversion treatment

Before conversion treatment, the average dose of MMF was 928.6 ± 152.7 mg/d, including 30 cases of 500 mg/d, 30 cases of 750 mg/day and 60 cases of 1000 mg/d. At day 0, all patients converted to equimolar EC-MPS treatment, and the average dose of EC-MPS was 700.3 ± 108.7 mg/d (Fig. 1). At week 7, 115 of the 120 patients (96%) tolerated the conversion treatment and were given a dose increment of 180 mg/d. At every evaluation time point after the beginning of conversion treatment, the dose of EC-MPS had significant increase ($P < 0.001$); the average dose of EC-MPS was 793.7 ± 148.8 mg/d, 1031.6 ± 165.2 mg/d and 1002.4 ± 207.9 mg/d at week 7, week 28 and month 12, respectively (Fig. 1). The results of the final follow-up showed that 110 patients who were treated with MMF in a dose below 1500 mg/d previously tolerated one or more times of dose increment of 180 mg/d.

Relief of diarrhea

In 114 of the 120 patients diarrhea was completely relieved after the conversion treatment; 75 out of 114 patients had relief within one week and 39 within two weeks. Four patients had significantly relieved diarrhea symptoms after converting to EC-MPS treatment, shown as reduced defecation amount and lower frequency of defecation. Therefore, the relief rate of diarrhea of patients within two weeks after

the beginning of the conversion treatment was 95%.

Evaluation of drug tolerance

The average GSIS score decreased from 2.43 ± 0.78 to 2.26 ± 0.68 at week 7 ($P = 0.265$). At month 12, the average GSIS score remained stable except for the increase of dose (Fig. 2A). Diarrhea was relieved after the initiation of the conversion treatment. At day 0, the average score of diarrhea was 2.94 ± 1.57 points; at week 7, the score turns was 2.28 ± 1.51 points ($P = 0.058$ compared to day 0); at month 12, the score was 2.31 ± 1.62 points ($P = 0.091$ compared to day 0, Fig. 2B). No adverse events were observed during the 12-month follow up.

Comparison of various indexes before and after conversion treatment

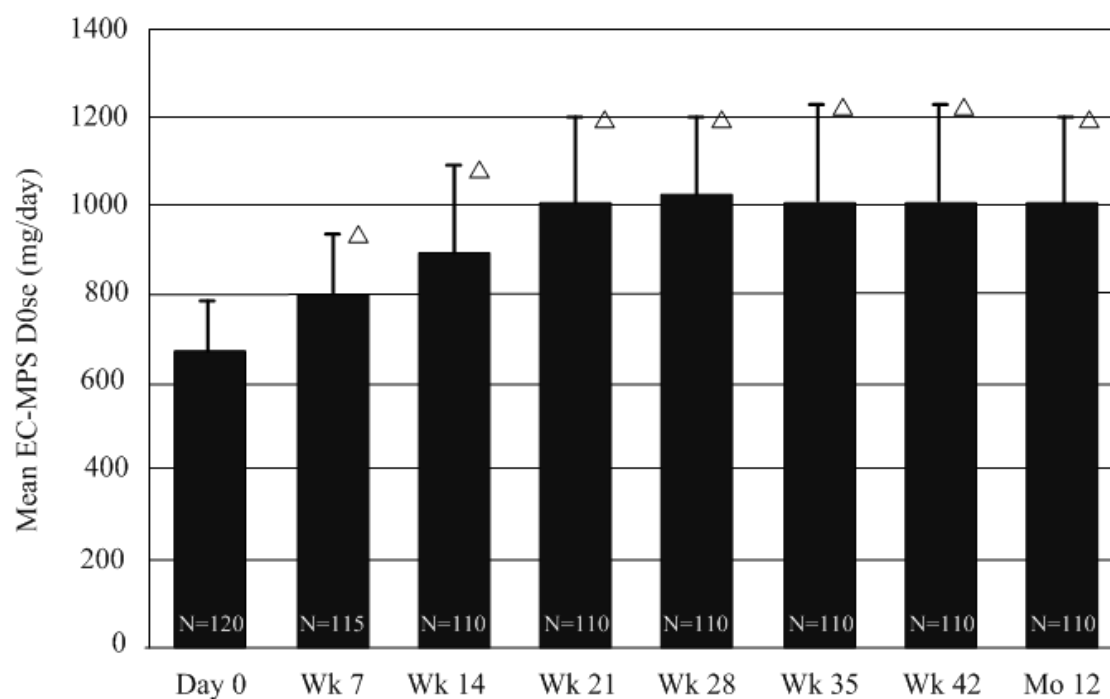
The levels of CO_2CP , serum sodium and serum potassium at month 12 were much higher compared to before conversion treatment ($P < 0.01$); moreover, the levels of 24-h urine protein and serum Scr of patients at month 12 were much lower than those before conversion treatment ($P < 0.01$; $P < 0.05$) (Table I).

DISCUSSION

The causes for the long-term chronic diarrhea of patients who undergo renal transplant is usually associated with drugs, especially MMF (6). When MMF-associated chronic diarrhea occurs, it is important to ensure sufficient exposure of MPA in the blood for the long-term survival of transplanted kidney (7). MMF is transformed to MPA after being absorbed into the blood from the intestines; and MPA is transformed to mycophenolic acid glucuronide under the catalytic action of glucuronyl transferase which is afterwards secreted to the enteric cavity along with bile as the metabolite. Mycophenolic acid glucuronide is an inflammatory factor precursor which can inhibit the proliferation of cells on the intestinal wall and induce long-term local inflammation; therefore, it is easy to induce chronic diarrhea (8). EC-MPS (9, 10) can only be disintegrated in an environment with pH value lower than 5; hence, its absorption efficiency in the small intestine is quite low. As a result, it can extend the

Table I. *Changes of various indexes before and after conversion treatment.*

Time point	Before	Month 12	T	P
CO ₂ CP (mmol/L)	16.48±3.96	22.75±3.77	12.58	<0.01
Serum sodium (mmol/L)	131.43±3.07	143.34±3.87	26.26	<0.01
Serum Scr (mmol/L)	122.46±51.75	101.02±32.46	47.24	<0.05
Serum potassium (mmol/L)	3.21±0.96	4.86±0.56	16.09	<0.01
24-h urine protein (g)	0.75±0.38	0.44±0.25	7.75	<0.01

(Mean±SD)**Fig. 1.** Mean enteric-coated mycophenolate sodium (EC-MPS) daily dose from day 0 to month 12. □ $P < 0.001$ for EC-MPS dose at time point versus day 0 (paired *t*-test).

difference, suggesting that the different groups were comparable. The differences of specific indexes are shown in Table I.

ADMA, eNOS and NO levels in groups

The ADMA, eNOS and NO levels of the four groups were recorded and compared. The comparison results demonstrated that the ADMA level of group 1 was much higher than that of the other three groups, and the eNOS and NO levels of group were much lower than those of the other three groups ($p < 0.01$); the ADMA level of group 2 and group 3 was significantly higher than that of the control

group and the eNOS and NO levels of group 2 and group 3 were much lower than those of the control group ($p < 0.01$); but the differences of the indexes between group 2 and group 3 were not statistically significant. The results indicated that ADMA could inhibit the activity of eNOS, reduce the generation of NO, induce endothelial function damage, and promote the occurrence of hypertension and its relevant complications. The differences of specific indexes are shown in Table II.

The levels of ADMA, eNOS and NO of the males and females in the four groups are shown in Table III. No obvious differences were found in the values

Table II. *The levels of ADMA, eNOS and NO in the four groups.*

Group	n	ADMA($\mu\text{mol/L}$)	eNOS(U/ml)	NO($\mu\text{mol/L}$)
Hypertension combined with myocardial infarction group	50	3.96 $\pm$ 0.85	43.21 $\pm$ 15.35	54.26 $\pm$ 11.25
Hypertension group	50	1.42 $\pm$ 0.26	55.36 $\pm$ 12.88	62.54 $\pm$ 10.84
Myocardial infarction group	50	1.75 $\pm$ 0.33	58.23 $\pm$ 16.24	65.71 $\pm$ 15.36
Healthy control group	50	0.56 $\pm$ 0.17	69.12 $\pm$ 25.64	78.86 $\pm$ 8.94

Table III. *The levels of ADMA, eNOS and NO of the males and females in the four groups.*

Group	Hypertension +myocardial infarction		Hypertension		Myocardial infarction		Healthy control	
	Male	Female	Male	Female	Male	Female	Male	Female
ADMA ($\mu\text{mol/L}$)	3.93 $\pm$ 0.89	3.89 $\pm$ 0.78	1.45 $\pm$ 0.23	1.41 $\pm$ 0.25	1.76 $\pm$ 0.32	1.75 $\pm$ 0.33	0.55 $\pm$ 0.18	0.55 $\pm$ 0.16
eNOS (U/ml)	44.16 $\pm$ 14.40	42.36 $\pm$ 14.50	51.45 $\pm$ 16.78	54.47 $\pm$ 11.99	58.29 $\pm$ 16.18	58.19 $\pm$ 16.20	68.98 $\pm$ 25.78	68.49 $\pm$ 25.00
NO ($\mu\text{mol/L}$)	55.21 $\pm$ 10.30	54.19 $\pm$ 11.18	62.24 $\pm$ 11.14	62.35 $\pm$ 10.65	64.96 $\pm$ 16.11	63.67 $\pm$ 13.32	79.05 $\pm$ 8.75	78.41 $\pm$ 8.49

between the males and females in different groups ($p > 0.05$).

Correlation between ADMA and the severity of hypertension

The comparison of serum NO, eNOS and ADMA levels between the patients with grade 1 ~ 3 hypertension suggested that the patients with grade 3 hypertension had the highest level of serum ADMA, followed by the patients with grade 2 and 1 hypertension, and the patients with grade 1 hypertension had the highest levels of serum NO and eNOS, followed by the patients with grade 2 and 3 of hypertension. It indicated that the serum ADMA level was in a positive correlation with the severity of hypertension, and the ADMA level of the patients with different grades of hypertension had remarkable differences. The results are shown in Fig. 1.

DISCUSSION

The incidence of hypertension has shown an increasing tendency in recent years. Hypertension,

a cardiovascular disease featured by increased arterial pressure, is accompanied with dysfunction of multiple organs such as heart, brain and blood vessels; other cardiovascular complications, such as acute myocardial infarction which has a high death rate, may also occur with hypertension. Recent studies found that vascular endothelial dysfunction could induce the occurrence of some cardiovascular diseases and relevant complications. Vascular endothelium, a psychological barrier between circulating blood and vascular smooth muscle (14), plays an important physiological function. When vascular endothelial dysfunction occurs, for example, endothelial-dependent vasodilation functions weaken and inflammatory reactions occur, and atherosclerosis (15) will form to induce cardiovascular disease. The cause for vascular endothelial dysfunction is complex, and the significantly reduced generation of NO is one of the major causes.

NO, a strong and effective endogenous vasorelaxation factor, can relax blood vessels and involve multiple physiological and pathological processes. Therefore, the abnormal change of the

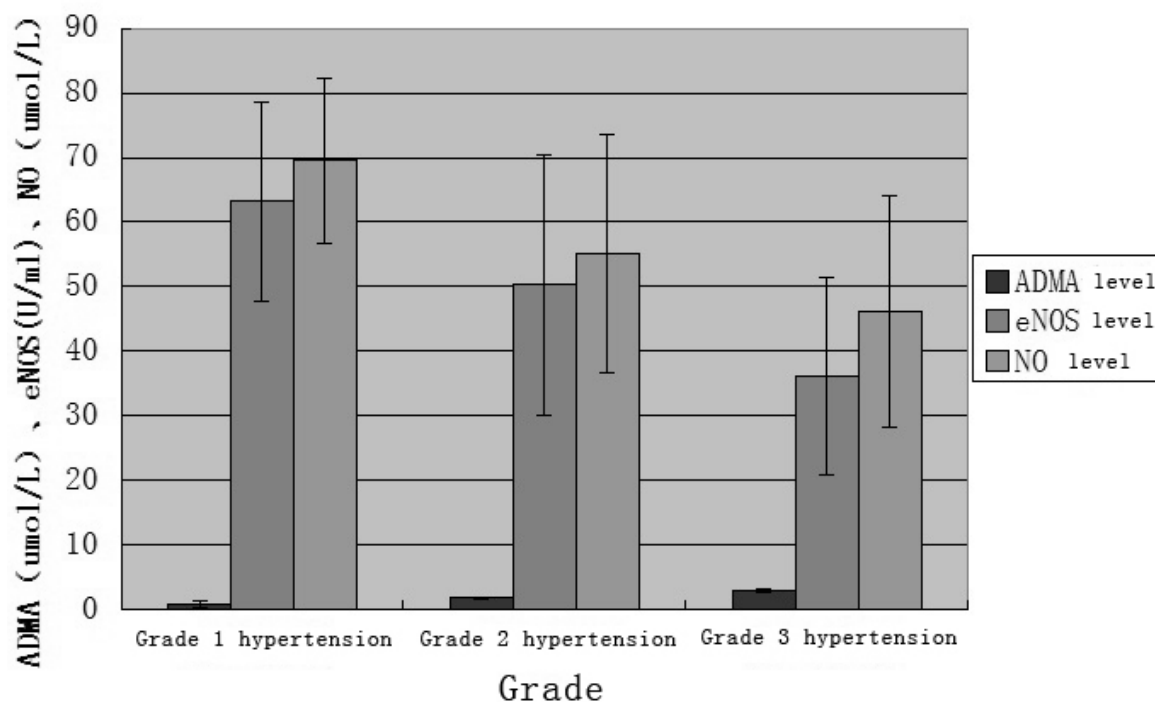


Fig. 1. Comparison of serum ADMA, NO and eNOS levels between patients with different grades of hypertension.

LETTER TO THE EDITOR

NEUROCHEMICAL EFFECTS OF PHOTOBIOSTIMULATION IN THE TRIGEMINAL GANGLION AFTER INFERIOR ALVEOLAR NERVE INJURY

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Orofacial pain is associated with peripheral and central sensitization of trigeminal nociceptive neurons. Nerve injury results in release of chemical mediators that contribute to persistent pain conditions. The activation of the transient receptor potential vanilloid 1 (TRPV1), promotes release of calcitonin gene-related peptide (CGRP) and substance P (SP) from trigeminal nerve terminals. CGRP and SP contribute to the development of peripheral hyperalgesia. The expression of SP and CGRP by primary afferent neurons is rapidly increased in response to peripheral inflammation. CGRP receptor activation promotes activation of AMPA receptors, leading to increased firing of neurons which is reflected as central sensitization. In this study we investigated whether inferior alveolar nerve (IAN) injury influences AMPA receptors, CGRP, SP and TRPV1 expression in the trigeminal ganglion (TG). The relative expression of the protein of interest from naive rats was compared to those from injured rats and animals that received low level laser therapy (LLLT). IAN-injury did not change expression of GluA1, GluA2 and CGRP, but increased the expression of TRPV1 and SP. LLLT increases GluA1 and GluA2 expression and decreases TRPV1, SP and CGRP. These results, together with previous behavioral data, suggest that IAN-injury induced changes in the proteins analyzed, which could impact on nociceptive threshold. These data may help to understand the molecular mechanisms of pain sensitization in the TG.

To the Editor,

Trigeminal nerve injury is a common cause of orofacial pain and can lead to a permanent sensory disturbance (1). The inferior alveolar nerve (IAN) is related to dental practice and is susceptible to damage during dental procedures (2). Following trigeminal nerve injury the trigeminal system shows altered expression of neuropeptides, ion channels and

receptors. Chronic pain involves the interaction of all the alterations mentioned above and can be enhanced by transmitters and receptors such as transient receptor potential vanilloid 1 (TRPV1), substance P (SP), calcitonin gene-related peptide (CGRP) (3) and glutamate (4). Glutamate is a neurotransmitter which plays an important role in peripheral pain mechanisms (5). One study showed that glutamatergic

Key words: inferior alveolar nerve injury, laser therapy, trigeminal ganglion, neurotransmitters, receptors

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transmission in TRPV1 neurons, together with SP and CGRP, all contributed to the development of mechanical hyperalgesia (6), and the TRPV1 can interact with NK-1 receptors for SP and enhance the hyperalgesic state (7); SP and CGRP expression by primary afferent neurons is increased in response to peripheral inflammation (8). Moreover, CGRP can facilitate nociception by delaying the metabolism of SP, by CGRP competing with SP for catabolism by endopeptidases (9) and also by increasing the release of glutamate and other neuropeptides in the central endings of primary afferent fibers (10). Orofacial pain is common and difficult to manage and treat. Low level laser therapy (LLLT) has positive effects, and one study shows that LLLT affected expression of biochemical related to pain (11). Our group has also had positive experience with LLLT, and its effectiveness was reported in a previous paper (12). In this study, we analyze the possible alterations in the expression of neuropeptides, such as, SP, CGRP, TRPV1 and glutamate AMPAR subunits (GluA1 and GluA2) induced by LLLT treatment in animals with IAN injury.

MATERIALS AND METHODS

Animals

Twenty male Wistar rats, weighing between 200 and 220 g (2 months old) were used in all experiments (5 per group). They were housed in a temperature and humidity-controlled animal facility, five animals per cage and maintained on a 12 h dark/light cycle. All animals were tested during the light cycle at about the same time of the day (9:00 am–12:00 a.m.). All procedures were approved by the Institutional Animal Care Committee of the University of São Paulo (protocol number 150 – book number 02/2010). Efforts were made to minimize the number of animals used and their suffering. To reduce stress, rats were daily adapted to the experimental environment for 3 days before the experiments.

Surgical procedure

Inferior alveolar nerve injury. IAN crush was performed as described previously (12), under deep anesthesia with ketamine (5mg/100g body weight, ip) and xylazine (1mg/100g body w, ip). The skin was sterilized

around the inferior jaw with povidone-iodine, and a small incision was made on the surface of the facial skin over the masseter muscle about 1 cm long. The alveolar bone was carefully removed and the IAN was exposed on the right side. The IAN was crushed for 30 seconds. Then muscle fibers were reapproximated and the incision was sutured. A control group of false-operated animals (sham), without the nerve crush, was also organized.

The animals were put back into the cages and observed until the moment they recovered, when they were brought back to the animal facility.

Laser therapy. The treatment was similar to that used in a previous study (12). Briefly, rats were randomly divided into 4 groups (5 rats per group). Two of these groups were irradiated with GaAs laser (Gallium Arsenide, Laserpulse-Laser, Ibramed Brazil) emitting a wavelength of 904 nm and an energy density of 6 J/cm². The treatment with the LLLT was initiated 2 days after surgery for IAN injury or in sham animals. The laser treatment was performed every other day, involving 10 sessions. After sterilization, the laser was lightly placed on the skin surface directly above the neural pathway of the IAN, without contact with the skin. A point near the temporomandibular joint and four points in the region of the inferior jaw body were irradiated, with duration of 18s per point. Each point was irradiated with intervals of 30s and each session had a total duration of 4 minutes.

Tissue preparation

Rats were euthanized by decapitation under isoflurane anesthesia and the TG was quickly removed and homogenized in an extraction buffer containing 100 mM Tris, pH 7.4, 1 % SDS, 10 mM EDTA, 2 mM PMSF, and 10 µg/mL aprotinin. After extraction, the homogenates were centrifuged at 12,000 rpm at 4°C for 20 min and the protein concentration of the supernatant was determined using the Bradford protein assay with albumin as a standard (Bio-Rad, Melville, NY) (13). Samples containing 75 µg of protein were loaded on a 12% acrylamide gel and electrotransferred to nitrocellulose membranes using a Bio-Rad miniature transfer apparatus for 1.5 h at 120V. After transfer, the membranes were treated for 2 h at room temperature with a blocking solution containing 5% powdered milk, washed and incubated overnight at 4°C with an anti-GluA1 (F30, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA,

USA), anti-GluA2 (1:5000; Sigma, St. Louis, MO, USA), anti-TRPV1 (rabbit polyclonal antibody, ACC-030, Alomone labs, Jerusalem, Israel), anti-Substance P (rabbit polyclonal antibody, AB962 Chemicon International, Temecula, CA, USA) and anti-CGRP (rabbit polyclonal antibody, ab47027, Abcam INC.), diluted 1:1000. The membranes were then washed and incubated for 2 h at room temperature with peroxidase-conjugated anti-rabbit (GE Healthcare) secondary antibody, diluted 1:5000. An anti- β -actin antibody (Sigma) was used to quantify β -actin as a loading control. The specifically bound antibody was visualized using a chemoluminescence kit (Amersham Biosciences). The blot was analyzed densitometrically using NIH-Scion Image 4.0.2 and quantified by optical densitometry of the developed autoradiographs (Scion Corporation, Release Beta 3b, NIH). Density values of bands were normalized to the total β -actin present in each lane and expressed in percentage of control.

Statistical analysis

Results are presented as means $\pm$ SEM. Statistical analyses of data were generated using GraphPAD Prism, version 4.02 (Graph-Pad Software Inc., San Diego, CA, USA). Statistical comparison of more than 2 groups was performed using analysis of variance (ANOVA);

differences between means were evaluated by the Tukey's test. In all cases, $p \leq 0.05$ was considered to be statistically significant (14).

RESULTS

Effects of IAN injury and laser therapy on protein expression

We did not observe differences of GluA1 expression between naive, sham +laser and IAN injury groups, but detected an increase of GluA1 levels on the side ipsilateral to the lesion in IAN-lesioned animals treated with laser (IAN injury + laser) when compared to the same side from naive rats, taken as a control and designated as 100%, and with the other groups. Densitometric analysis revealed that GluA1 level did not show significative changes in sham + laser and IAN injury groups. On the other hand, rats that received treatment with laser therapy (IAN injury + laser) had an increase of GluA1 expression (59% above the control; $p \leq 0.001$) (Fig. 1A). No statistically significant differences of GluA1 expression were observed between sham + laser and naive animals.

In the same way, our results also showed no

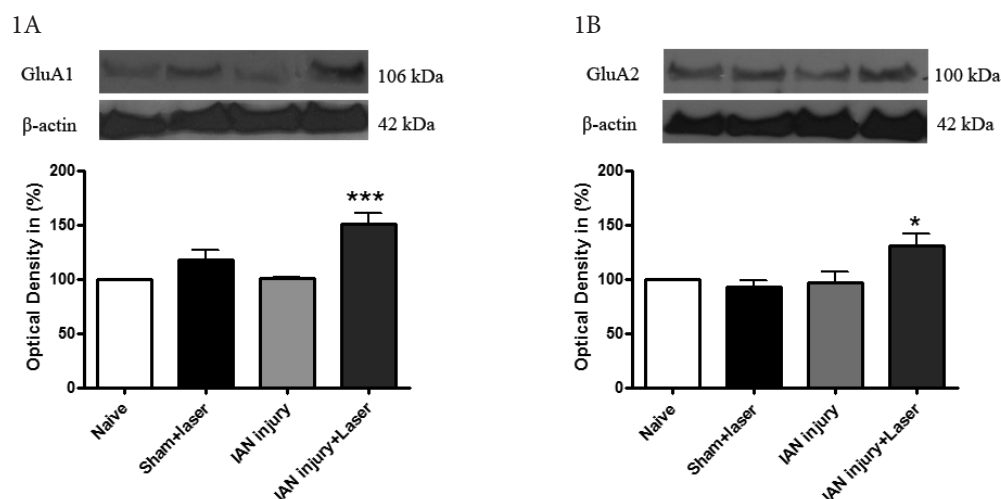


Fig. 1. Densitometric analysis of GluA1 and GluA2 protein levels. **A, B**) Expression level of GluA1 and GluA2, respectively, after the last session evaluated. Twenty days after injury and 20 days after LLLT. Data are normalized in relation to those from naive rats. Data are reported as mean $\pm$ SEM of 5 animals per group. * $p \leq 0.05$ and *** $p \leq 0.001$ compared to other groups.

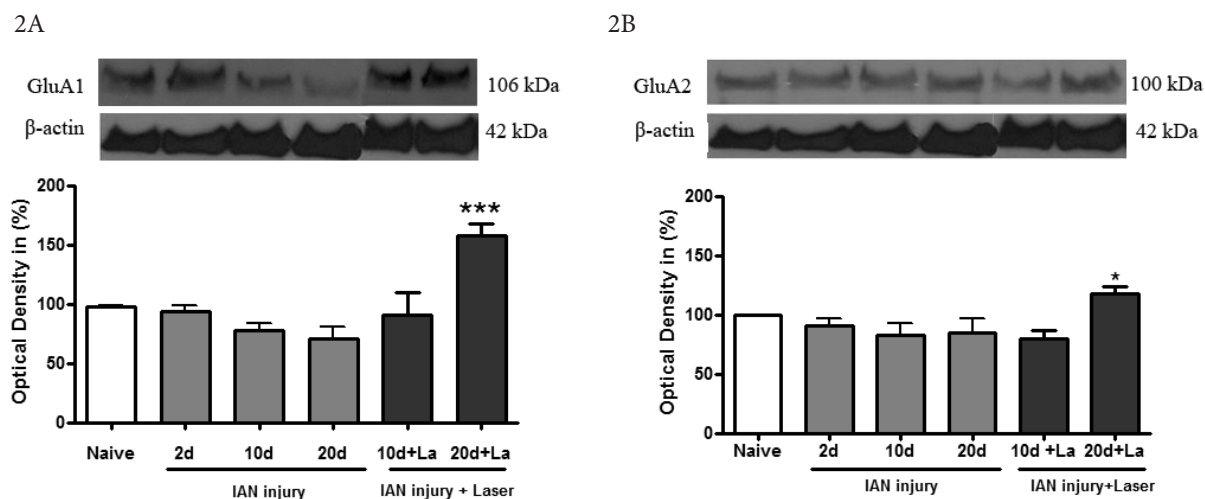


Fig. 2. Temporal densitometric analysis of GluA1 and GluA2 protein levels. **A,B)** Expression level of GluA1 and GluA2, respectively, at 2 days, 10 days and 20 days after injury and 10 days and 20 days after LLLT. Data are normalized in relation to those from naive rats. Data are reported as mean $\pm$ SEM of 5 animals per group. * $p \leq 0.05$ and *** $p \leq 0.001$ compared to other groups.

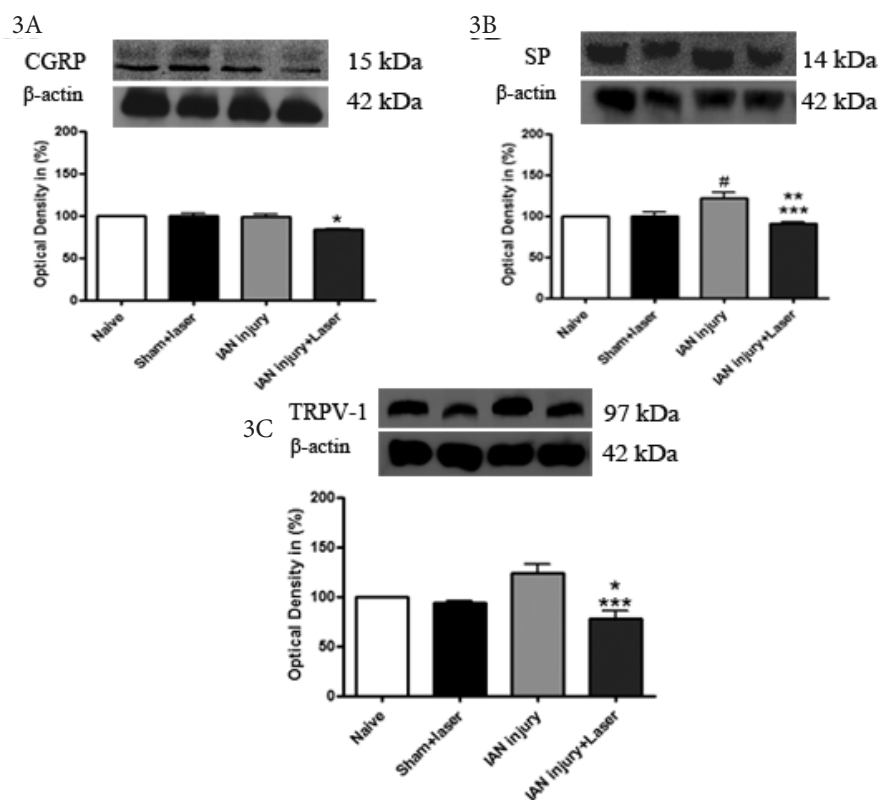


Fig. 3. Densitometric analysis of CGRP (3A), SP (3B) and TRPV1 (3C) protein levels. Data are normalized in relation to those from naive rats. Data are reported as mean $\pm$ SEM of 5 animals per group. * $p \leq 0.05$ compared to control animals, * $p \leq 0.05$ compared to control groups and compared to IAN injury group, ** $p \leq 0.001$ and *** $p \leq 0.0001$ compared to IAN injury group.

differences in expression levels of GluA2 in sham + laser and IAN injury groups when compared to naive animals. There was, however, an increase (14% above the control; $p \leq 0.05$) after laser therapy treatment (IAN injury + laser). No differences were observed between naive and sham + laser animals, used as controls (Fig. 1B). To clarify this similar profile of expression between GluA1 and GluA2 and for measuring changes in AMPAr subunit protein levels temporally, we also evaluated its protein levels at different time points during the treatment [2 days after lesion; 10 days after lesion (5^a session); and 20 days after lesion (last session)]. Similar to the results above, the temporal analysis showed that GluA1 expression did not change at 2 days, 10 days and 20 days after injury when comparing to the naive group. However, we observed an increase at 20 days after injury plus laser (last time evaluated) (Fig. 2A). Analysis of GluA2 temporal expression shows that at 2 days, 10 days and 20 days after injury there was no significant change compared to the naive group. Twenty days after injury plus laser we saw an increase (Fig. 2B).

No changes were observed in the expression of CGRP after IAN injury compared with the control group (naive and sham + laser). On the other hand, after 10 sessions of laser therapy, the IAN-injury + laser group showed a decrease in the CGRP expression protein level when compared to the bands of the naive group [16% below the control ($p \leq 0.05$)]. No differences were observed between naive and sham + laser animals, used as controls (Fig. 3A).

Our results also showed an increase of SP levels (21% above the control; $p \leq 0.01$) after IAN injury when compared to naive animals. There was, however, a decrease (10% below the control; $p \leq 0.05$) after laser therapy treatment (IAN injury + laser). No differences were observed between naive and sham + laser animals, used as controls (Fig. 3B).

Our data show an increase of TRPV1 levels on the side ipsilateral to the lesion in IAN-injured animals (IAN-injury) when compared to the same side from naive rats, taken as controls and designated as 100%. Densitometric analysis revealed that the TRPV1 average increase was 24% ($p \leq 0.05$) in the IAN-injury group above the control group. On the other

hand, rats that received treatment with laser therapy (IAN injury + laser) had a decrease of expression [23% below the control ($p \leq 0.05$) and 47% below the NAI-injury ($p \leq 0.0001$)]. No statistically significant differences of TRPV1 expression were observed between sham + laser and naive animals (Fig. 3C).

DISCUSSION

IAN crush produced sustained modification in sensory processing, resulting in mechano-allodynia in an area adjacent to the area innervated by the injured IAN. In the present study we observed an increase in TRPV1 and SP protein level, and no changes of AMPA GluA1 and GluA2 subunits and CGRP after IAN injury in the TG. On the other hand, we observed that treatment with laser therapy increased the expression of AMPAr subunits compared to controls, and decreased the expression levels of TRPV1, SP and CGRP compared to controls. Our results showed the presence of GluA1 and GluA2 proteins in TG in all groups and at all times evaluated. Similarly, the IAN injury did not significantly alter the expression of either. But LLLT resulted in statistically significant up-regulation of AMPA subunits GluA1 and GluA2 protein levels in a time-dependent manner.

Some data propose that the peripheral GluAs are responsible for nociceptive responses via TRPV1 activation (15). One study showed that persistent pain states are mediated by cooperative actions of TRPV1, glutamate, SP and CGRP (6). Our findings also reveal a probable cooperative role for nociceptive processing from TRPV1 expression mediating mechano-allodynia associated with IAN injury. However, the interactions between GluAs and TRPV1 remains poorly documented, especially in the trigeminal system. Based on our observations, we hypothesized that the possible mechanism of mechanical allodynia after IAN injury was not affected by AMPAr and CGRP expression; this behavior may be due to a higher SP expression level which leads to sensitization of TRPV1 receptors in trigeminal afferents.

The present results point out to a critical role of the involvement of AMPAr subunits in trigeminal

chronic pain mechanisms. The AMPA receptor trafficking dynamics in the trigeminal system under orofacial pain conditions is needed to understanding the nociceptive actions of AMPA receptor in TG following nerve injury.

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LETTER TO THE EDITOR

ROLE OF DIODE LASERS IN ORO-FACIAL PAIN MANAGEMENT

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With the increasing use of low level laser therapy (LLLT) in clinical dentistry, the aim of the present study was to assess the effectiveness of diode lasers in the management of orofacial pain. Indexed databases were searched without language and time restrictions up to and including July 2016 using different combinations of the following key words: oral, low level laser therapy, dental, pain, diode lasers, discomfort and analgesia. From the literature reviewed it is evident that LLLT is effective compared to traditional procedures in the management of oro-facial pain associated to soft tissue and hard tissue conditions such as premalignant lesions, gingival conditions and dental extractions. However, it remains to be determined which particular wavelength will produce the more favorable and predictable outcome in terms of pain reduction. It is highly recommended that further randomized control trials with well-defined control groups should be performed to determine the precise wavelengths of the diode lasers for the management of oro-facial pain. Within the limits of the present review, it is concluded that diode lasers therapy is more effective in the management of oro-facial pain compared to traditional procedures.

To the Editor,

One parameter that determines the success of any clinical therapy is management of pain associated with the disorder. A variety of scales have been developed to assess the severity of pain, which include the Likert scale, Visual Analog Scale (VAS), Numerical Rating Scale and the orofacial Pain Scale for non-verbal individuals (1-3). One intervention, which is effective in the mediation of acute and chronic pain, is low-level laser therapy (LLLT) (4, 5). It has been suggested that diode lasers play a role in the mediation of analgesia by affecting the synthesis, metabolism and release of neurochemicals, such as endorphins and enkephalin, which alter nerve

conduction and modify pain response (6). Diode lasers have been used to reduce oro-facial pain associated with dental extractions (7), orthodontic treatment (8) and trigeminal neuralgia (9).

With the increasing use of LLLT in clinical dentistry, the aim of the present review was to assess the effectiveness of diode lasers in the management of oro-facial pain.

MATERIALS AND METHODS

Indexed literature in PubMed/Medline (National Library of Medicine, Washington, DC), Cochrane Register of Systematic Reviews, EMBASE, ISI Web

Key words: lasers, low-level light therapy, oro-facial, pain

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of Knowledge and Google-Scholar databases were searched using the following key words: (a) oral; (b) low level laser therapy; (c) dental; (d) pain; (e) diode lasers; (f) discomfort; and (g) analgesia. Letters to the editor, commentaries, and conference abstracts were excluded.

RESULTS

Some studies (10-13) have reported that there is no difference in the severity of pain perception following conventional surgery and CO₂ laser in the treatment of leukoplakia; whereas, Kharadi et al. (13) showed complete lesion removal and minimal pain intensity according to the VAS scale after one month of treatment. Basha et al. (14) reported that neodymium-doped yttrium aluminium garnet (Nd:YAG)-laser, when used for gingival excoriation, presented significant improvement in VAS pain scores compared to surgical stripping after the first post-operative week. Braun et al. (15) compared the intensity of pain on the VAS among patients treated with Er:YAG laser and sonic scaling, and reported a greater decrease in pain scores in the group treated with lasers. Kara et al. (16) and Haytac et al. (17) reported that patients' experience of pain recorded on the VAS was lower following Nd:YAG- and CO₂-laser frenectomy compared to scalpel surgery, respectively. In the study by Ferrante et al. (18), patients who received LLLT did not experience a significant reduction in pain compared with the 15 controls (No LLLT)

DISCUSSION

With reference to the effect of lasers in pain reduction among patients with oral premalignant lesions, controversial results have been reported (10-12). It is pertinent to mention that in the study by Ribeiro et al. (12) most of the lesions were between 20 mm and 50 mm. Post-operative pain is affected by the size of the excised lesion as the time between the inflammatory and granulation phase is longer in larger lesions.

With reference to the role of Nd:YAG lasers in gingival excoriation, it is hypothesized that the reduction in pain may be related to the sealing of the

sensory nerves and coagulum formed on the wound surface following laser irradiation. Diode laser treatment of gingival hyperpigmentation has been demonstrated to be less painful than electrosurgery in the immediate post-operative period. In relation to the reduction in postoperative pain using Er:YAG lasers compared with sonic scaling, it has been suggested that the vibratory and acoustic component of the sonic scaling may have contributed to the increased pain perceived by these patients. The usual management of low frenum insertion consists of frenectomy using scalpel and blade under local anesthesia. However, laser use has demonstrated efficient cutting with no bleeding, no need of anesthetic, antibiotic or analgesics, faster recovery and reduced pain (19). It is likely that the decreased edema, possible sterilization of tissues which could reduce post-operative infections and decreased requirement for sutures, may contribute to reducing the overall pain experienced following diode laser therapy. As far as the role of lasers in reducing pain following third molar extraction is concerned, it is speculated that the difficulty level of the extractions could have influenced these conflicting outcomes. It is hypothesized that more complicated extractions, which require significant bone and tissue removal, may not be as responsive to laser irradiation for pain control. However, further studies are needed in this regard.

From the literature reviewed it is evident that LLLT is effective in the management of oro-facial pain compared to traditional procedures. However, it remains to be determined which particular wavelength will produce the more favorable and predictable outcome in terms of pain reduction. Moreover, it is highly recommended that further randomized control trials with well-defined control groups should be performed to determine the precise wavelengths of the diode lasers for the management of oro-facial pain. Within the limits of the present study, diode lasers therapy is more effective in the management of oro-facial pain compared to traditional procedures.

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LETTER TO THE EDITOR

OSTEOGENESIS-PROMOTING ACTIVITY OF COMPOSITES SBA-15 MESOPOROUS PARTICLES CARRYING OXYTOCIN *IN VITRO* AND *IN VIVO*L. CHENG¹, X. HE¹, Y. CHE², H. CHE³ and M. QIAN¹¹Department of Prosthodontics, School of Stomatology, Jilin University, Changchun, China;²Department of Science and Education, School of Stomatology, Jilin University, Changchun, China;³Department of Periodontics, School of Stomatology, Jilin University, Changchun, China

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This study analyzes the performance of SBA-15 mesoporous particles carrying oxytocin (OT) in promoting osteogenesis *in vitro* and *in vivo*. The SBA-15 particles synthesized in the previous studies (about 30 µm in diameter and containing 10 nm deep pores) were loaded with the drug oxytocin and cultured with human osteosarcoma MG-63 cell line *in vitro*. The influence of particles on cell proliferation was studied. The level of the osteogenic marker (alkaline phosphatase and type I collagen) was measured. For *in vivo* studies, the connectivity defects of rabbit skull were prepared, and SBA-15 suspensions were regularly injected at the defect sites. The changes in the defect site calcium salt deposition were measured, and morphological changes were observed by microscopy. The material had to promote effect on osteogenesis-related indicators such as alkaline phosphatase and collagen I in bone sarcoma cell line MG-63. *In vivo*, the calcium salt deposition in OT/SBA-15 group was significantly higher than in the blank group. SBA-15 carriers appeared to persist in the region of the defect after the injection and release the drugs slowly, thus playing a more distinct role in promoting bone repair of local bone defects. The results showed that SBA-15 particles with OT could slow the release drugs and could help in promoting osteogenesis.

To the Editor,

Repair of a bone defect is a relatively slow process. It takes 2-4 months for fresh blood to fill the bone defect and early osteogenesis to finish, and between 6 months and two years for the mature bone to form (1, 2). To speed up this process, some biological agents can be used. For example, bone morphogenetic proteins (BMPs) (3), and vascular endothelial growth factor (VEGF) can be administered. However, the drug utilization ratio through systemic drug administration is very weak, and therefore these factors can only be administered locally.

Even with local drug administration, the problem of drug retention and sustained release in the defect site needs to be solved (4, 5). For example, although platelet rich plasma (PRP) contains a lot of effective growth factors, most of them are lost soon after local injection to the bone defect site, and thus cannot be used effectively. However, the growth factors-containing platelet rich fibers with spongy structure can slow down the speed of factor release and has thus been widely used in the field of bone defect repair (3, 6, 7).

We previously reported (8) that our experimental

Key words: osteogenesis, oxytocin, SBA-15 mesoporous particles

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group had synthesized mesoporous material SBA-15 by hydrothermal treatment. SBA-15 has a particle diameter of 30 microns and a highly orderly internal pore structure with pores of 10 nm in diameter. The pores can be used to load and release small drug molecules that are less than 10 nm in size.

We then prepared SBA-15 composite containing small molecular drug oxytocin (OT) (OT/SBA-15). OT was used as a model drug to obtain preliminary data on the biological safety of the material *in vitro*. In this study, we further validated the performance of SBA-15/OT composites in promoting osteogenesis *in vitro* and *in vivo*.

MATERIALS AND METHODS

Effect of OT/SBA-15 on the proliferation of MG63 cells

WST-1 powder (6.52 mg) and 2 ml of electron coupling reagent (Abnova) were thoroughly mixed and placed into a water bath (37°C) for 3 min until complete dissolution of WST-1. The resulting solution was stored at 4°C in the absence of light. The MG63 cells were plated on the 96-well cell culture plate (2000 cells per well in 100 μ L of culture medium) and incubated at 37°C, 5% CO₂, humidity culture incubator for 8 h till the cells were adherent. Three groups (control group, SBA-15 group, OT/SBA-15 group) were set, with six wells per group. The cells were incubated for 1, 2, 3, 4, 5, 6, or 7 days. At the end of the incubation period, 10 μ L of WST-1 solution were added to each well. After 2 h of incubation, the cells were shaken for 1 min to mix the system. The absorbance was measured at 450 nm with microplate reader

Testing for alkaline phosphatase and type I collagen

Specimens were tested for alkaline phosphatase (ALP) levels by double antibody sandwich assay. The microtiter plate was coated with purified ALP antibody. Then the antibody was fixed with ALP and combined with horseradish peroxidase (HRP)-labeled goat anti-human antibody to form antibody-antigen-enzyme labeled antibody complexes. After washing, tetramethylbenzidine (TMB) substrate was added to display color. Using the commercially available kit (Ebioscience), TMB was transformed into a blue colored substance under catalysis by HRP, and then to a final yellow substance. The depth of color was positively correlated with the level of ALP in

the sample. Microplate reader was used to measuring the absorbance (OD) at 450 nm, and ALP concentration was calculated using the standard curve. The samples were respectively divided into the control group, SBA-15 group, and OT/SBA-15 group, the testing cycle was set at 3 days, seven days, and 14 days.

Purified antibodies to type I collagen were used to coat the microtiter plate. The measurements of collagen I levels were taken using the same method as described above.

Preparation of animal skull defect model and administration

Five rabbits, weighing 2.5 kg each and aged three months, were anesthetized (1.5 mg of mebubarbital per kg of body weight), conventionally disinfected, sliced in the middle of the skull, and the soft tissue was separated to expose the top of the skull. Penetrating defect (5mm x 5mm) was prepared in four distal quadrants at the top of the skull (Fig. 1). Picric acid was used to mark the site of the defect after suturing and shaving. Subcutaneous injections were given to the corresponding parts of the defect once a week for seven weeks.

GE scanning

After 2, 3, 4, 5, 6, 7 or 8 weeks post-surgery, GE energy spectrum CT was used to scan the animals. Specifically, the rabbits were anesthetized and fixed with a special head rest and scanned by GE spectrum CT in the GSI mode. CT was set at a GSI-Helical voltage (80 kVp/140 kVp), pitch 0.984:1, detector collimation 64 x 0.626 mm, ball tube speed 0.8s/turn, scanning and interval thickness - 5 mm, the reconstruction layer thickness - 1.25 mm. All data analysis and image processing were carried out using AW4.6 GE workstation and analyzed by Viewer GSI software to obtain three-dimensional reconstruction image and calcium (water) and water (calcium) map. First of all, the defect site was calibrated on a 3D reconstruction image, to divide the defect area at the corresponding level, then measure the area of calcium based value/water-based values. Each site was measured three times, the average values were taken, and the index of calcium salt deposition was calculated.

HE staining

Eight weeks after the operation, animals from the experimental group were sacrificed by intravenous

injection of air, and the skulls were separated. Firstly, the bones were processed for seven days in 50% formic acid and 10% aluminum chloride, followed by 15 days in 10% EDTA for decalcification, and then washed overnight. The specimens were gradient dehydrated as follows: 60% butanol for 2 h, 70% butanol for 2 h, 80% butanol for 2 h, 90% butanol for 16 h, 95% butanol for 2.5 h, absolute alcohol butanol for 1.5 h, butanol for 16 h, xylene for 45 min. Then the specimens were dipped in paraffin, embedded, and cut at the bone defect center along the sagittal position to obtain 5- μ m complete paraffin sections, and analyzed by microscopy after the HE staining.

RESULTS

WST - 1 test results

The cell proliferation WST-1 test results for non-drug loaded SBA-15, OT/SBA-15 and control group from day 1 to day 7 are shown in Fig. 1.

The experimental results show that the cell proliferation of SBA-15 was slightly higher than the control group, but the effect did not reach statistical significance. It illustrates that SBA-15 has to promote effect on cell proliferation. The OT/SBA-15 group was slightly higher than the control group at the initial stage, but no significant difference was observed compared to the control group and SBA-15 group. From the fourth day, however, the cell

proliferation activity in the OT/SBA-15 group was significantly higher than in the control and SBA-15 groups. Slow release of OT by SBA-15 material may be the reason for this effect.

Alkaline phosphatase and collagen type I detection

The test results for the level of alkaline phosphatase and type I collagen in no-drug loaded SBA-15, OT/SBA-15 and control group at days 3, 7 and 14 are shown in Figs. 2 and 3.

Detection results for alkaline phosphatase showed that at days 3 and 7 alkaline phosphatase activity in SBA-15 group did not significantly change compared with the blank group, and there was no significant difference compared with the control group. Compared with the alkaline phosphatase activity in the blank and SBA-15 groups, the ALP activity in the OT/SBA-15 group was significantly increased. The difference gradually disappeared by day 14, resulting in no significant differences between all three groups.

Detection results for the level of collagen I showed that there were no significant differences between the blank group and SBA-15 group at day 3, but the OT/SBA-15 group had significantly higher level of collagen than the other groups. At days 7 and 15, the three groups had no significant difference in collagen level.

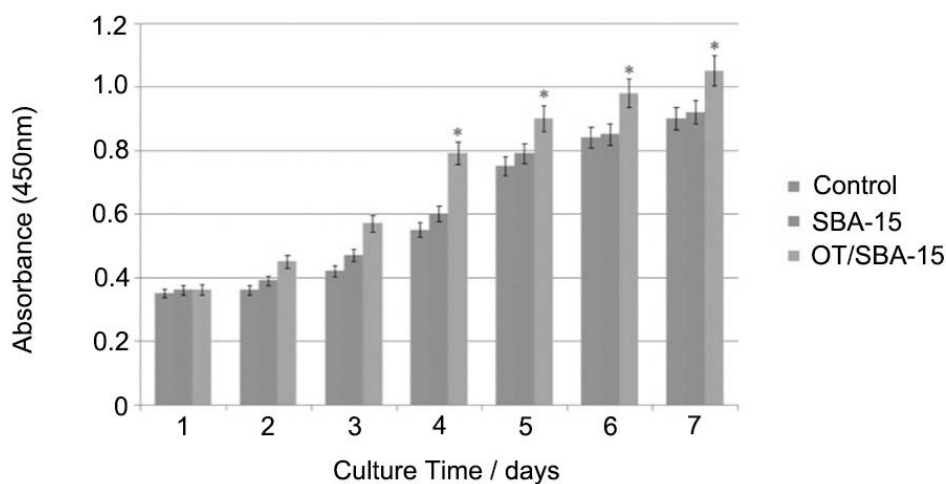


Fig. 1. WST-1 test results for non-drug loaded SBA-15, OT/SBA-15 and control group.

CT scan results of GE energy spectrum

Detection results for calcium salt deposition quantity showed that the calcium salt precipitation amount in each group gradually increased with the passing of time. The control group and the other groups had no significant difference at week 2; the three groups had no significant differences from week 3 to week 6, but the calcium salt deposition index in the OT/SB-15 group was significantly higher than in the above three groups. There was no significant difference between the four groups at weeks 7 and 8.

Results of HE staining

New bone formation with continuous healing was observed in the control group and the OT/SBA-15 group under low magnification. In the high-power field, a large amount of new bone formation near the edge of the defect area and osteoblasts around the new bone were visible. Near the center of the defect area, new bone formation was less prominent, but a large number of active osteoblasts were aggregated. There were no obvious inflammatory cells present in the control group and the OT/SBA-15 group.

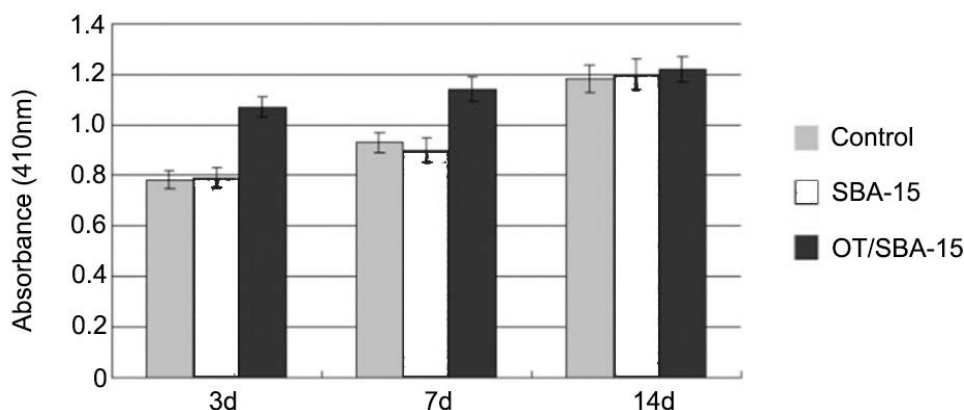


Fig. 2. Detection results for alkaline phosphatase.

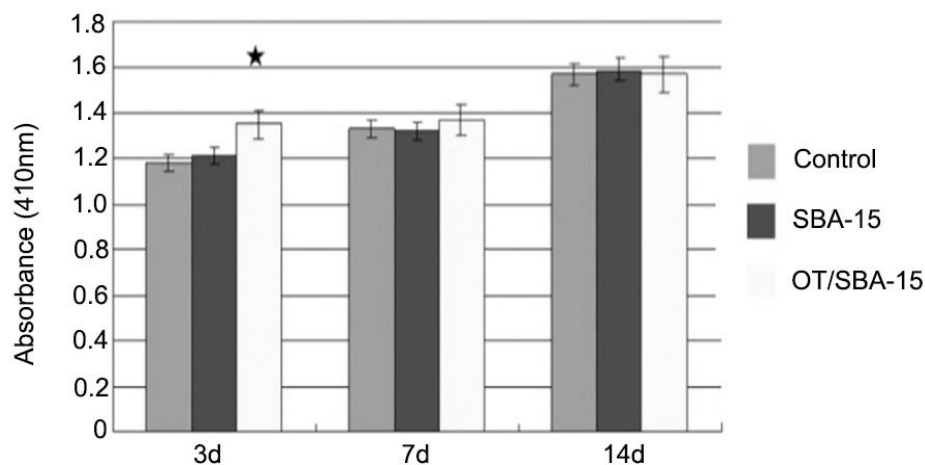


Fig. 3. Detection results for collagen I.

DISCUSSION

Commonly used scaffold materials at present include organic, biodegradable materials, unlike the earliest artificial bone scaffold materials that were non-biodegradable. Materials used then were characterized by a high mechanical strength. Due to the problems associated with the second surgery, researchers began to study the use of biodegradable and bioactive materials. In fact, if a material has good biological compatibility and enough space not to hinder tissue growth, non-biodegradable materials can also be used for tissue engineering (3, 9-11)

In this study, we used previously synthesized silicate crystals SBA-15 to prepare the SBA-15 particles loaded by the drug, and use them to verify their effect on promoting osteoblast proliferation and differentiation *in vivo* and *in vitro*. Studies *in vitro* showed that the material has a distinct promoting effect on osteogenesis-related indicators. SBA-15 has no harmful effects on the healing of bone defects, however, it can persist in the region of defect after the injection and release the drugs slowly, thus playing a more obvious role in promoting bone repair of local bone defects. *In vivo*, the defects produced in animals in this experiment were not finalized by the end of our experiment and healed around week 8. This was the reason why the OT/SBA-15 group and the blank group demonstrated no obvious advantages at the late stages of bone repair.

On HE stained slides we found that inflammatory reaction during the late bone defect repair in the OT/SBA-15 group, as well as new bone formation, exhibited no significant difference compared to controls at week 8, which highlights potential inevitableness of SBA-15 implementations to the defect site.

ACKNOWLEDGEMENTS

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LETTER TO THE EDITOR

UP-REGULATION OF miR-888-5p IN HEPATOCELLULAR CARCINOMA CELL LINES AND ITS EFFECT ON MALIGNANT CHARACTERISTICS OF CELLSE. HAO¹, J. YU², S. XIE¹, W. ZHANG² and G. WANG¹¹Department of General Surgery, ²Institute of Virology and AIDS Research, The First Hospital of Jilin University, Changchun, Jilin, China*Received September 1, 2016 – Accepted February 17, 2017*

MicroRNA (miRNA) expression has been linked to the molecular pathogenesis of hepatocellular carcinoma (HCC). The aberrant expression of miRNA is involved in the processes of tumorigenesis and cancer progression. According to the latest research, miR-888-5p is associated with strong cancer-promoting effect. For instance, miR-888-5p is up-regulated in prostate cancer and breast cancer. Nevertheless, the role of miR-888-5p in HCC has not been investigated to date. In this study, we found that miR-888-5p levels in four HCC cell lines (SMMC7721, HepG2, Huh-7 and Bel7402) were significantly up-regulated compared with human hepatocyte cell line (HHL-5). After transiently transfected with miR-888-5p mimic, our results demonstrated that miR-888-5p plays a major role in promoting the proliferation and metastatic potential of HCC cells. Moreover, miR-888-5p also increased the expression of MMP-2 and MMP-9 proteins which account for cell migration and invasion, and decreased the expression of p53 protein which further promoted malignance of HCC. Therefore, miR-888-5p may be considered a potential biomarker for diagnostics and prognosis of HCC.

To the Editor,

Hepatocellular carcinoma (HCC) is the fifth most prevalent tumor and the third cause of cancer-related mortality worldwide. Most patients undergo recurrence and/or develop metastatic disease after surgery, and their prognosis remains poor (1).

microRNAs (miRNAs) have gained importance in the processes of tumorigenesis (2). Depending on the role of the target gene in tumor development and progression, miRNAs can function as tumor oncogenes or suppressor genes. The use of miRNAs as potential biomarkers for the diagnosis, therapeutic treatment and prognosis of HCC has recently increased. It has been found that miR-21, miR-155, miR-664, miR-485, miR-495 (5-7), which regulate the expression of many genes, are up-regulated during carcinogenesis and progression of HCC. The

miR-21 can enhance the tumor-suppressor function of programmed cell death protein 4 (PDCD4) in HCC (3). Aberrant expression of miR-155 may accelerate cell proliferation by targeting sex-determining region Y box 6 in HCC (4).

According to recent data, miR-888-5p is associated with strong cancer-promoting action. miR-888-5p was shown to be up-regulated in prostate (5) and breast cancer (6, 7). The up-regulation of miR-888 contributes to aggressiveness in MCF-7 SP cells. This microRNA was shown to repress the adherent junction (AJ) pathway in MCF-7 cells (9). However, the role of miR-888-5p in HCC has not yet been investigated.

In this study, we focus on the expression and function of miR-888-5p in HCC. To determine whether miR-888-5p could be used as a potential

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diagnostic marker, we analyzed miR-888-5p levels in four human HCC cell lines and one human hepatocyte cell line by using qRT-PCR assay, and evaluated the effect of miR-888-5p on tumorigenesis.

MATERIALS AND METHODS

Cell cultures and materials

Human HCC cell lines HepG2, Huh-7, SMMC7721, and Bel7402 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HHL-5 cell line was a gift from Prof. Y-H. Gao (Harbin Medical University). All cells were cultured in DMEM supplemented with 100 mL/L fetal bovine serum at 37°C in a 5% CO₂ tissue culture incubator.

RNA isolation and qRT-PCR

TRIZOL reagent (Invitrogen, USA) was used according to the manufacturer's instructions to extract the total RNA (including miRNAs). NanoDrop 2000 (NanoDrop Technologies, USA) was used for RNA quantification. MiRNA Reverse Transcription Kit (Takara, Japan) was used according to the manufacturer's instructions for reverse transcription of miRNAs. The relative expression levels of miR-888-5p in each cell line were assayed by qRT-PCR and the data analysis was performed using the 2^{-ΔΔCt} method. The results were standardized using U6 as a control. The commercial primers for miR-888-5p and U6 were bought from RiboBio Co. (Guangdong, China). Each experimental group consisted of 3 wells adjacent to each other, and the tests were repeated three times.

Transient transfection

Commercially available miR-888-5p mimic and negative control miRNA (NC) were purchased from RiboBio Co. (Guangdong, China). Cells were seeded in 6-well culture plates (Corning, USA) at a density of 6 × 10⁵ cells/well and transfection of miRNA was performed 24 h later with lipofectamine 2000 (Thermo, USA) according to the manufacturer's protocol. qRT-PCR was used to examine the transfection efficiency. RNA isolation was conducted 72 h after transfection.

MTT analysis

A total of 2 × 10³ cells per well were plated into a 96-well plate. Cells were cultured for 24 h after miRNA

transfection, and the cell growth was measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Promega, USA). Percentage of viable cells was evaluated by measuring the absorption at 490 nm using microplate reader (Epoch, BioTek) at the same time points each day (days 1, 2, 3, 4, 5, 6, and 7), as per the manufacturer's instruction.

Plate clone formation assay

After being cultured for 24 h post-transfection, about 100 cells were added to each well of a 6-well culture plate, and each group contained three wells. After incubation at 37°C for 14 days, the cells were washed twice with 1X PBS and stained with Crystal Violet Staining Solution. The number of colonies containing at least 50 cells was counted under a microscope. The plate clone formation efficiency was calculated using the formula: plate clone formation efficiency = (number of colonies/number of cells inoculated) × 100%.

Wound healing assay

After cells had been transfected with miRNA in serum-free medium for 24 h, the cells were seeded at 2.0 × 10⁵ cells/well in 24-well plates and allowed to reach 100% confluence. A scratch was created on the surface of cell monolayer using a micropipette tip. The scratch area was photographed by microscopy at ×100 magnification every 24 h after scratching. The width of the scratch was

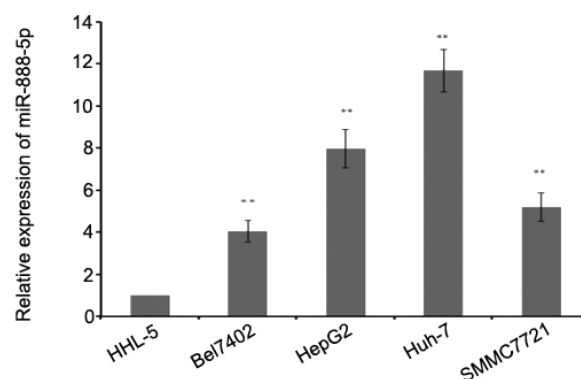


Fig. 1. The miR-888-5p levels in human HCC cell lines vs human hepatocyte cell line. Individual qRT-PCR reactions verified elevated level of miR-888-5p in aggressive human HCC cell lines vs human hepatocyte cell line (HHL-5) ($P < 0.01$). Huh-7 had the highest level of miR-888-5p. The results were standardized using U6 as a control.

measured, and the migration distance was calculated by Image J (Version 1.37v, Wayne Rasband, NIH, USA). Five separate visual fields were measured in each experiment.

Transwell assay

After cells had been transfected with miRNA in serum-free medium for 24 h, cell migration and invasion assay were performed using Transwell chamber (8 μ m, 24-well format; Corning, USA). In the migration assay, 1×10^5 cells in 0.2 ml of serum-free medium were added to the upper chamber, and 0.6 ml of medium containing 10% FBS were added to the lower chamber. Cells were then incubated for 24 h. For invasion assay, diluted Matrigel (BD Biosciences) was used to coat the insert chambers' membrane. Cells were cultured for 48 h under the same conditions. Finally, cells that migrated or invaded into the lower chambers were fixed with methanol, stained with crystal violet and counted in six random fields.

Western blot

MMP-2, MMP-9, p53 and GAPDH (Santa, USA) protein levels were determined by Western blot analysis. RIPA lysis buffer was used to extract proteins. The protein concentrations were measured by BCA Protein Assay Kit (Thermo, USA) and adjusted to the equal level. The samples were separated by electrophoresis on 12% SDS-PAGE gels (80 V, 30 min; 120 V, 90 min). The proteins were transferred to PVDF membranes (Life, USA) and blocked with 10% skimmed milk. Next, the corresponding primary antibodies (1: 500) were used to detect the proteins. The membranes were washed with TBST (Tris-buffered saline and Tween 20) thrice. Subsequently, the blots were incubated with the corresponding secondary antibody at 1:1,000 dilutions. Proteins were visualized using nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indoylphosphate and quantified using a scanner (Hp G4050; Hewlett-Packard, USA). The quantification

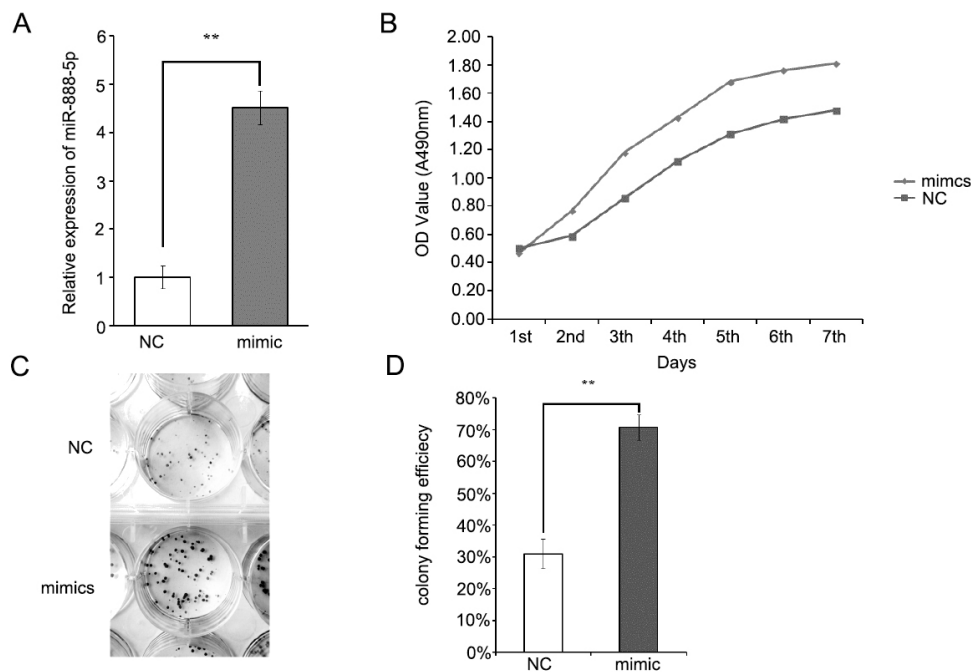


Fig. 2. Cellular proliferation analyses demonstrate that overexpression of miR-888-5p promoted the proliferation of HCC cells. **A)** Expression of miR-888-5p in each transfection group, the comparison between NC group (NC, negative control miRNA) and miR-888-5p overexpression group (mimic) ($P < 0.01$). **B)** MTT assay for cellular proliferation. Comparison between NC group and miR-888-5p overexpression group ($P < 0.01$). **C)** Colony formation assay showed that NC group displayed much fewer and smaller colonies ($31.0 \pm 3.5\%$) compared to miR-888-5p overexpression group ($70.7 \pm 4.5\%$) ($P < 0.01$). **D)** The plate clone formation efficiency was calculated using the formula: plate clone formation efficiency = (number of colonies/number of cells inoculated) $\times 100\%$.

was performed as follows: relative expression of the target protein=gray level of target band/gray level of the same sample reference.

Statistical analysis

All experiments were performed three times. Semi-quantitative analysis of the bands was measured with the Image J analysis software (Version 1.37v, Wayne Rasband, NIH, USA). The data were presented in the mean $\pm$ SD format and analyzed by independent-samples *t*-test (SPSS version 19.0). ***P*-values of <0.01 were considered statistically significant.

RESULTS

MiR-888-5p level is elevated in four human HCC cell lines

We analyzed miR-888-5p levels in four human HCC cell lines (SMMC7721, HepG2, Huh-7, and Bel7402) and one human hepatocyte cell line

(HHL-5) by using qRT-PCR assay. Compared with HHL-5, miR-888-5p was found to be up-regulated significantly in all human HCC cell lines. Among those HCC cell lines, Bel7402 showed the lowest expression levels of miR-888-5p while Huh-7 had the highest one (Fig. 1, $P < 0.01$). Among the above HCC cell lines, SMMC7721 cells had the highest transfection efficiency, as confirmed by our previous experiments, therefore we chose these cells to explore the function of miR-888-5p in HCC.

Overexpression of miR-888-5p promotes cellular proliferation

To explore the biological significance of miR-888-5p, the SMMC7721 cells were transiently transfected with miR-888-5p mimic miRNA (mimic) or negative control miRNA (NC) (RiboBio Co., China). The efficiency of the transfection was evaluated by RT-qPCR at 72 h after transfection. Compared with the NC group, miR-888-5p expression levels

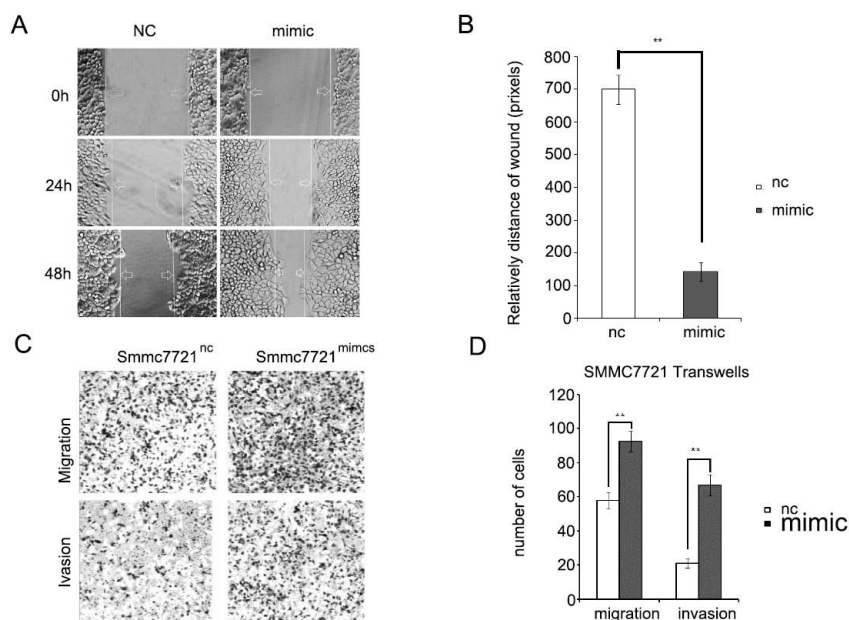


Fig. 3. The overexpression of miR-888-5p promotes cell migration and invasion. **A)** *In vitro* scratch assay. SMMC7721 cells were treated with miR-888-5p mimic (mimic) or negative control miRNA (NC) for 24 h, and cell migration ability was examined by scratch assay. **B)** The average migration distances of the scratch edge were quantified by Image J (Version 1.37v, Wayne Rasband, NIH, USA), ** $P < 0.01$ compared to NC group. **C)** The effect of miR-888-5p on the migration and invasion ability of SMMC7721 cells in the Transwell assay. **D)** The number of migrated or invaded cells increased in cells transfected with miR-888-5p mimic compared with the NC-transfected cells. The number of migrated or invaded cells counted in 6 randomly selected fields of view (magnification, $\times 400$) is presented as a histogram. ** $P < 0.01$ compared to NC group.

were increased nearly four-fold in the mimic group (Fig.2A). There were no evident morphological changes in the transfected miR-888-5p mimic cells.

To detect the effect of miR-888-5p on cellular proliferation, we monitored cell growth using MTT assay after miRNA transfection. MTT analyses demonstrated that the overexpression of miR-888-5p promotes cellular proliferation, and the mimic groups showed increased cellular proliferation [about 1.11-, 1.37- and 1.47-fold on the 2nd, 3rd and 4th day comparing with the NC groups at the same time points, respectively (Fig. 2B, $P < 0.01$)].

Also, to further elucidate the effect of miR-888-5p on the proliferation of HCC cells, the colony formation assay was performed. After being cultured for 24 h post miRNA transfection, about 100 SMMC7721 cells were counted and added to one well of a 6-well culture plate, with each group consisting of three wells. After incubation at 37°C for 14 days, the number of colonies containing 50 or more SMMC7721 cells was counted under a microscope. The results showed that the mimic groups displayed much more and bigger colonies compared with the NC groups (Fig. 2C). The plate clone formation efficiency was calculated using the formula: plate clone formation efficiency = (number of colonies/number of cells inoculated) $\times$ 100%. The results showed that plate

clone formation efficiency of the mimic group was $70.7 \pm 4.5\%$, while in the NC group it was $31.0 \pm 3.5\%$ (Fig. 2D, $P < 0.01$). The colony formation rate in the mimic group was increased approximately twice over that of NC group. These data confirm that miR-888-5p does promote the proliferation of HCC cells.

Overexpression of miR-888-5p promotes cell migration and invasion

Migration and invasion abilities are two essential features of cancer metastasis. We examined whether miR-888-5p could regulate cell motility by performing the scratch assay *in vitro*. SMMC7721 cells were transfected with miR-888-5p mimic or NC in serum-free medium for 24 h and monolayers were scratched with pipette tips. Fresh medium was replaced. The scratch closing process was observed for 48 h, and photographs were taken every 24 h. After 48 h, we observed that the cells in the mimic group had migrated into the scratch zone and the boundary area had become indistinct. In contrast, the scratch area was still distinct in the NC group (Fig. 3A). Using the data from Fig. 3A, we calculated the scratch width in both groups after the 4- h period of re-growth. The data for the mimic group indicate an increased capacity for cell migration (156.6 ± 28.3 pixels scratch width) compared with the cells in the

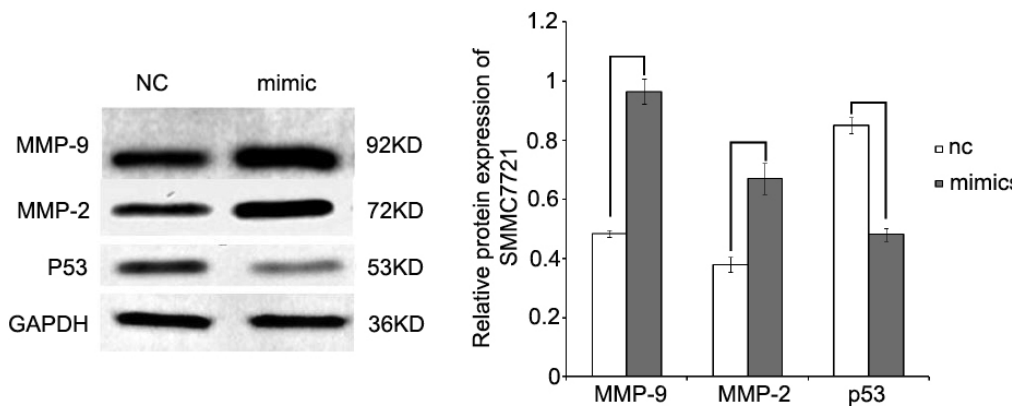


Fig. 4. The overexpression of miR-888-5p increases the expressions of MMP-2 and MMP-9 and significantly inhibits the p53 expression. **A)** The protein expression of MMP-2, MMP-9 and p53 was determined by Western blotting after miR-888-5p mimic (mimic) and negative control miRNA (NC) transfection; GAPDH was used as loading control. **B)** Levels of MMP-2, MMP-9 and p53 were quantified by Image J (Version 1.37v, Wayne Rasband, NIH, USA) ($P < 0.01$).

NC group (scratch width of 700.0 ± 44.0 pixels) (Fig. 3B, $P < 0.01$). The average migration distances of the wound edge in the independent experiments were quantified by Image J (Version 1.37v, Wayne Rasband, NIH, USA).

To further investigate whether miR-888-5p possesses a positive effect on migration and invasion ability of SMMC7721 cells, the Transwell assay was employed after the cells were transfected with the miRNA in the serum-free medium for 24 h. The cells that migrated or invaded into the lower chambers were fixed with methanol, stained with crystal violet and counted in five random fields (Fig. 3C). The results showed that the number of cells that passed through chamber membrane in the mimic group of migration (92.5 ± 8.5 /hp) or invasion (63.5 ± 6.0 /hp) was significantly higher than those in the NC groups (56.5 ± 4.5 /hp; 22.0 ± 3.0 /hp) (Fig. 3D, $P < 0.01$). Our data suggest that in contrast with the NC group, application of miR-888-5p mimic promotes the cell migration and invasion.

Effect of miR-888-5p on the expression of MMP-2 and MMP-9 involved in cell migration

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases (8) involved in the processes of angiogenesis and tumorigenesis, cancer metastasis, and invasion. We hypothesized that stimulating the effect of miR-888-5p on migration and invasion of SMMC7721 cells may be mediated through regulation of MMPs. To confirm this hypothesis, the expression levels of MMP-2 and MMP-9 were determined at the protein level by Western blotting. The results demonstrate that overexpression of miR-888-5p leads to the increasing expression of MMP-2 and MMP-9 at the protein levels (Fig. 4). Our findings indicate that the increase of MMP-2 and MMP-9 may be the consequence of miR-888-5p transfection which influences the invasiveness of HCC cells.

Effects of miR-888-5p on expression of p53 in SMMC7721 cells

We also studied the effect of miR-888-5p transfection on the level of protein p53. Western blot analysis revealed that miR-888-5p transfection significantly inhibited the p53 expression at the protein levels (Fig. 4). These results indicate that miR-888-

5p probably influences the malignant properties of HCC via p53 pathways.

DISCUSSION

The microRNAs (miRNAs) are important regulators of gene expression (12). The development of HCC is a multistep process that involves various changes in gene structure and proteins expression. Aberrant miRNA expressions have been linked to the molecular pathogenesis of HCC (9).

The role of miR-888-5p was insufficiently reported in the liver. Our studies indicate that miR-888-5p is over-expressed in HCC cells, and is involved in carcinogenesis and tumor development via multiple mechanisms.

To understand how miR-888-5p promotes cell migration and invasion, we detected the protein expression after transfection of miR-888-5p. Also, we found that MMP-2 and MMP-9 were expressed in an SMMC7721 cell line and that SMMC7721 cells with a higher level of miR-888-5p tend to have high levels of MMP-2 and MMP-9 expression. Overexpressed miR-888-5p in HCC might promote cancer metastasis through up-regulating the MMP-2 and MMP-9 expressions in HCC. p53 has been shown to regulate the expression of miRNAs involved in the cellular senescence and tumor suppression (10, 11). In this study, we also found that miR-888-5p decreased the level of p53 expression. Therefore, it is reasonable to conclude that miR-888-5p exerts a tumorigenic role in HCC by inhibiting p53 expression, which further promotes the malignant properties of HCC.

To conclude, miR-888-5p is overexpressed in HCC cells and is involved in the carcinogenesis and tumor development. miR-888-5p, due to its carcinogenic effects, can be considered as a potential new biomarker for diagnostics and prognosis of HCC. Studies on patients should be performed to check the clinical significance of elevated miR-888-5p level.

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LETTER TO THE EDITOR

DECITABINE TREATMENT FOR ACUTE MYELOID LEUKEMIA RELAPSE AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATIONX-L. LIU¹, X. ZHAO², C. WANG¹, S-J. GAO¹ and Y-H. TAN¹¹*Cancer Center, The First Hospital of Jilin University, Changchun, P.R. China;* ²*Department of Pediatrics, The First Hospital of Jilin University, Changchun, P.R. China**Received August 28, 2016 – Accepted February 21, 2017*

Therapeutic options for patients with relapse of acute myeloid leukemia (AML) after allo-SCT are limited. Here, we present a case of a 49-year female with AML who underwent myeloablative allo-SCT from a matched sibling donor. Seven months after transplantation she developed cGVHD and suffered from extramedullary plus concurrent medullary relapse. The presence of CNS extramedullary disease is unique. Our patient was treated with decetabine. After one cycle the patient achieved complete remission and full donor chimerism without severe side effects or the occurrence of GVHD. Our case report, together with previous studies, provides strong evidence that decitabine may be a suitable treatment option for AML relapse after allogeneic transplantation, especially in patients who developed GVHD.

To the Editor,

Allogeneic hematopoietic stem cell transplantation (Allo-HSCT) (1) is commonly used for reconstitution of damaged bone marrow. The approach relies on the use of a donor whose HLA type closely matches the recipient. Allo-HSCT is a potentially curative approach for acute myeloid leukemia (AML), but relapse post-HSCT is one of the main reasons for treatment failure. The relapse rate after transplantation varies from 20–70% (2), and the median time to relapse has been reported to be 104–175 days after transplantation (3). Relapse after transplantation is mainly related to the disease state at the time of transplantation, AML subtype, the donor source, the conditioning regimen, patients age, and the T-cell content of the graft (2). Without treatment, the relapse is associated with poor prognosis, with median survival of just 3–4 months. Relapse after transplantation includes isolated medullary relapse, extramedullary (EM)

relapse, and EM plus concurrent medullary relapse, with incidence rates of 29%, 10%, and 1%, respectively (2).

Currently, there is still no standard treatment for AML relapse after transplantation. Therapeutic strategies include withdrawal of immunosuppressive agents, conventional salvage chemotherapy, supportive care, donor lymphocyte infusion (DLI), and second Allo-HSCT (4). The DLI and second Allo-HSCT have good efficacy, and patients who underwent these treatments have a 3-year survival of approximately 30% after AML relapse (5). In contrast, the efficacy of conventional chemotherapy is limited by the high toxicity and low remission rate (4). Studies have shown that the DNA methyltransferase inhibitor Azacytidine can effectively suppress AML relapse after transplantation, with very few toxic side effects and without exacerbation of graft-versus-host disease (GVHD) (3). However, there is little information on the use of another hypomethylating drug,

*Key words: AML, allo-HSCT, decitabine, relapse**Mailing address:*

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decitabine, in the treatment of post-HSCT AML relapse. Here, we present a case of AML relapse after HSCT treated with decitabine and review the relevant literature. In Table I we summarize the clinical characteristics of AML patients who relapsed after allo-SCT with decitabine alone.

Case report

A 49-year female was admitted to our hospital based on symptoms of fatigue, fever, and joint pain. Laboratory findings on admission revealed a white blood count (WBC) of $50.14 \times 10^9/L$, a hemoglobin (Hb) level of 76 g/L, and a platelet count of $19 \times 10^9/L$ which indicated a bone marrow biopsy.

Bone marrow aspiration showed 54% myeloblasts and 29% immature monocytes. Flow cytometry revealed positivity for CD117, CD33, CD13, CD14, HLA-DR, C123, and CD11. The karyotype was normal. MLL gene, C-KIT (exon 8, exon 17), CBFB/MYH11 and AML1/ETO mRNA were negative, NPM1 and FLT3/ITD were positive. Based on these findings, the patient was diagnosed with acute myelomonocytic leukemia. She achieved complete remission (CR) after induction chemotherapy with a DA regimen, followed by consolidation therapy which included one cycle of the DA regimen and two cycles of high-dose cytarabine (2 g/m², over 3 hours every 12 h on days

1, 3, and 5). On the lumbar puncture, cerebrospinal fluid cytology was negative. Prophylactic intrathecal chemotherapy with cytarabine, methotrexate, and dexamethasone was administered three times before HSCT. The allogeneic transplantation was performed following a myeloablative conditioning regimen (Bu/Cy regimen), the donor was a matched brother. The dose of CD34(+) cells was $2.49 \times 10^6/kg$, and that of the mononuclear cells was $12.89 \times 10^8/kg$. The times of leukocyte and platelet engraftment were respective. The evaluation of bone marrow aspiration and flow cytometric analysis were performed 14, 28, 42, 60, and 90 days after transplantation, and the results showed no abnormalities. The chimerism was full donor chimerism, according to dual-color interphase fluorescence *in situ* hybridization (I-FISH).

The dosage of cyclosporine was reduced gradually 75 days after transplantation (10% reduction each week). The patient developed chronic GVHD (cGVHD) with oral ulcer and liver involvement 120 days after transplantation. At that time, the patient was receiving a dosage of cyclosporine of 25 mg, once daily. Therefore, the dosage of cyclosporine was adjusted to 25 mg, twice daily, and prednisone was administered at 30 mg/day (0.5 mg/kg·d). With rapid improvement of liver function and the oral cGVHD, the prednisone was tapered off and ended 6 months

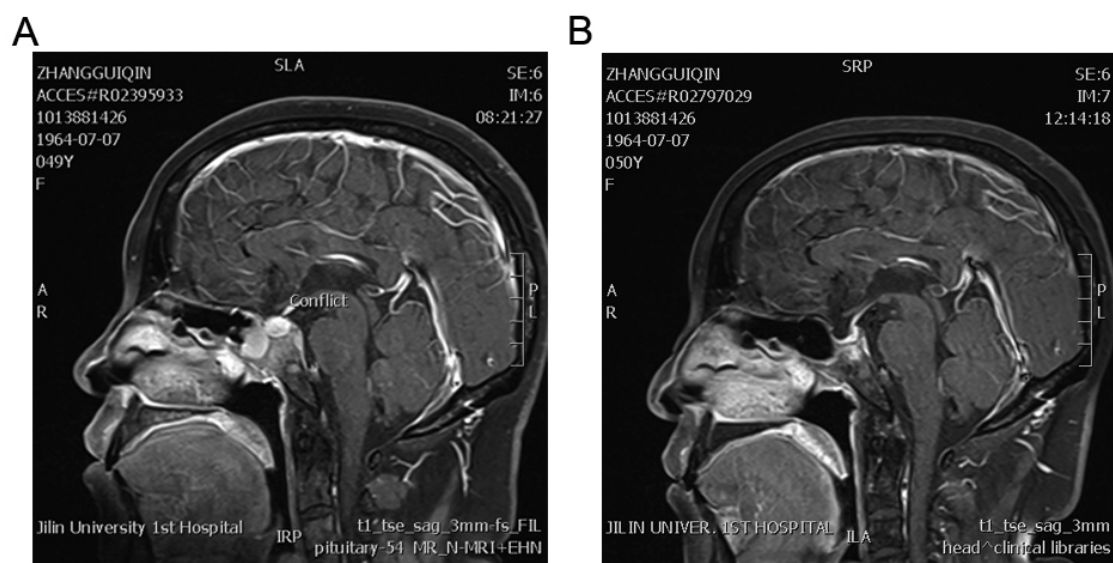


Fig. 1. Brain MRI analysis. **A)** Brain MRI before decitabine treatment. **B)** Brain MRI after two cycles of decitabine treatment.

after HSCT. However, oral mucosal lichenoid changes reoccurred, and prednisone was again administered (10 mg, twice daily). The patient experienced headaches and hallucinations 7 months after HSCT. Brain MRI showed abnormal signals in the pituitary gland and left sphenoid bone (Fig. 1A), but the cerebrospinal fluid was normal. Bone marrow aspiration showed 10.5% myeloblasts and 15.5% immature monocytes. The flow cytometric analysis showed 3.5% abnormal immature cells, and the donor chimerism was 29% according to I-FISH (Fig. 2A). The percentage of regulatory T cells (Tregs) was 0.17%. These data indicated that the patient experienced AML relapse after HSCT.

At the time of relapse, the patient had ongoing chronic oral GVHD. Consequently, immunosuppression was discontinued, and decitabine was administered at a dose of 15 mg/m² intravenously daily for 5 days per monthly cycle. During the treatment, the patient had febrile neutropenia and mild oral infection, but these symptoms improved after administration of anti-inflammatory therapy. The cGVHD had also improved. At 18 days after decitabine treatment, bone marrow aspiration showed that the morphology was normal, and the donor chimerism was 100% by I-FISH (Fig. 1B). Moreover, the flow cytometry showed 0.1% abnormal cells.

Considering the possibility of central nervous system leukemia, intrathecal chemotherapy with

cytarabine, methotrexate, and dexamethasone was performed seven times. After two cycles of decitabine treatment, brain MRI showed that pituitary lesions significantly reduced (Fig. 1B). After four cycles of monthly decitabine treatments, the percentage of Tregs was 7.45%, and decitabine was switched to a tri-monthly schedule. The patient received, in total, seven cycles of therapy with no serious side effects. She remained CR with normal blood counts and full donor chimerism without cGVHD at 14 months after the relapse of AML.

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

DISCUSSION

The 2-year survival rate for EM relapse after transplantation is only 11–38%, whereas the 6-month survival rate for EM plus concurrent medullary relapse is only 8% (2). Demethylating drugs can inhibit DNA methyltransferase 1 (DNMT1), reversing the methylation of tumor suppressor genes to activate them. Azacitidine and decitabine can be incorporated into DNA and trap DNMTs, which results in their degradation, hypomethylation and reactivation of silenced genes in the daughter cells.

Azanucleotides increase the immunogenicity of AML (3). Likewise, azacitidine and decitabine are effective for advanced myelodysplastic syndrome

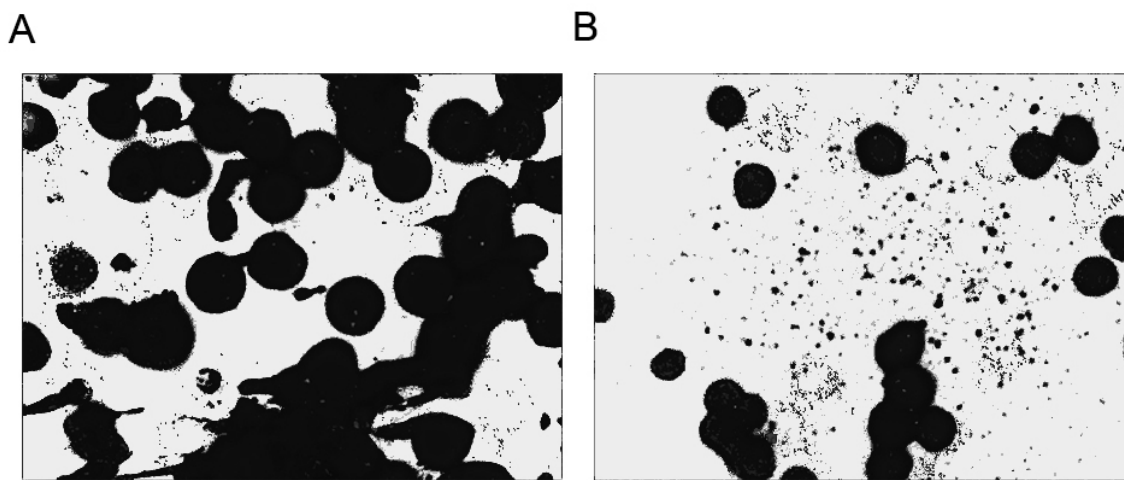


Fig. 2 I-FISH analysis. **A)** Sex chromosome chimerism at the time of relapse after transplantation. **B)** Sex chromosome chimerism after two cycles of decitabine treatment.

Table 1. Baseline clinical patient's characteristics of AML patients who relapsed after allo-SCT with decitabine alone.

Case No.[ref]	Age/ Gender	Dignosis	Type of transplant	Conditioning regimen	Post-relapse decitabine	Patient status
1	55/M	MDS	MUD PBSCT	Bu-Flu-ATG	Decitabine (two cycles)	Alive with remission
2	29/M	AML	Double cord blood	Flu-Cy-TBI	Decitabine (one cycle)	Died with progression
3	48/M	AML	MUD PBSCT	Flu-TBI	Decitabine (two cycles)	Died from progression
4	48/F	AML	Double cord blood	Flu-Cy-TBI	Decitabine (two cycles)	Alive with disease
5	64/F	AML	MUD PBSCT	Flu-Bu-ATG	Decitabine (five cycles)	Alive with remission
6 [44]	57/M	AML	MSD PBSCT	Mel-TBI	Decitabine (thirteen cycles)	Alive with remission

AML: acute myeloid leukemia; MDS: myelodysplastic syndrome; MSD: matched sibling donor; MUD: matched unrelated donor; PBSCT: peripheral blood stem cell transplant; TBI: total body irradiation; Flu: fludarabine; Cy: cyclophosphamide; ATG: anti-thymocyte globulin; Bu: busulphan; Mel: melphalan

(MDS) and relapsed/refractory AML. Azacytidine prolongs the survival of high-risk MDS patients and of elderly patients compared with conventional therapy (3). Decitabine treatment had a significant response rate in relapsed/refractory acute leukemia patients. The best responses were observed after a median of 2.5 cycles, while bone marrow suppression and febrile neutropenia were the main side effects (6). Azacytidine was also reported to be effective for relapsed AML patients after transplantation (7). As the active GVHD usually precludes taper of immunosuppression, symptoms in our patient were mild enough to permit immunosuppression taper.

The use of Azacytidine in the management of post-transplantation relapse was recently presented by the French ATU program (8). Ganguly et al. reported five patients with AML who relapsed after allo-SCT and were treated with a standard dose of decitabine. Three patients achieved remission with full donor chimerism with decitabine treatment (9). Ritchie et al. (10) reported the efficacy of repeated IV cycles of decitabine. Hantel et al. (3) reported the p-value < 0.05, but with low numbers in a retrospective fashion, so these findings are only hypothesis generating. Interestingly, secondary AMLs were 7.8 times more likely to achieve a response compared to *de novo* AML (9, 10). Currently, the mechanism by which

demethylation inhibitors achieve efficacy in treating relapse after transplantation is not fully understood (14, 15). To our knowledge, few cases in which decitabine was used to treat AML relapse after transplantation have been reported.

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LETTER TO THE EDITOR

**DETECTION OF COMBINED PROCALCITONIN AND C-REACTIVE PROTEIN
APPLIED IN THE DIAGNOSIS OF BACTERIAL INFECTIONS**

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In recent years, procalcitonin and C-reactive protein have been used as important indexes in the detection of inflammation. In order to analyze the combined detection of procalcitonin and C-reactive protein in infected patients, 57 subjects in the Clinical Laboratory of Zhengzhou Maternal and Child Health Hospital with a bacterial infection were selected as the observation group. Correspondingly, 57 non-infected subjects were selected for the control group. The procalcitonin and C-reactive protein levels in the included cases were analyzed and compared by extracting peripheral blood. The results showed that the two indexes of C-reactive protein (46.13 ± 8.24 mg/L) and procalcitonin (6.61 ± 3.45 ug/L) of the observation group were significantly higher than those of the control group ($P < 0.05$). The positive rates of C-reactive protein (71.93%) and procalcitonin (91.23%) of the observation group were significantly higher than those of the control group ($P < 0.05$). Within the observation group, the C-reactive protein and procalcitonin levels in the infected patients after 2 and 3 days of treatment, decreased significantly ($P < 0.05$). This study indicates that the combined detection of procalcitonin and C-reactive protein in patients with bacterial infections is effective and can be used in clinical settings.

To the Editor,

U patients have an especially high risk of infection. If they can be diagnosed early and given effective, timely treatment, the number of drug-resistant bacteria can be reduced, giving the patients a better prognosis (1). In recent years, procalcitonin, a type of protein, and C-reactive protein, an acute-response protein, have played important clinical roles (2). Procalcitonin levels can rise along with the levels of infectious fungi, bacteria, etc. Thus, it can be used as an important index in blood tests for the detection of diseases caused by infection, such as sepsis and pneumonia. C-reactive protein levels will rise when the body has a bacterial infection, and can also be used

as an important index for detecting infection. This study explores the effect of the combined detection of procalcitonin and C-reactive protein.

MATERIALS AND METHODS*General information*

Fifty-seven patients in the Clinical Laboratory of Zhengzhou Maternal and Child Health Hospital with bacterial infections from May 2014 to December 2015 were selected as the observation group. They all conformed to the diagnostic criteria set by the Deterioration of Critical Care Medical Association. The diagnostic criteria were as follows: i) temperature $< 36^{\circ}\text{C}$ or $> 38^{\circ}\text{C}$; ii) heart rate $>$

Key words: bacterial infection, procalcitonin, C-reactive protein

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90 times/min; iii) white blood cell count $> 12 \times 10^9/L$ or $< 4 \times 10^9/L$; iv) $PaCO_2 < 32$ mmHg; v) respiratory rate > 20 times/min. But what should be noticed is that these indexes are not specific. For example, the body's white blood cell count will decrease if it has a serious infection, making the white blood cell count unable to reflect the true severity of the infection. Among the 57 cases in the treatment group, there were 8 cases of severe urinary tract infections, 28 cases of severe pneumonia, 14 cases of severe sepsis, and 7 cases of septic shock. Correspondingly, 57 cases of non-infected people were chosen as the control group. All the included cases and their family members gave their informed consent. This study was approved by the Ethics Committee of our hospital. There were no statistical differences in gender, or age, or history of disease ($P > 0.05$), so the cases were comparable (Table I).

Methods

All the included cases had 6 ml of peripheral venous blood extracted and divided into two different tubes for procalcitonin or C-reactive protein, both with heparin. A Cobas E601 enzyme immunoassay analyzer (Roche, Basel, Switzerland) used electrochemiluminescence to detect procalcitonin; a BC-6800 Automatic Blood Analyzer (Mindray, Shenzhen, China) and kit (Diop Biological Medicine, Shanghai, China) used the antibody-sandwich ELISA method to detect C-reactive protein.

All the experiments were conducted according to the manufacturers' instructions.

Observational indexes

Procalcitonin and C-reactive protein levels in all the included cases were analyzed and compared before treatment, after 2 and 3 days of treatment, and the day before the patients were discharged.

If the C-reactive protein level was below 10 mg/L, it was considered positive. If the procalcitonin level was below 0.5 $\mu g/L$, it was considered positive.

Statistical analysis

SPSS 11.5 software was used to analyze the data in this study. Standard Deviation ($\bar{x} \pm S$) was used to express the data, t -test was used to check the data, the χ^2 test was used to compare the measurements, and the rank sum test was used to compare ranked data. $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of the C-reactive protein and procalcitonin level of the two groups

The C-reactive protein level of 46.13 ± 8.24 mg/L and procalcitonin level of 6.61 ± 3.45 $\mu g/L$ in the observation group were much higher than those of

Table I. General data of the two groups.

Groups	Gender		Age	BMI ($\bar{x} \pm S$) kg/m ²
	Male	Female		
Observation group (n=57)	34	23	40.79 $\pm$ 4.24	23.15 $\pm$ 3.91
Control group (n=57)	30	27	41.04 $\pm$ 3.96	22.78 $\pm$ 4.04
Statistical value		0.5700	0.3253	0.4969
P value		0.4503	0.7455	0.6203

Table II. Comparison of the C-reactive protein and procalcitonin levels of the two groups ($x \pm S$).protein and procalcitonin between the two groups.

Groups	Cases	C-reactive protein (mg/L)	Procalcitonin (ug/L)
Observation group	57	46.13 $\pm$ 8.24	6.61 $\pm$ 3.45
Control group	57	4.24 $\pm$ 2.07	0.31 $\pm$ 0.15
T-test		37.2248	13.7736
P		< 0.05	< 0.05

Table III. Comparison of the positive rates of C-reactive protein and procalcitonin between the two groups.

Groups	Cases	+C-reactive protein	+Procalcitonin
Observation group	57	41 (71.93 %)	52 (91.23 %)
Control group	57	8 (14.04 %)	7 (12.28 %)
χ^2		38.9783	71.1402
P		< 0.05	< 0.05

Table IV. Comparison of the C-reactive protein and procalcitonin levels of the two groups at different periods ($x \pm S$).

Time	Cases	C-reactive protein (mg/L)	Procalcitonin (ug/L)
Before treatment	57	46.13 $\pm$ 8.24	6.61 $\pm$ 3.45
After 2 d treatment	57	34.13 $\pm$ 6.02	4.72 $\pm$ 3.02
After 3 d treatment	57	26.21 $\pm$ 3.82	2.65 $\pm$ 1.87
The day before discharge	57	16.89 $\pm$ 2.76	0.31 $\pm$ 0.08

the control group, and the difference was statistically significant ($P < 0.05$, Table II).

Comparison of the positive rates of C-reactive protein and procalcitonin between the two groups

The positive rates of C-reactive protein and procalcitonin in the observation group were much higher than those of the control group, and the differences were statistically significant ($P < 0.05$, Table III).

Comparison of the C-reactive protein and procalcitonin levels of the two groups at different periods

The C-reactive protein and procalcitonin levels after 2 and 3 days of treatment and on the day before being discharged of the observation group were much lower than the levels before treatment, and the difference was statistically significant ($P < 0.05$, Table IV).

DISCUSSION

ICU patients are at a very high risk of bacterial infection, which can cause organ failure or even death (3). At present, sputum cultivation pathogens are often used to detect bacterial infections, but it takes a long time to cultivate, separate, and detect the infection, so its use is limited (4, 5). The leukocyte counting method is also used to detect severe infection, but it can be influenced by a multitude of factors, therefore, is not a helpful method in accurately detecting the infection in a timely manner (6). At present, certain infectious markers are used to help detect infections early in clinical settings.

Procalcitonin levels rise when the body has an infection, but is otherwise stable. Other studies have shown that when a patient had a serious infection, the procalcitonin level would rise within 3-6 h, peak from 8-24 h, and would enter its half-life period from 22-25 h. The procalcitonin level has a high specificity and sensibility (6, 7). Procalcitonin has good stability, and can be kept in a blood sample with a degradation of 12% when kept at room temperature for one day, and a degradation of only 6% when kept at 4°C for one day (8, 9). Therefore, procalcitonin has a high potential for providing a diagnostic index during the early stages of a bacterial infection. In addition, it cannot be metabolized by the kidneys, so it can be used with patients who have artificial kidneys or kidney failure. However, procalcitonin tests can produce a false-negative, so patients with a high level should be rechecked within 6-12 h (10, 11). Procalcitonin, as a critical index, can be used to help doctors diagnose early stage bacterial infections and create a specific treatment for each patient. The comparison of procalcitonin levels between infected and non-infected patients can help detect infections, and thus can be taken as a critical index in patients with severe infections (12, 13).

The C-reactive protein level will rise when the body responds to an infection or injury. It is mainly comprised of *Streptococcus pneumoniae* C-polysaccharide and is produced by the liver. Its levels will rise sharply when the body has an infection, peaking within 2-3 h, and then declining when the infection clears up (14, 15). Therefore,

C-reactive protein is very sensitive and can be used as an important index for detecting local infections. However, compared with PCT, CRP has some limitations, such as slower clearance and induction kinetics. Therefore, the combined detection of C-reactive protein and procalcitonin can make a more accurate diagnosis for patients with severe infections.

The findings in this study show that the procalcitonin level (6.61 ± 3.45 ug/L) and C-reactive protein level (46.13 ± 8.24 mg/L), and the positive rates of 91.23% and 71.93%, respectively, of the observation group were both significantly higher than those of the control group. In addition, the procalcitonin and C-reactive protein levels of the infected patients after 2 and 3 days of treatment and on the day before they were discharged all declined, indicating that the procalcitonin and C-reactive protein had a short half-life period and high specificity. Together, this study indicates that the combined detection of procalcitonin and C-reactive protein is effective in diagnosing the early stages of bacterial infections and has a high clinical value.

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LETTER TO THE EDITOR

BENEFICIAL EFFECTS OF PROTEIN HYDROLYSATES IN EXERCISE AND SPORTS NUTRITION

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Protein hydrolysates (PH) are rich sources of proteins that supply the need of exercising muscles. PHs are enriched in di- and tripeptides and are better than free amino acids or intact proteins when muscle anabolic effect is considered. Digestion, absorption and muscle uptake of amino acids are faster and more efficient when PH is ingested in comparison to the respective intact protein. PHs not only enhance endurance in high intensity exercise regimen, but also help in faster post-exercise recovery of muscle by promoting glycogen synthesis, although the latter effect requires more convincing evidence. PHs have been shown to exhibit insulinotropic effect as it enhances the secretion of insulin and the hormone, in turn, exerts muscle anabolic effect.

To the Editor,

Protein hydrolysates (PH) are prepared from purified protein by heating with acid or treating with proteolytic enzymes followed by purification. PH is a mixture of peptides of varying chain length and free amino acids and is defined by the degree of hydrolysis which is a measure of the fraction of peptide bonds cleaved in the purified protein. Based on the methods used, PH could be either a mixture of oligopeptides with free amino acids or a mixture mainly of di- and tripeptides. The degree of hydrolysis could be similar between these two types of PH, however, the absorption kinetics of the two vary significantly (1).

Peptides longer than four amino acids are poorly absorbed, if at all. Absorption of di- and tripeptides are favoured in the small intestine (2, 3). Only a very minor fraction of these peptides enter the blood intact (4).

In this review, we critically discuss the effects

of PHs in amino acid and peptide absorption and their rates, muscle building, insulin secretion, body composition (whether gain in lean mass is achieved), performance enhancement and post-exercise recovery.

Most widely used PHs

Whey protein (WP) and casein are widely used sources of PHs. Conformation and functionality of WPs are interdependent and influenced by alterations in folding of their globular structure.

Major steps involved in the preparation of PH are shown in Fig. 1. Whey proteins (WP) are by-products of cheese production. The major compositions of WP include β -lactoglobulin, α -lactalbumin, immunoglobulin and bovine serum albumin, and biological functions of these proteins have been extensively studied (2). Among these proteins, β -lactoglobulin, a globular and amphiphilic protein, is the most abundant constituent and maximally

Key words: bone and joint, casein, insulin, muscle anabolic, whey protein

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Table I. Common endopeptidase used in producing PH.

Name	Preferential cleavage sites
Cysteine proteases	
Papain	Arg, Lys, Phe-X
Bromelain	Lys, Arg, Phe, Tyr
Ficin	Phe, Tyr
Cathepsin B	Arg, Lys, Phe-x
Serine proteases	
Trypsin	Arg, Lys
Chymotrypsin	Tyr, Trp, Phe, Leu
Aspartic proteases	
Pepsin	Aromatic Leu, Asp, Glu
Chymosin	Phe ₁₀₅ -Met ₁₀₆ bond in κ -casein
Metallo proteases	
Neutral proteinase	Phe, Leu, and others
Thermolysin	Leu, Ileu, Val, Phe

contributes to the effect of WP (5-7).

For producing whey PH (WPH), a variety of enzymes are used, including digestive enzymes (trypsin, chymotrypsin and pepsin) (8), bacterial proteases from *Bacillus subtilis* and *Bacillus licheniformis* (9, 10), plant enzymes (papain and bromelain) or a mixture of some of the enzymes (3, 11). Table I lists commonly used endopeptidases for the preparation of PH (8).

Casein is a mixture of phosphoproteins and its extensive enzymatic hydrolysis is a prerequisite for ensuring complete removal of antigenic (allergen) attributes of the protein. Enzymatic hydrolysis of casein is carried out by trypsin (1.2×10^5 U/g). The degree of enzymatic hydrolysis by trypsin is rapid, as most hydrolyses are completed in 20 min, and yields increased contents of α -helix and β -turn compared to non-hydrolysed protein. However, β -sheet content is decreased after enzymatic hydrolysis such that the ratio of α -helix to β -sheet is lower than the non-hydrolysed casein (4).

In addition to WPH and casein, gluten, fish

proteins, collagen and rice bran proteins represent high quality protein sources and PH made from them are available. However, most of the studies on exercise and sports have been carried out with WPH and casein hydrolysates.

Effect on amino acid absorption

PH consisting of di- and tripeptides are considered to be absorbed faster than intact protein. However, there exists a lack of consensus regarding this theory (5).

Effect on muscle protein synthesis

Increasing blood glucose and insulin levels during long-duration variable intensity exercise by supplementing with carbohydrate (CHO) has been considered to conserve muscle glycogen and increase aerobic endurance. Addition of protein to CHO supplement will result in greater insulin response of a CHO supplement. Long duration cycling is an energy demanding sport, and CHO + protein ingestion may increase time-to-exhaustion compared with CHO alone (6, 7).

Effect of PH on body composition and exercise performance

When L6 myotubes were treated with WPH containing dipeptides that were made predominantly of branched chain amino acids, a significant upregulation of glucose uptake was observed. Glucose plus WPH significantly increased skeletal muscle glycogen content after a glycogen depleting exercise regimen (8). These results suggested greater glycogen restoration ability of WPH after exercise and thus improve exercise performance. WPH was also found to accelerate recovery of peak isometric torque after an eccentric exercise (isometric contractions of knee extensors) (9). WPH appears to serve as an ergogenic aid for athletes however, more studies are required to firmly establish this.

Insulin secretion and muscle anabolic effect

Although glucose is its most potent physiological secretagogue, amino acids are also known to stimulate insulin secretion. Insulin stimulates phosphorylation of glycogen synthase to activate this rate-limiting enzyme in glycogen synthesis pathway.

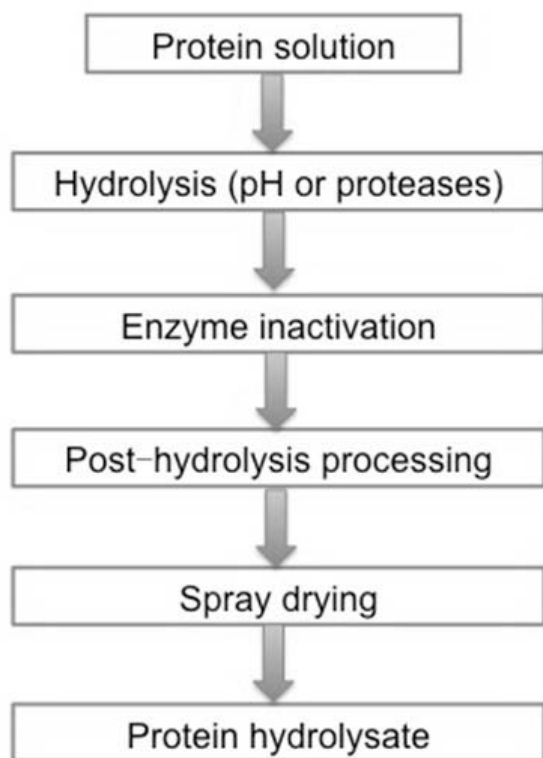


Fig. 1. Flow diagram for producing PH.

Muscle glycogen is the most important fuel used during moderate and high intensity exercise, and a direct relationship between fatigue and fall in muscle glycogen level has been reported (10, 11). Insulin is considered as a muscle anabolic as it enhances muscle protein synthesis. Direct activation of protein translation in muscle cells by insulin is mediated by PI3K–AKT and mTORC1 pathway (12). Protein + CHO ingestion in healthy individuals as well as type 2 diabetic patients (13) resulted in increased plasma insulin levels. In a study involving 80 healthy adult men, PHs from various plant and animal sources were individually compared in combination with CHO and the data showed that the plasma insulin levels were significantly higher in the co-ingested group compared with the CHO group alone. At present, it appears that insulin acts as a priming signal for muscle protein synthesis.

Glycogen

Muscle glycogen synthesis after high-intensity exercise occurs in two phases. The first phase

occurs at a higher rate than the second. In the first, insulin-independent phase, glucose transporter-4 (Glut-4) is translocated to sarcolemma in response to muscle contraction which increases glucose uptake by myocytes together with increase in the activity of glycogen synthase. The second phase of glycogen synthesis occurs at a lower rate than the first and is insulin dependent (14). Although, CHO has been shown to stimulate post-exercise muscle glycogen synthesis, various reports have shown that co-ingestion of protein or amino acids with CHO accelerates muscle glycogen synthesis over CHO alone (15).

Overall, the present state of knowledge suggest that insulin has a permissive effect in muscle protein synthesis in young adults and that insulinotropic effect of PHs in combination with CHO achieves a muscle anabolic effect via the vasodilatory effect of the hormone (16).

Mechanism of action

The kinetics, rather than net absorption of amino acids, determine the nutritional values of protein and their anabolic muscle effect (1). Ingesting a PH which is rapidly broken down and absorbed (so called fast-acting) results in inefficient splanchnic uptake and allows a rapid rise in plasma amino acid levels and increases tissue amino acid delivery.

In comparison to intact proteins, PHs appear to absorb faster and display greater tissue utilization. By keeping nitrogen (N) load comparable, administration of PH or di/tri-peptides, resulted in increased plasma levels of amino acids and dipeptides as well as greater amino acid delivery over intact protein and free amino acids. However, some studies failed to show faster and greater absorption of amino acids and dipeptides after the administration of PH over intact protein. Types of PH and the manufacturing process could influence absorption. For example, plasma amino acid level was reported to be higher with WPH than soy PH. Likewise, enzymic methods used, duration of hydrolysis and pH conditions in manufacturing PH could significantly influence the composition of di- and tripeptides which eventually impact their biological effects (1).

Insulin has protein anabolic effect and PH

has been shown to exhibit greater insulinotropic effects compared to intact protein. At the tissue level, increased circulating insulin stimulates protein synthesis, inhibits protein catabolism and enhances tissue uptake of branched-chain amino acids which are responsible for stimulating protein synthesis. Again, the extent of insulinotropic effect is dependent on the source and degree of hydrolysis of protein as well as amino acid composition and average amino acid chain length. In addition, PHs elicit substantially higher gastric secretion compared to intact proteins, thereby resulting in higher plasma levels of an incretin hormone, glucose-dependent insulinotropic polypeptide (GIP) (17). However, the mechanism by which PH increases insulin secretion is unresolved.

Bone and joint health and the effect of collagen hydrolysate

Degenerative joint and spine disease is one of the most debilitating diseases and one of the biggest causes of poor athletic performance. Collagen hydrolysis results in the generation of small peptides (average MW < 5 kD) from collagen rich tissues of pigs and cows. Collagen hydrolysate (CH) is generally regarded as having an inferior nutritional value because it does not contain all of the essential amino acids. Also, Cys content is small and Trp is absent. However, CH has an excellent digestibility and when combined with high-efficient protein carriers such as casein could be advantageous for nutritional therapy. Upon feeding mice with ^{14}C -labeled CH or ^{14}C -labelled Pro, a significantly higher radioactivity was located in the cartilage in the former than the latter group. Given the preferential uptake of CH-derived peptides in cartilage, a therapeutic role of CH in osteoarthritis and joint diseases could be envisaged. Indeed, CH has been shown to increase bone mineral mass, bone mineral content and quantity of type I collagen and proteoglycans in the bone matrix. *In vitro*, CH stimulates type II collagen biosynthesis by chondrocytes (from bovine articular cartilage). However, when human osteoarthritic articular explants were treated with bovine CH, no increase in collagen synthesis was observed, thus raising concern regarding the efficacy of bovine CH

in the treatment of bone-joint diseases.

In the last three and half decades, seven human studies (four open-label and three double-blind) have been carried out assessing the role of CH on joint pain and recovery from the symptoms of osteoarthritis. Bello and Oesser (18) reviewed the results of these studies which suggested pain reduction, greater ease in movement and improvement in general conditions by CH, however, the major drawback of the studies was the lack of statistical analyses. CH (10 g/day), when fed to young athletes for 24 weeks, reduced the symptoms of joint pain. Results of six randomized clinical trials (RCT) on the efficacy of CH ingestion in osteoarthritis patients have been reviewed. The results suggest that although CH was safe as only mild adverse effects were reported, efficacy in mitigating symptoms of osteoarthritis was minimal, largely due to moderate-to-low quality of the evidence provided. At present, high quality RCTs using standardized and validated instruments for data collection are required to establish the therapeutic effects of CH in osteoarthritis and chondroprotective effect in joints. In addition, although CH appears to be promising in mitigating bone and joint pains and defects in athletes involved in high endurance sports, appropriate studies on athletes with CH are lacking (19).

Oxidative stress and the effect of fish protein hydrolysates

Fish protein hydrolysates (FPH) have shown health benefits. Partially hydrolysed pacific whiting fish containing glutamine in its di/tripeptide forms prevented villus damaging effect of indomethacin, a non-steroidal anti-inflammatory drug on small intestine in both animal and human studies. PH made from salmon fish protein with a desirable composition of di-tripeptides is capable of significantly increasing BMI values in mal-nourished children (age group 6-8 years), suggesting that it has rich nutrition value. FPH prepared by pepsin hydrolysis is a rich source of peptides containing 2-16 amino acids having the anti-oxidant property that is related to their sequence, composition, and hydrophobicity. The presence of hydrophobic amino acids and one or more residues of Pro, Cys, His, Phe, Trp, Tyr, and Met has been

reported to augment anti-oxidant activity of peptides. Tyr and Phe act as direct radical scavengers and serve as hydrogen donors due to phenolic groups. Radical scavenging activity of His is due to the imidazole group that has proton donating function, and Cys could donate sulfur hydrogen. Antioxidant mechanisms of other amino acids are not clearly understood. Pro-Arg from salmon protamine hydrolysate, a dipeptide, demonstrated hydroxyl radical scavenging activity.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are presumably produced by muscle cells after intense and prolonged exercise, although heart, lung and white blood cells could also contribute to their production. ROS and RNS could cause oxidative damage to lipids and proteins of exercising muscle cells.

Anti-oxidant supplements including vitamin C, vitamin E, and carotenoids have been proposed in preventing muscular fatigue during sustained exercise, although data are not sufficiently high quality to warrant regular use for athletes. Considering prevention of fatigue and muscle cell damage from oxidative stress due to sustained exercise to be of some merit, FPH could be put to use after carrying out high-quality clinical trials (20).

Future direction

The most widely used PH in sports nutrition is derived from whey or casein protein. The proportion of di- and tripeptides in PH critically influences absorption kinetics, which in turn determines the nutritional value of PHs. Therefore, increase in net absorption of amino acids by PHs should not be considered as its nutritional efficacy. Human studies correlating absorption kinetics with exercise performance enhancement by various PHs need be compared.

Generally speaking, PH has shown great potential in enhancing exercise performance and post-exercise recovery. However, further research is needed to conclude whether PHs stimulate post-exercise net muscle anabolism and its underlying mechanism.

Furthermore, studies are lacking in healthy athletes regarding skeletal muscle anabolic effect comparing a) WPH and casein PH, and b) WPH and casein PH with their respective intact protein.

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LETTER TO THE EDITOR

SCREENING AND COMPARISON OF POLYCHROMATIC AND MONOCHROMATIC
IMAGE RECONSTRUCTION OF ABDOMINAL ARTERIAL ENERGY SPECTRUM CT

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We screened the suitable image reconstruction to observe the abdominal artery and compare the quality between the polychromatic and the monochromatic reconstruction images of the abdominal artery spectrum CT. Eighty patients underwent Gemstone CT energy spectrum imaging to obtain an abdominal artery polychromatic image (140 kVp) and a monochromatic image from 40 ~ 140 keV. The CT value of region of interest (ROI) was measured on the polychromatic image and the single energy image. The signal-to-noise ratio (SNR) and contrast-to-noise ratio (CNR) of the abdominal aorta and hepatic artery were determined. The images in each group underwent image quality subjective scoring by three experienced radiologists using a blinded method. Finally, comprehensive comparisons and image quality subjective scorings were performed on the CT, SNR, and CNR values of the abdominal aorta. The obtained data were statistically analyzed by SPSS 19.0 software. When the keV value was reduced, the CT value of the abdominal artery gradually increased, and the image noise also changed. The comprehensive comparisons and subjective scorings were finalized for each single energy image based on the abdominal artery image quality objective indicators (CT value, SNR, and CNR). Results revealed that the abdominal artery image quality in the 50 ~ 60 keV monochromatic group was better compared to the polychromatic group. Furthermore, monochromatic imaging had different impacts on the abdominal aorta and hepatic artery image qualities. In different types of abdominal arterial reconstruction images obtained using abdominal energy spectrum CT conventional enhanced scanning, the image quality of the 50 ~ 60keV monochromatic reconstruction was higher when compared with the polychromatic reconstruction. Thus, it is recommended to apply the conventional reconstruction for abdominal artery energy spectrum CT scanning.

To the Editor,

Abdominal artery energy spectrum CT scanning (Gemstone Spectral Imaging, GSI) can obtain polychromatic (QC), monochromatic (MONO), and multiple reconstruction images. Due to the numerous reconstruction images, a more efficient image reconstruction mode was needed to shorten

the image processing time, improve image quality, and improve the clinical efficiency. This study utilized abdominal energy spectrum CT conventional enhanced scanning to reconstruct different images of the abdominal artery. Through the reconstruction quality comparison study of polychromatic and monochromatic imaging, this research screened the

Keywords: abdomen, tomography scanners, X-ray computed, energy spectrum imaging, CT angiography, reconstruction mode

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most efficient energy spectrum CT images to observe the abdominal artery.

MATERIALS AND METHODS

General information

Eighty patients who underwent upper abdomen GSI enhanced CT scanning in The First Affiliated Hospital of Zhengzhou University were randomly selected. Of these, 48 were males and 32 females, aged from 21 to 84 years, with the average age being 53.6 years. All patients had no allergic history to iodinated contrast agents. Exclusion criteria included patients with aortic dissection, abdominal artery intervention, and thrombosis and cancer embolus of the abdominal aorta and its branch arteries. The study was approved by the hospital ethics committee and all patients signed informed consent.

Image acquisition and reconstruction

Eighty cases were examined using the GE Discovery CT 750 HD spectrum CT for a routine abdominal scanning. Fast switching spectrum scanning modes with single source dual voltage (80 and 140 kVp) to perform the enhanced scan on liver arterial phase and portal venous phase were used. The scan scope was from the diaphragmatic dome to the area 2 cm beneath the right inferior kidney. The X-ray current of energy spectrum CT scanning was fixed at 600 mA, the screw pitch was 0.984, and the layer thickness and interlamellar spacing was 5 mm. The enhanced contrast agent was Ultravist (300 mg/ml), at a dose of 75 ml, the injection flow rate was 2.5 ml/s, and the binoculars high-pressure injector was adopted. Polychromatic energy (140 keV) and monochromatic energy (40~140 keV) horizontal section images had a 0.625 mm layer thickness and 0.625 mm interlamellar spacing, and were reconstructed based on the artery energy phase spectrum CT scanning data. The GSI viewer was used to reconstruct the monochromatic images, and 11 kinds of different energy monochromatic images with 10keV space were reconstructed in the 40 ~ 140 keV group.

Image evaluation and arterial phase selection

The quality of the polychromatic and monochromatic images of the abdominal aorta and hepatic artery was measured and evaluated. The unified bandwidth, window level, and threshold value were adopted for image

measurement and evaluation. CT values were measured by an experienced radiologist using a blinded method. CT image scoring was performed by an experienced chief physician and two attending physicians. The score was required to be chosen unanimously.

Image quality was objectively evaluated through the calculation of the CT value of the abdominal aorta and hepatic artery, signal-to-noise ratio (SNR), and contrast-to-noise ratio (CNR). $SNR = SI_{\text{tissue}} / SD_{\text{background}}$, $CNR = (SI_1 - SI_2) / SD_{\text{background}}$. SI_{tissue} and SI_1 were the CT value of the abdominal aorta and hepatic artery, SI_2 was the CT value of the psoas major and liver parenchyma, $SD_{\text{background}}$ was the standard deviation of the surrounding air. The same region of interest (ROI) value was used for image measurement in each group.

The subjective evaluation of the artery image quality was graded scores using the vascular maximum intensity projection (MIP) reconstruction and cathode ray tube (CRT) image. According to the differences of the vascular density, the definition of intravascular and vascular boundaries, the grade to which blood vessel branches reached, the construct ratio between blood vessels and surrounding tissue, and image noise. The scoring criteria standard was as follow: 5 for excellent, 4 for good, 3 for average, 2 for poor but still could be used on diagnosis), and 1 for unusable.

Statistical analysis

SPSS19.0 software was used on data statistical analyses. The comparison of the artery CT value, SNR, CNR, and image quality subjective evaluation (first processed into sequence) of the polychromatic (QC) and monochromatic (MONO) images in each group were analyzed using a single factor variance analysis of totally random design. If the overall difference between the groups was statistically significant, the comparison of the QC and MONO images between each group were conducted using a multiple pair comparison *t*-test (Dunnett method). The average was displayed as $\bar{x} \pm s$, and $P < 0.05$ was considered statistically significant.

RESULTS

There was a statistical significance in the overall difference of CT value, SNR, CNR, and image quality subjective evaluation of the abdominal aorta

and hepatic artery in the images in each group, $P < 0.05$. Results are shown in Table I.

The CT values of the abdominal aorta and hepatic artery in each MONO group decreased gradually as the energy increased (Fig. 1).

Through the comprehensive comparison of the objective index (CT value, SNR, and CNR) and subjective evaluation of the abdominal aorta and hepatic artery image quality, the 50 ~ 60 keV group had a better image quality when compared with the QC group. Most small arterial branches can be seen clearly in the QC group. Also, the abdominal aorta and its major branches were shown with more clarity (Fig. 2A, 2B). Meanwhile, the study results indicated that the monochromatic images had different effects on abdominal artery and hepatic artery image quality. Although the monochromatic images of the low energy phase (40 ~ 60 keV) can increase the hepatic

artery CT value and CNR, the SNR almost did not change. Also, the abdominal artery CT value, SNR, and CNR increased in the low energy phase (4 ~ 60 keV) monochromatic images.

DISCUSSION

Gemstone spectral CT imaging is a novel technology for CT energy imaging. It uses the absorption curve of a relevant base substance to capture the 101 virtual monochromatic images in a certain range (40~140 keV) through a single source instantaneous double kVp (80/140) syntropy collection technique scan. The base substance pairs images were acquired from the original high and low energy scan data and conventional polychromatic images (QC). Under polychromatic mode, tissue CT values were constant, and the comparison of different tissues in

Table I. Comparisons of the objective indicators and subjective indicators of images between QC group and each MONO group.

groups		Abdominal aorta			Hepatic artery			Subjective
		CT value	SNR	CNR	CT value	SNR	CNR	ratings Image Quality
QC	140 kVp	193.7±36.2	30.9±14.6	21.0±10.9	123.6±31.9	20.1±10.7	8.7±6.8	3.0±0.5
	40 keV	654.6±131.1	43.2±18.0	35.8±15.3	304.0±64.1	20.2±8.3*	13.2±6.1	2.8±0.4*
	50 keV	435.1±84.5	47.7±22.1	37.8±18.1	212.6±43.3	23.5±10.5*	13.8±7.0	3.7±0.8
	60 keV	301.7±56.3	44.1±21.6	32.7±16.6	157.6±32.3	23.2±11.4*	12.0±7.0	4.0±0.7
	70 keV	212.4±36.2*	33.2±16.3*	21.8±12.4*	118.7±22.3*	18.7±8.9*	7.9±4.7*	3.1±0.5*
	80 keV	161.8±26.5	18.3±7.0	11.0±4.7	95.5±16.4	11.0±4.4	3.5±2.1	2.3±0.5
MONO	90 keV	126.2±19.0	12.1±4.2	6.2±2.6	78.7±13.5	7.7±2.8	1.8±1.2	1.6±0.7
	100 keV	101.5±13.9	9.7±3.4	4.1±1.8	68.2±11.9	6.7±2.5	1.1±0.8	1.1±0.3
	110 keV	84.0±10.3	8.0±2.7	2.5±1.2	60.9±10.8	5.9±2.2	0.8±0.5	1.0±0.0
	120 keV	71.6±8.0	6.9±2.3	1.5±0.9	55.5±10.4	5.4±2.0	0.7±0.5	1.0±0.0
	130 keV	62.3±6.2	6.0±2.0	0.8±0.6	51.7±9.7	5.0±1.9	0.7±0.6	1.0±0.0
	140 keV	55.0±5.3	5.2±1.8	0.5±0.3	48.1±10.0	4.7±1.8	0.9±0.7	1.0±0.0

Note: The overall difference of above-mentioned variables between both groups is significant ($P = 0.000 < 0.05$). The QC group and MONO group were pairwise compared. *: $P > 0.05$. The values without asterisk indicate the corresponding variable comparison of P values is less than 0.05, and the difference was statistically significant.

the images were relatively constant. As the X-ray absorption curves were different for the same tissues under different energy levels, tissue CT values can change relatively as the energy changes. Each tissue had a unique X-ray absorption curve. The comparison of different tissues in the images also can change as the energy changes. Also, the changes in energy level can change the image noise and affect image quality. High energy X-rays are able to improve photon ability to penetrate tissue, so more photons can reach the detector and reduce the quantum noise. Because low energy X-rays reduced their penetration ability, there was a reduced number of photons that reached the detector, and the noise increased. Therefore, the energy spectrum CT improved the image quality through the adjustment of feasible energy levels (2-3).

Spectrum CT imaging is a relatively new technology, and its clinical application is still in the exploratory stage (4-8). This study utilized abdominal energy spectrum CT conventional contrast enhanced scanning to reconstruct the polychromatic and monochromatic images of the abdominal artery and comparatively evaluate the image quality based on the objective

and subjective indicators, and discuss the clinical application value of energy spectrum CT imaging of the abdominal artery. CT value, SNR, and CNR were the key objective indicators of abdominal artery image quality evaluation. The artery CT value was the most important indicator of the degree of artery strengthening. A higher degree of blood vessel strengthening indicated the possible acquisition of a better artery image. A good SNR was the basic requirement for a CT to clearly show abdominal artery anatomy structure and lesions. Under certain SNR conditions, an adequate CNR played a fundamental role in displaying abdominal lesions. The subjective evaluation of the abdominal artery image was evaluated based on MIP and volume-rendering technique (VRT) images.

This study took the example of the abdominal aorta of the main abdominal artery and hepatic artery and used arteriole to comparatively investigate different reconstruction imaging qualities of the abdominal aorta with larger vessel diameters and the hepatic artery with smaller vessel diameters. The results show that the CT value of the abdominal aorta and hepatic artery increased with a decrease in energy

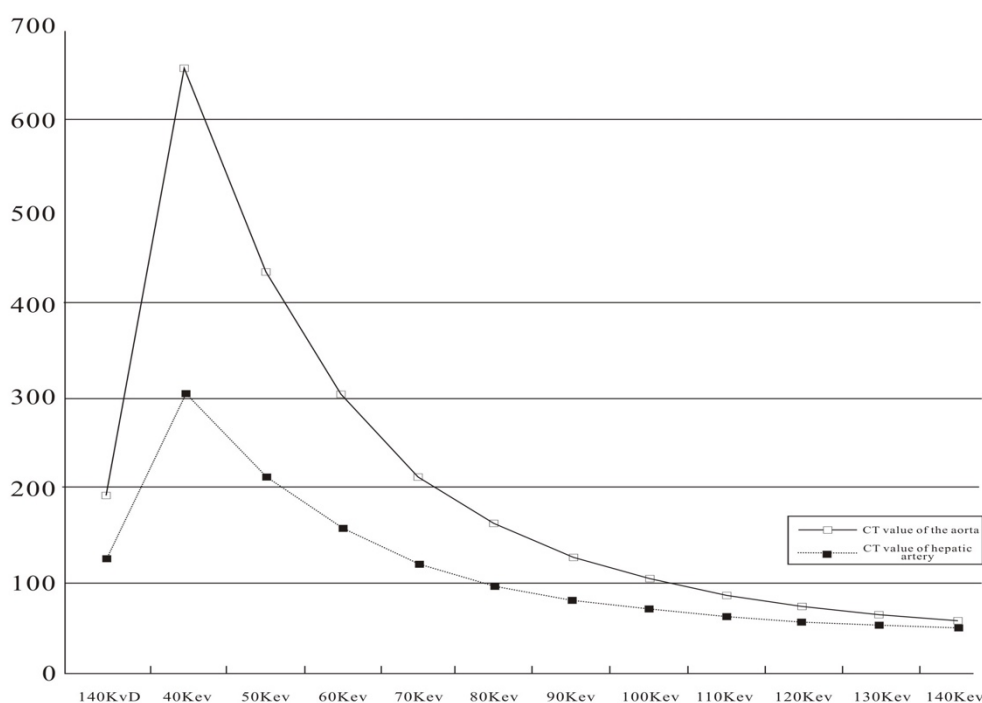


Fig. 1. Energy-density curve. CT values of hepatic artery and abdominal aorta decreased with the increase of energy. CT values of a mixture of both energy image (140 kvp) and mono-energetic image (70 keV) are very close.

level. In low energy phase images, the strengthening degree of the abdominal aorta and hepatic artery increased, the contrast enhanced, but the noise also increased. However, in high energy phase images, the strengthening degree of the abdominal aorta and hepatic artery obviously decreased, the contrast weakened, and the image artifact and noise decreased. In the comparison of the MONO and QC images of objective indicators and subjective evaluations, the display of the monochromatic images in the 70 keV group was as same as the QC group ($P>0.05$). The image quality of the 80 ~ 140 keV group was worse than the QC group ($P<0.05$). In the monochromatic images in groups under 70 keV, although the objective indicators of the abdominal aorta and most of the objective indicators of the hepatic artery were higher when compared with the QC group ($P<0.05$), there were no differences between the images in the two groups in hepatic arterial SNR and the objective evaluation of the abdominal artery and hepatic artery ($P>0.05$). Meanwhile, the 40keV image noise increased significantly, and affected the observation of the MIP and VRT images. With the increase of the CT value of the surrounding tissue, the abdominal aorta and hepatic artery were increasingly covered by

surrounding tissue in VRT images, and could not be clearly displayed. Therefore, we were unable to prove that the image quality of the 40 keV group was superior to the QC group. The 50~60 keV group image quality was higher when their objective indicators of the abdominal aorta, the hepatic artery partial objective indicators, and subjective image evaluation were compared with the QC group ($P<0.05$). Although there were no differences in the SNR between the 50~60 keV group and QC group ($P>0.05$), the hepatic arterial CNR of the first group was higher when compared with the latter group ($P<0.05$). This indicated that most sub branch arteries in the latter group cannot be seen clearly. Comprehensively, considering the objective indicators and subjective evaluations, the display of the images in the QC group were more pronounced when compared with the 50~60 keV group. Our results demonstrated that the difference value of the abdominal aorta and hepatic artery increased with a decrease in energy level. Low energy phase (40~60 keV) monochromatic images had unique effects on image quality. This was illustrated mainly by differences in arteriole SNR. Low energy phase monochromatic images increased the abdominal aorta SNR, but had almost no effect on the

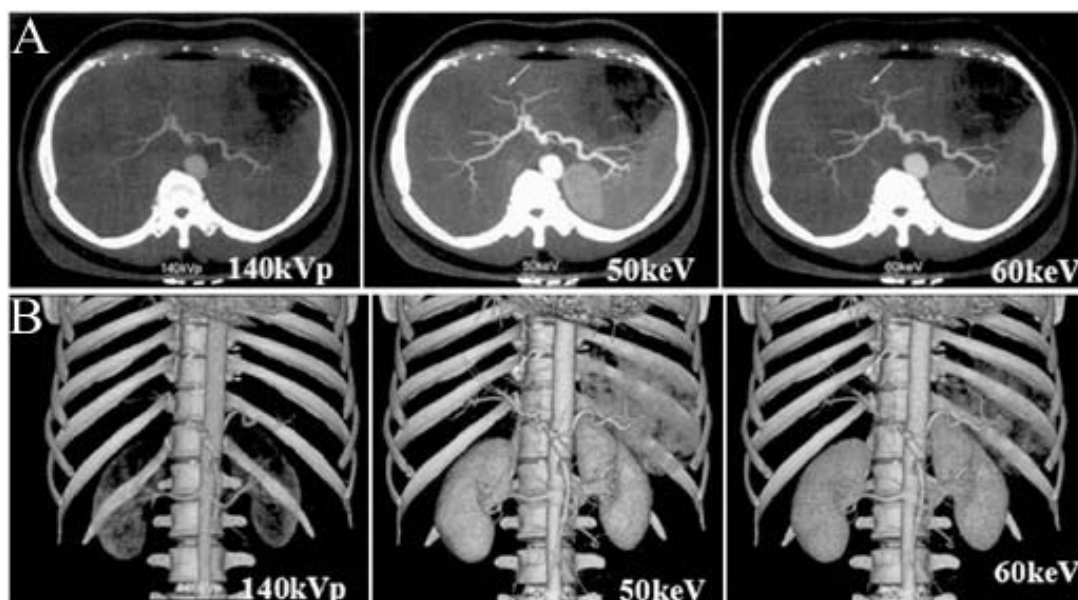


Fig. 2. A) MIP images; B) VRT images. Note: Compared with the 140 kvp image, 50 keV and 60 keV images of abdominal aorta and hepatic artery had a higher CT value and clarity (the same window width, window level, and threshold). The arrows in 50 keV and 60 keV of MTP, VRT images pointed out more small intrahepatic arterial branches.

hepatic artery SNR. This may be because the hepatic artery had a small diameter and the contrast was less. Thus, the increased range of the hepatic artery CT value was far less than in the range of the abdominal aorta. However, image noise increased to a certain degree as the energy decreased. The abdominal aorta and hepatic artery had the same noise at same energy levels. Therefore, the increased range of the hepatic artery CT value was counteracted by the increased noise level. The hepatic artery SNR had almost no change. The increased range of the abdominal aorta CT value was higher than the increased range of the noise. Low energy phase monochromatic images can significantly boost the abdominal aorta SNR.

In different image reconstruction of abdominal artery spectrum CT scanning, monochromatic (50~60 keV) reconstruction image quality was significantly better than the polychromatic reconstruction image quality. Also, the abdominal aorta and its large branch were displayed better than in conventional polychromatic imaging, and certain fine artery branches could be clearly seen. It is recommended to be the conventional reconstruction imaging for abdominal artery CT scanning. Further research into this field is required to decrease the morbidity of contrast-induced nephropathy through the reduction of contrast agent concentration, dosage, and injection rate without affecting the abdominal vascular image quality and obviously increasing radiation dose.

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LETTER TO THE EDITOR

**IDIOPATHIC INTRACRANIAL HYPERTENSION IN A PAEDIATRIC POPULATION:
A RETROSPECTIVE OBSERVATIONAL STUDY ON EPIDEMIOLOGY, SYMPTOMS
AND TREATMENT**

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Idiopathic intracranial hypertension (IIH) is a disorder of unknown origin, which is characterized by elevated intracranial pressure (ICP) without underlying etiological evidence of neurological disease. The purpose of the current study was to evaluate epidemiological features, clinical presentation, diagnostic findings and treatment of sixteen children (7 males and 9 females) with IIH. Medical records of the patients were obtained from the University Paediatric Hospital of Catania, Italy. Clinical features, investigations and treatment approaches were retrieved. The mean age of the sixteen children at onset of symptoms was 9 years (range: 4 to 16 years). Most of the patients were classified as pre-pubertal. Mean BMI was 28.9 kg/m². In 93.75% of patients headache was the presenting clinical symptom; and in the same percentage papilledema was detected as the accompanied sign during diagnostic flow-chart. The mean lumbar puncture opening pressure (LPOP) was 350 mm H₂O. Fifty percent of the cases had normal brain imaging, while 12.5% showed enlarged optic nerve diameter and one patient had an intraocular protrusion of the optic nerve on MRI. Two patients (12.5%) had venous sinus stenosis, and one case showed an abnormal spinal MRI. With regard to therapeutic approaches, 93.75% of the cases were successfully treated with Acetazolamide. None of the patients required surgical procedures, and all neuroimaging findings disappeared after receiving treatment. In the present study we investigated the association of IIH with venous sinus stenosis. We also found ocular ultrasound to be a useful non-invasive alternative method for determining papilledema in paediatric IIH, specifically in an emergency setting.

To the Editor,

Idiopathic intracranial hypertension (IIH) (also known as *Pseudotumor cerebri*) is a disorder of unknown etiology, characterized by signs and symptoms of increased intracranial pressure without

evidence of any space-occupying lesions. Since it is a diagnosis of exclusion, secondary causes such as cerebral infection, vasculopathy or inflammation should be investigated and ruled out (1, 2).

The prevalence of IIH is estimated to be

Key words: idiopathic hypertension, paediatric age, clinical outcome, diagnosis, treatment

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approximately 1 case per 100,000 women. However it rises up to 19 per 100,000 in obese women of childbearing age (3). After puberty, women are affected 9 times more often as men (3.5/100,000 in 15–44-year-old women; and 19.3/100,000 in 20–44-year-old women who are 20% above the ideal body weight). In spite of this, the association with obesity and female gender is not reflected in the paediatric population, as IIH is uncommon in children under 10 years of age, and extremely scarce in toddlers under 3 years of age (4).

This condition has uncertain pathogenesis. In particular, the reason for lack of an increase in ventricular size [despite the raised intraventricular cranial pressure (ICP)] is still unclear. Three main mechanisms enumerated for this phenomenon include increased venous sinus pressure, low CSF drainage across the arachnoid granulations or lymphatics, and increased CSF production at the choroid plexus (5).

IIH in children appears to have different epidemiological and clinical aspects when compared to adults. The Modified Dandy criteria for diagnosis of IIH in adults were also used for the diagnosis of paediatric cases. However, new diagnostic criteria for IIH in pre-pubertal ages were proposed in 2007 (5) which were revised in 2010 and again in 2013 (1). Available treatment options of IIH are the topic of scientific debate and their efficiency in childhood has been demonstrated in only few randomized controlled trials.

This is therefore an observational retrospective study reviewing epidemiological features, clinical presentation and management of IIH in a pediatric setting in order to identify the most efficient diagnostic and therapeutic follow-up of the disorder in this age group.

MATERIALS AND METHODS

This is a retrospective observational clinical study on children admitted to the Paediatric Acute and Emergency Unit of the Policlinico-Vittorio Emanuele University Hospital (Catania, Italy) due to signs and symptoms of IIH. The Authors reviewed the medical records of 16 consecutive patients with IIH aged between 0 and 16 years who were diagnosed with IIH between 2005 and 2015.

Data of presenting symptoms, comorbid conditions, investigations and treatments were collected. Files from neuroimaging (Computed Tomography (CT) or Magnetic Resonance Imaging (MRI)), lumbar puncture and ophthalmological assessment (ophthalmoscopy, visual perimetry and acuity) were reported. Moreover, further tests were requested according to the medical history and the results of the initial analyses.

Demographic information (including gender, height, weight, BMI and age) of patients were gathered. Children aged 11 years or younger were considered as pre-pubertal. According to an international standard growth chart, overweight was defined as a BMI at or above the 85th percentile and below the 95th percentile for children and adolescents of the same age and sex. Obesity was defined as a BMI at or above the 95th percentile for children and adolescents of the same age and sex (8).

Children were diagnosed according to the modified Dandy criteria (1): a) papilledema; b) normal neurological examination with the exception of cranial nerve abnormalities; c) normal brain parenchyma without any evidence of hydrocephalus, mass, or structural lesion and no abnormal meningeal enhancement or venous sinus thrombosis on neuroimaging; d) elevated Lumbar Puncture Opening Pressure (LPOP); e) normal fluid parameters, namely cytology and chemistry values. The current study considered LPOP elevated if 280 mm H₂O, or 200 mm H₂O plus clinical signs and symptoms were consistent with IIH or anamnestic history of previous IIH. If papilledema was absent but criteria from b to e were fulfilled, a suspected diagnosis could be made when three or more of the following neuroimaging signs coexisted: flattening of the posterior globe, empty sella turcica, perioptic subarachnoid space distension with or without optic nerve tortuosity, and transverse venous stenosis.

Statistical analysis

For statistical evaluation, we used dedicated software: JMP (produce of SAS Institute Inc. Cary, NC 27513-2414, USA) and GraphPad 5.0 (La Jolla, CA, USA).

Categorical data are described as absolute and relative frequencies. Parametric data are expressed as mean $\pm$ standard deviation (SD). A frequency for distribution test was used to calculate the distribution in percentages of the studied groups.

RESULTS

The demographic information, clinical presentation, laboratory and neuroimaging features are shown in detail in Table I.

Patient cohort

This study included 16 confirmed diagnoses of

IIH, of which 7 were male and 9 were female. Mean age at the time of presentation was 9 (ranging from 4 to 16 years). Most patients (12/16: 75%) were pre-pubertal (age 11 years). BMI measurements were available for all children; mean BMI at presentation was 28.9 kg/m², with no significant difference between male and female patients (29.35% and 28.47%, respectively).

Table I. Summary of the diagnostic, demographic, and clinical features.

N	Age/sex	BMI (kg/m ²)	Clinical presentation	Clinical examination	Lumbar puncture opening pressure (LPOP) (mm CSF)	Neuroimaging
1	10 years/F	34.7	Headache, vomiting, paresthesias, confusion	Papilledema, hemiplegia, gait imbalance, visual field defect, aphasia, abducens nerve dysfunction	400	Normal brain CT and MRI
2	9 years/M	22	Headache	Papilledema	350	Normal brain CT and MRI
3	7 years/M	34	Headache, vomiting, asthenia, sleepiness	Papilledema, abducens nerve dysfunction, impaired visual acuity, gait imbalance, retinal hemorrhages,	420	Normal brain CT and MRV Brain MRI: arachnoid cyst
4	7 years/M	30.5	Headache, photophobia	Papilledema, impaired visual acuity	380	Brain CT and MRI: bilateral enlarged optic nerve diameter; intraocular protrusion of the optic nerves
5	10 years/F	26.6	Headache, neck pain	Papilledema	400	Normal brain and spinal cord MRI
6	16 years/F	33	Headache	Visual field defect	275	Normal brain MRI
7	12 years/F	25	Headache, blurry vision, diplopia	Papilledema	350	Normal brain MRI
8	10 years/F	33	Headache, vomiting	Papilledema	320	Normal brain CT Brain MRI and MRA: bilateral enlarged optic nerve diameter
9	8 years/F	23	Headache	Papilledema, abducens nerve dysfunction, impaired visual acuity, oculomotor nerve dysfunction	310	Normal brain MRI
10	13 years/F	31	Paresthesias, dizziness, asthenia	Papilledema, facial nerve dysfunction	400	Normal MRA Spinal cord MRI: a raise of CSF in C4-L2; spinal cord compression and dislocation at thoracic region
11	8 years/M	31	Headache	Papilledema	320	Normal brain and spinal cord MRI MRV: transverse venous sinus narrowing
12	5 years/F	24	Headache, vomiting	Papilledema	550	Normal brain MRI
13	10 years/M	32	Headache, vomiting	Papilledema	250	Normal brain MRI and MRA
14	12 years/F	26	Headache	Papilledema	220	Normal brain MRI
15	11 years/M	33	Headache	Papilledema	400	Normal brain MRI
16	4 years/M	23	Headache	Papilledema	250	Brain MRI: arachnoid cyst; turbulence of CSF flow within the inferior aspect of the arachnoid cyst and at foramen magnum region Brain MRA: horizontal (A1) segment of the right anterior cerebral artery (ACA) not visualized; Brain MRV: transverse venous sinus narrowing

Clinical presentation

The delay between the onset of symptoms and first visit to the clinic ranged from 1 day to 6 months.

All patients had clinical symptoms of increased intracranial pressure at presentation to the Emergency Unit. The most common presenting clinical symptoms were headache (15/16: 93.75%), visual complaints (5/16: 31.25%) and vomiting (5/16: 31.25%). Headache was the sole complaint reported by 6 children (37.5%), while the others reported more than one symptom (62.5%). The visual complaints included decreased visual acuity (as reported below), photophobia, blurry vision and diplopia. Other associated symptoms were paresthesia (2/16: 12.5%) (peri-oral in one case, limb paresthesia in the other), asthenia (2/16: 12.5%), dizziness (1/16), sleepiness (1/16), confusion (1/16) and neck pain (1/16).

Clinical examination

All patients had documented neurological and ophthalmological examinations. Papilledema was present in 15 patients (93.75%), and one child had retinal haemorrhage. The single patient without papilledema had presented with headache and a previous history of IIH. Unilateral or bilateral Abducens nerve dysfunction (cranial nerve VI) was detected in 3 cases (18.75%). Impaired visual acuity was present in 3 (18.75%) patients while visual field defects were detected in 2 (12.5%). Other symptoms were gait disturbance (2/16: 12.5%), hemiplegia (1/16: 6.25%), aphasia (1/16: 6.25%), asymmetry of mouth (dysfunction of the cranial nerve VII) (1/16: 6.25%) and bilateral palpebral ptosis (dysfunction of the cranial nerve III) (1/16: 6.25%).

Comorbidity

Associated comorbidities (either probable cause or coincidental finding) included systemic hypertension (1/16), previous HSV-1 (Herpes simplex virus) meningitis (1/16), use of estroprogestinic medications for Polycystic ovary syndrome (PCOS) (1/16), hyperinsulinemia (1/16), use of Topiramate and Valproic acid for epilepsy (1/16), CMV infection (documented by serologic assays for specific IgM and IgG antibody) (1/16), subclinical hypercortisolism (biochemical cortisol

excess without the classical signs of Cushing's syndrome) (1/16).

Investigations

Lumbar puncture was performed on all patients. The LPOP ranged from 220 to 550 mm H₂O, with a mean pressure of 350 mm H₂O.

All patients had CT or MRI as part of their evaluation; five of these (31%) underwent a magnetic resonance angiography (MRA) and four patients (25%) had MRI of their spinal cord. Eight patients (50%) had normal brain imaging. Two patients (12.5%) had enlarged optic nerve diameters and one had an intraocular protrusion of the optic nerve. Two patients (12.5%) had venous sinus stenosis, one of who, had unilateral stenosis of the sigmoid sinus without flow reduction, and the other had bilateral transverse venous sinus narrowing.

Two patients (12.5%) had an arachnoid cyst: one in the temporal lobe, and the other in the posterior fossa (that appeared extended) generating a mass effect on the adjacent cerebellar tissue and remodelling the adjacent occipital squama. In the latter, cerebellum, medulla oblongata and pons were dislocated before the cyst. The last patient was the same child with transverse venous sinus narrowing. MRA was not able to visualize the horizontal (A1) segment of the right anterior cerebral artery (ACA); and a turbulence of CSF flow within the inferior aspect of the arachnoid cyst and at the foramen magnum region was also detected.

One patient showed an abnormal spinal MRI, plus CSF rise in C4-L2 intra canal epidural posterior aspect with the compression and the dislocation before the dural sac and the spinal cord at the thoracic region. It should be noted that this neuroimaging finding disappeared after therapy.

Ocular ultrasonography was performed on two children (12.5%). In both patients papilledema was detected; and in one case the optic nerve sheath diameters (ONSD) were dilated (6.7 mm in the right eye and 6.63 mm in the left eye).

A retinal fluorescein angiography was performed on the child who had retinal haemorrhages that confirmed papilledema and retinal haemorrhagic vasculopathy.

Management

After initial diagnosis of IIH, most patients ($n = 15$; 93.75%) were started on Acetazolamide, which was effective in all cases. The average treatment duration with Acetazolamide was 6 months. Two patients received treatment with Topiramate; one of whom received it as an additional therapy. No patients had optic nerve fenestration, shunting or venous sinus stenting.

Follow-up

All patients were symptom-free after 6 months of Acetazolamide. The patients with optic impairment (detected by optic consultation) underwent a second revision after initial therapy; which resulted in improvement of their clinical condition and reduction of their optic disease.

DISCUSSION

Our study was a retrospective observational study on epidemiology, clinical, and therapeutic aspects of IIH in paediatric patients. We included 16 children with IIH, most of whom (75%) were pre-pubertal. The high incidence of IIH in subjects under 11 years of age in our study might indicate that different risk factors are more likely to be leading in our region. The youngest patient was four years old. Since IIH is extremely rare in infants and toddlers, evaluating patients for secondary causes of intracranial hypertension is particularly important in the first years of life.

Boys were nearly equally affected as girls in the present study. This finding is in agreement with previous studies suggesting that the association with female gender is not as prevalent in paediatric populations as it is in adult IIH patients. In contrast to prior studies which reported no association between obesity and IIH in younger children (11), in our study all patients were overweight (3/16: 18.75%) or obese (13/16: 81.25%) with no significant difference between ages. Moreover, no difference was reported between male and female regarding BMI, suggesting that an increase in BMI represents a risk factor for IIH in childhood in general.

Similar to previous literature (7, 8), the most

common baseline symptom in our series was headache, which was accompanied with a large degree of variability in duration, severity and clinical features. Paraesthesia, asthenia, dizziness and unilateral cranial nerve VII impairment were the clinical features in the only patient who presented without headache. The next most common clinical presenting symptoms were vomiting (often associated with headache) and visual complaints (decreased visual acuity, photophobia, blurry vision, diplopia). Vision loss in IIH is believed to be due to transmission of high ICP to the head of optic nerve, resulting in axoplasmic stasis and microvascular compromise. Diplopia can be secondary to unilateral or bilateral sixth nerve palsies. In contrast with previous studies, transient visual obscuration was not present in this series (8). This seems to be relatively common in older children but rarely reported on routine questioning. The mentioned obscuration is possibly due to transient ischemia of the optic nerve head, and is mostly triggered by changes in position. This could be either unilateral or bilateral; and often lasts for few seconds.

A lumbar puncture to a patient with IIH displays an increased CSF opening pressure in addition to a normal composition (9). In our study, LPOP ≥ 280 mm H₂O (or ≥ 200 mm H₂O, if papilledema was present in addition to other clinical signs and symptoms consistent with IIH, or anamnestic history of previous IIH) was considered as abnormal, therefore it was recommended for the diagnosis of IIH. The LPOP of the studied patients in our study ranged from 220 to 550 mm H₂O.

Neuroimaging is mandatory to detect any potential secondary cause of elevated ICP (10, 11). Two of the patients were identified with arachnoid cysts. In one case, the cyst was located in the posterior fossa and generated a mass effect on the adjacent cerebellum, medulla oblongata and pons that were consequently displaced.

Due to scarcity of randomized, controlled and double-blind prospective studies, the best management strategy for IIH in children has not yet reached an unanimous decision. Therefore the current treatment is largely established on evidence obtained from adult populations. Decreasing ICP is the main component of treatment and aims to

preserve vision and control symptoms. Asymptomatic patients with normal vision and minimal papilledema could be monitored without therapy.

Carbonic-anhydrase inhibitors are among medications that are currently used to decrease ICP. Acetazolamide (an example of the mentioned agents) is believed to adjust CSF secretion at the choroid plexus, therefore is preferred for treating IIH. Topiramate and steroids have also been suggested as IIH treatment strategies (12).

As far as mechanical treatment of IIH is concerned, it has been demonstrated that non-invasive mechanical ventilation could be useful to improve IIH clinical condition, since it decreases systemic CO₂ as well as its secondary central diffusion (13, 14).

IIH represents an uncommon problem in the paediatric population. Its clinical presentation is usually characterized by the symptomatic triad: headache, papilledema and visual complaints. There are no prospective or randomised controlled trials in paediatric group; therefore diagnostic criteria and medical management of IIH in children is faced with uncertainty. Our study considers ocular ultrasound as a useful non-invasive alternative method for determining the presence or absence of papilledema (secondary to raised ICP) in a child with headache, or a child with positive risk factors for IIH in an emergency setting or when fundoscopy cannot be adequately performed.

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LETTER TO THE EDITOR

ADJUVANT TREATMENT WITH A SYMBIOTIC IN PATIENTS WITH INFLAMMATORY NON-ALLERGIC RHINITIS

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Inflammatory non-allergic rhinitis (INAR) is characterized by the presence of an inflammatory infiltrate and a non-IgE-mediated pathogenesis. This retrospective, controlled, multicentre study investigated whether a symbiotic, containing *Lactobacillus acidophilus* NCFM, *Bifidobacterium lactis*, and fructo-oligosaccharides (Pollagen®, Allergy Therapeutics, Italy), prescribed as adjunctive therapy to a standard pharmacological treatment, was able to reduce symptom severity, endoscopic features, and nasal cytology in 93 patients (49 males and 44 females, mean age 36.3±7.1 years) with INAR. The patients were treated with nasal corticosteroid, oral antihistamine, and isotonic saline. At randomization, 52 patients were treated also with symbiotic as adjunctive therapy, whereas the remaining 41 patients served as controls. Treatment lasted for 4 weeks. Patients were visited at baseline, after treatment, and after 4-week follow-up. Adjunctive symbiotic treatment significantly reduced the percentages of patients with symptoms and endoscopic signs, and diminished inflammatory cells. In conclusion, the present study demonstrates that a symbiotic was able, as adjuvant treatment, to significantly improve symptoms, endoscopic feature, and cytology in patients with INAR, and its effect may be long lasting.

To the Editor,

Itching, sneezing, rhinorrhoea, and nasal obstruction are symptoms characterizing non-allergic rhinitis (NAR), which is caused by a non-IgE-mediated pathogenesis. Different types of NAR exist: the presence of inflammatory cell infiltrate

characterizes inflammatory NAR (INAR). INAR classification is based on the predominant infiltrating cell: eosinophil (NARES), mast cell (NARMA) or combined pattern, such as NARESMA, which is signed by contemporary predominance of eosinophil and mast cell (1). However, the exact pathogenic

Key words: inflammatory non-allergic rhinitis, probiotics, symbiotic, symptoms, turbinate, eosinophil, mast cell

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mechanism of INAR is still unknown, even though there is evidence of a close relationship between inflammation intensity and symptom severity (2). Nasal hyper-reactivity commonly accompanies INAR: the exposure to non-specific stimuli (i.e. cool and dry air, vapours, perfumes, irritants, spicy foods, etc.) elicits nasal symptoms.

As there is no definite cause, the medications for INAR are prescribed on an empirical basis: indeed, the medications indicated for allergic rhinitis, such as antihistamines and intranasal corticosteroids, are usually used also for INAR. These compounds may be generally effective, but sometimes may be ineffective or have adverse events. For this reason, the number of doctors prescribing also other therapies, including non-pharmacologic products, is growing. In this regard, probiotics might represent an interesting option in the management of rhinitis.

Probiotics are live microorganisms able to provide a health benefit to the host (3). Prebiotics are non-digestible compounds, mainly of vegetable origin (e.g. fructans), enhancing the growth and/or activity of probiotics (4). Frequently, probiotics are combined with prebiotics: this association is defined 'sybiotic' (5). This subject is up-to-date in the prevention and treatment of respiratory disorders (6). Two recent meta-analyses highlighted the role of probiotics in the management of allergic rhinitis and prevention of upper respiratory infection (7, 8).

It is worthy of note that the effectiveness of probiotics is usually specific for each single strain. In this regard, there is a body of evidence that *Lactobacillus acidophilus* NCFM and *Bifidobacterium lactis* BL-04 exert different anti-inflammatory effects and modulate allergic reaction, diminishing the Th2-polarization, promoting Th1 proliferation, and restoring the defective Treg function (9-11). Therefore, these strains have been fruitfully used in the treatment of pollen-induced allergic rhinitis (12). Recently, a food supplement sybiotic, including these two probiotics and the prebiotic fructo-oligosaccharides (FOS), was initially tested in the treatment of perennial and seasonal allergic rhinitis (13).

Therefore, the current retrospective, controlled, multicentre study aimed to investigate whether

Pollagen®, administered as adjuvant therapy to a common pharmacological treatment, could improve symptoms, endoscopic features, and cytological parameters in patients with INAR.

MATERIALS AND METHODS

Globally, 93 patients (49 males and 44 females, mean age 36.3 ± 7.1 years) were considered in this retrospective study. The inclusion criteria were: i) either gender and adult age; ii) documented diagnosis of INAR; iii) presence of nasal symptoms for at least one month, documented by a one-month run-in period. Exclusion criteria were: i) concomitant comorbidities, including allergy and respiratory infections; ii) chronic illness; iii) hypersensitivity to Pollagen®.

The Review Boards approved the procedure and written informed consent was given by all participants.

All patients were treated with a common therapy including: intranasal mometasone furoate (1 puff per nostril twice a day), rupatadine fumarate (1 tablet daily), and nasal lavage with isotonic saline solution (1 puff per nostril twice a day). The treatment lasted for 4 weeks. At baseline, the patients were randomly subdivided in two groups: 52 patients were adjunctively treated with the active medication (Group A), and 41 were treated only with the standard therapy and consequently considered as a control (Group B). Active medication was the sybiotic Pollagen®, containing per dose: *Lactobacillus acidophilus* NCFM $> 1 \times 10^9$ CFU; *Bifidobacterium lactis* BL-04 $\geq 3 \times 10^9$ CFU; fructo-oligosaccharides 1 g; fasting patients assumed 1 sachet daily in the morning.

The patients were visited at baseline (T0), after treatment (T1), and after a 4-week follow-up (T2). During the visits, the patients were evaluated by clinical assessment, endoscopic exam, and nasal cytology, and adverse events were registered.

Clinical parameters

The presence of nasal obstruction and rhinorrhoea was assessed in every patient.

Endoscopy

Nasal endoscopy was carried out by a 3.4 mm diameter flexible fibroscope (Vision-Sciences® ENT-2000) to assess the presence of turbinate hypertrophy.

Nasal cytology

This procedure was performed at baseline and after follow-up by scraping the middle part of the inferior turbinate with a Rhino-Probe® device (Arlington Scientific). The sample was smeared onto a slide, air-dried, then stained with the May-Grünwald Giemsa preparation. The type and cell number were examined using microscopy (Nikon® E600). Cell types were identified, and intracellular components were studied at x1000 in oil immersion. The mean number per 50 fields was calculated and reported (14, 15).

Statistical analysis

Statistical analysis of the results was performed using χ^2 and Wilcoxon tests for intergroup and intragroup analysis at the different times. Clinical and endoscopic data were expressed as percentage of patients presenting the specific symptom or endoscopic feature. Nasal cytology data were expressed as absolute values of inflammatory

cells. The Δ of pre-treatment versus post-treatment values were calculated. Statistical software (Statistica 8.0) was used for analysis.

RESULTS

All patients completed the study; the treatments were well tolerated and no clinically relevant adverse event was reported. All recruited patients had NARSEMA.

Clinical parameters

Nasal obstruction: the percentage of patients with obstruction decreased during the study in Group A (T0= 84.6%; T1= 55.8%; T2= 40%) as well as in Group B (T0= 70%; T1= 63.4%; T2= 51.2%), as reported in Fig. 1A. Considering the Δ change, there were -34% between T1 and T0 and -52.7% between T2 and T0 in Group A ($p<0.01$), whereas

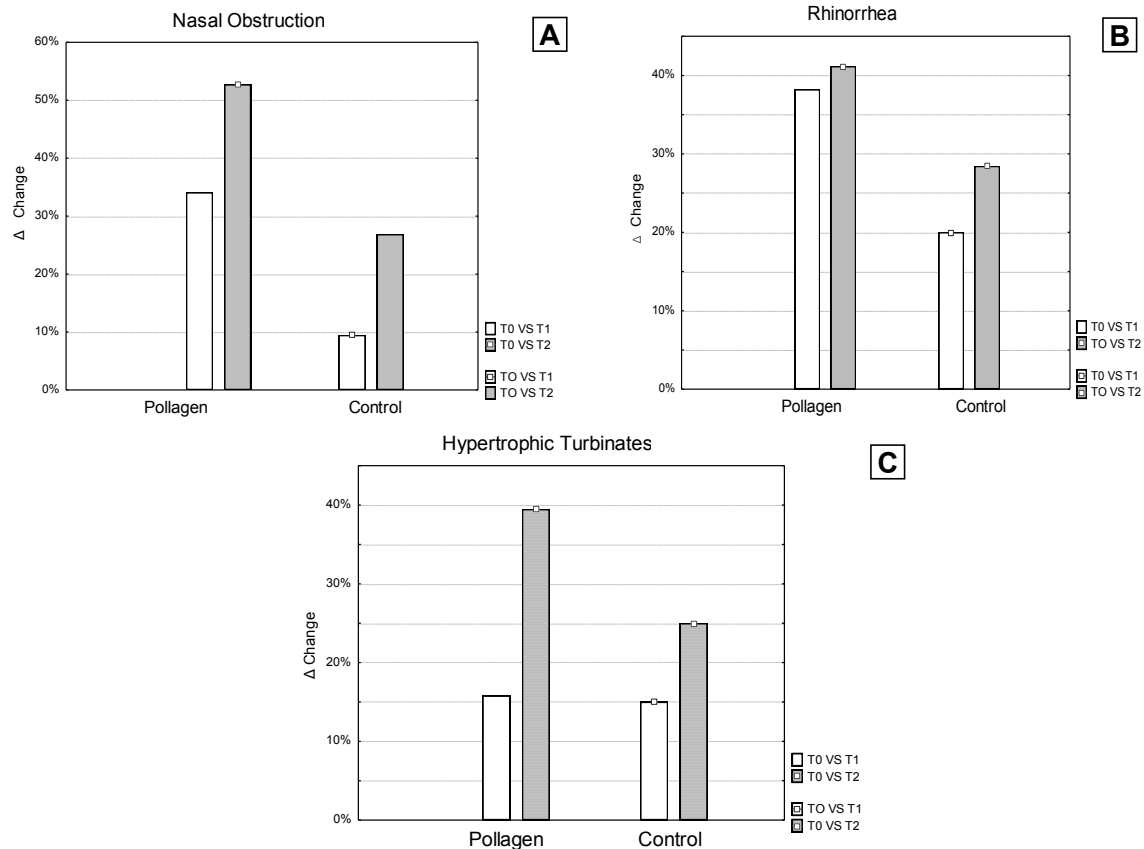


Fig. 1. A) Percentages of patients with obstruction in Pollagen group and Control group at T0, T1, and T2. B) Percentages of patients with rhinorrhoea in Pollagen group and Control group at T0, T1, and T2. C) Percentages of patients with hypertrophic turbinates in Pollagen group and Control group at T0, T1, and T2. (data are expressed as Change in percentage)

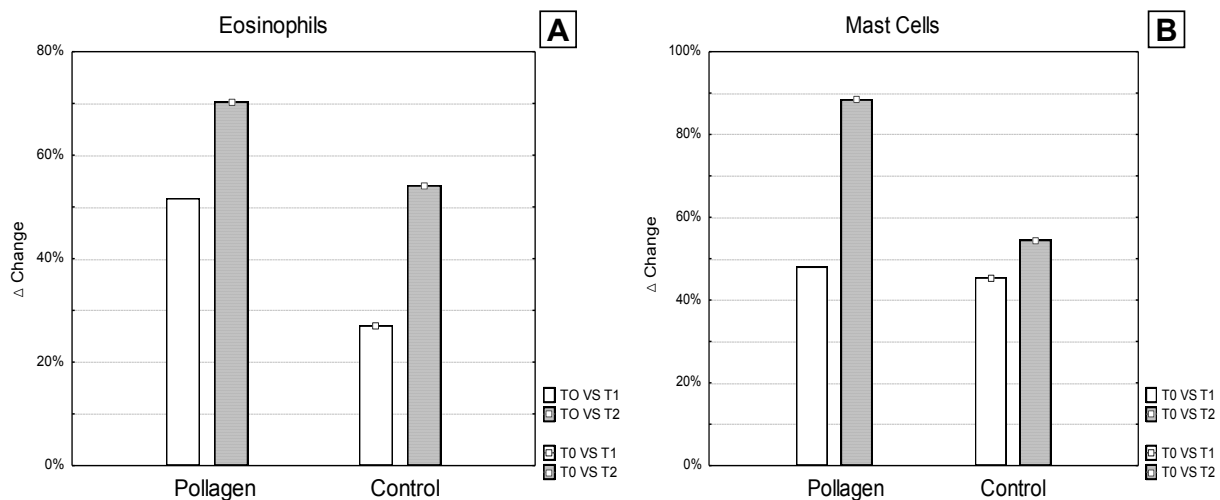


Fig. 2. A) Mean number of eosinophils in Pollagen group and Control group at T0, T1, and T2. B) Mean number of mast cells in Pollagen group and Control group at T0, T1, and T2. (data are expressed as Change in percentage)

there were -9.4% and -26.9% at the same times in Group B ($p < 0.01$). The intergroup analysis showed a significant difference considering both the T1-T0 and T2-T0 period ($p < 0.001$ for both).

Rhinorrhoea: the percentage of patients with rhinorrhoea reduced in Group A (T0= 65.4%; T1= 40.4%; T2= 38.4%) as well as in Group B (T0: 75%; T1= 60%; T2= 53.6%), as reported in Fig. 1B. Considering the Δ change, there were -38.2% between T1 and T0 and -41.2% between T2 and T0 in Group A, whereas there were -20% and -28.5% at the same times in Group B. The intergroup analysis showed a significant difference considering the T1-T0 period ($p < 0.01$).

Endoscopic features

Hypertrophic turbinates: the percentage of patients with hypertrophic turbinates were 73% in Group A at T0, 61.5% at T1, 44.2% at T2; in Group B: 48.8% at T0, 56.1% at T1, 61% at T2, as reported in Fig. 1C. Considering the Δ change, there were -15.8% between T1 and T0 and -39.5% between T2 and T0 in Group A ($p < 0.01$), whereas there were -15% and -25% at the same time points in Group B. The intergroup analysis showed a significant difference considering the T2-T0 period ($p < 0.05$).

Nasal cytology

Eosinophil: patients of Group A had a decrease (T0= 118; T1= 57; T2=35) as well as patients of Group B (T0= 48; T1= 35; T2= 22), as reported in Fig. 2A. Considering the Δ change, there were -51.7% between T1 and T0 and -70.4% between T2 and T0 in Group A ($p < 0.001$), whereas there were -27% and -54.1% at the same time points in Group B. The intergroup analysis showed a significant difference considering the T2-T0 period ($p < 0.001$).

Mast cell: Group A had at T0= 52; at T1= 27; at T2= 6. Group B had at T0= 11; at T1= 6; at T2= 5, as reported in Fig. 2B. Considering the Δ change, there were -48% between T1 and T0 and -88.5% between T2 and T0 in Group A, whereas there were -45% and -54.5% at the same time points in Group B. The intergroup analysis showed a significant ($p < 0.05$) difference considering the T2-T0 period ($p < 0.05$).

DISCUSSION

This retrospective, open study demonstrated that Pollagen® as adjunctive therapy to a common treatment (16, 17) was able to significantly affect the presence of nasal symptoms and endoscopic features and diminish cytological data in patients with INAR.

These findings may be explained by the complex anti-inflammatory and immune-modulating activity exerted by the present symbiotic. In fact, it was previously reported by *in vitro* studies that single strains exerted relevant immune-modulatory activities (9-11). Namely, these two strains were able to orient the immune response toward a physiological Th1-polarized balance and to induce an expansion of T regulatory cells, also able to exert anti-inflammatory activity. Interestingly, all patients had NARESMA, such as the INAR characterized by the most relevant inflammatory feature.

Even though the exact pathogenic mechanisms are still obscure, it is conceivable to hypothesize that eosinophilic and mast cell infiltrate could be dependent on unbalanced T-cell response in patients with INAR. Thus, a symbiotic could induce a more favourable balance between protective and inflammatory immunological pathways probably involved in INAR. This possible immune-modulatory effect might positively affect the clinical picture and the inflammatory frame.

In particular, the number of patients with symptoms and endoscopic signs significantly decreased after intranasal symbiotic adjunctive treatment. These positive effects are consistent with the parallel effect of nasal inflammation pattern. Indeed, symbiotic therapy was able to significantly reduce the grade of eosinophilic and mast cell infiltrate. This local anti-inflammatory activity may explain the relevant effect on symptoms (mainly obstruction) and signs (mainly turbinate hypertrophy) of rhinitis. Noteworthy, the effectiveness of the symbiotic tend to last also during the follow-up period. This fact is particularly relevant as it demonstrates the profound impact exerted on mucosal repair.

However, the present study has some limitations: it was a retrospective, open and uncontrolled study, had a restricted number of patients, and there was lack of the demonstration of effects on pathophysiologic mechanisms. In addition, mometasone was prescribed using high dosages, only nasal obstruction and rhinorrhea were considered as other symptoms, i.e. sneezing, loss of smell, difficulty in falling asleep, and nocturnal awakening are relevant symptoms. Furthermore, adequate scoring of symptoms, such

as assessing visual analogue scale, was missed as well as rhinitis control assessment. Finally, objective measurement of nasal obstruction was not performed. In this regard, further controlled studies should be conducted to confirm this preliminary experience and provide new information on the possible mechanism of action. On the other hand, this study had a follow-up period of observation.

Of course, it has to be noted that the symbiotic was prescribed as ancillary therapy in addition to standard treatment of rhinitis, such as intranasal corticosteroid, oral antihistamine, and nasal lavage.

In conclusion, the present retrospective study shows that a symbiotic was able, as adjuvant therapy, to significantly affect symptom severity, endoscopic features, and cytological parameters in patients with INAR, and its effect was long lasting.

DISCLOSURE: F. Guagnini is an employee of Allergy Therapeutics Italia. The other Authors have no conflict of interest to declare.

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LETTER TO THE EDITOR

ACTN3/ACE GENOTYPES AND MITOCHONDRIAL GENOME IN PROFESSIONAL SOCCER PLAYERS' PERFORMANCE

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Two nuclear genes, *ACTN3*, encoding for the α -actinin skeletal muscle isoform 3, and *ACE* encoding the angiotensin-converting enzyme, have both been associated with quantitative physical performance traits in the general population. The purpose of our study was to assess the association between the two nuclear gene variants, R577X (rs1815739) in *ACTN3* and I/D (rs4340) in *ACE*, with elite athletes' performance and the effect of training on the mitochondrial DNA (mtDNA) content in peripheral blood. We evaluated the genotypes and frequencies of *ACTN3* R577X and *ACE* I/D polymorphisms between soccer players (n = 43) and healthy non-athletic controls (n = 128). Total DNA was extracted from peripheral blood samples using the standard procedure. The genotypes were assessed by PCR-RFLP analysis and mtDNA cellular content by RT-PCR. The soccer players showed a tendency to a prevalence of *ACTN3*RR and *ACEDD* genotypes both independently and in co-occurrence. The effect of physical training on the mitochondrial DNA content in the athletic population was reflected strikingly in its increase in peripheral blood. Based on our results, we suggest that the analysis of *ACTN3* and *ACE* genotypes could predict talent in the soccer field and that knowledge of the genetic variants could determine types and training times for soccer players. In addition, the novelty of this work, never before described in the sports literature, is that the increase of mitochondrial content can be correlated with the training load, suggesting that the mtDNA copy number may be considered a viable bioenergetics biomarker.

To the Editor,

Skeletal muscle performance and physical fitness are influenced by environmental, training, metabolic and genetic factors that currently include 214 gene single nucleotide polymorphisms (SNPs) in nuclear DNA (nDNA) considered to be potential genetic markers associated with physical capacity (1). The most frequently investigated genetic markers are *ACTN3*, that encodes the α -actinin-3 protein (2) and

ACE, which encodes the angiotensin-converting enzyme (3). SNPs in both genes have been reported to be associated with elite athletic performance (4) and with normal, quantitative physical performance traits in the general population (5). α -actinin-3 protein (*ACTN3*) is almost exclusively expressed in type IIA fibers that are responsible for the generation of rapid contractions during activities such as sprinting (6). The *rs1815739* nucleotide polymorphism ([*Key words: ACE, ACTN3, mitochondrial DNA, soccer, athletic performance*](http://www.</p>
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ncbi.nlm.nih.gov/SNP/) truncates the 901aa protein at position 577. Intriguingly, this variation does not result in muscle disease due to the functional compensation of the α -actinin-2 (7) and occurs in approximately 40% of the global population (<http://www.1000genomes.org/>). The angiotensin-converting enzyme (*ACE*) is active in endothelial cells and has an important role in the regulation of blood pressure, which is necessary to sustain the increased metabolic and physiological demand of skeletal muscle during exercise (3). The *rs4340* polymorphism (<http://www.ncbi.nlm.nih.gov/SNP/>), consisting of a 287bp *Alu* sequence insertion within intron sixteen, is associated with a lower level of circulating enzyme (3) and lower levels of sprinting ability and muscle strength (8, 9). Previous studies demonstrated highly significant associations between both *ACTN3* and *ACE* genotypes with athletic performance (4, 10, 11). Elite sprint athletes have significantly higher frequencies of the *ACTN3RR* allele than the control population (11, 12). A genotype effect has also been reported in sprint and endurance athletes, with higher than expected frequencies of *ACTN3RX* heterozygotes among sprint athletes and lower than expected frequencies among endurance athletes (11) implying that a single *ACTN3R* allele is sufficient to enable power events (11, 13, 14). In team sports, diverging data have been reported (6, 15, 16). In addition, to the best of our knowledge, none of the previous studies examined the role of mitochondrial DNA in athlete populations. Exercise training induces metabolic changes in response to the energetic requests of the skeletal fibers, varying the mitochondrial content in agreement with aerobic metabolism (17). For this reason, the mitochondrial DNA, a multicopy genome that is essential for the translation of thirteen subunits belonging to the oxidative phosphorylation (OxPhos) system, has to be considered among the genetic factors contributing to physical performance as well.

We examined the distribution of *ACTN3* and *ACE* polymorphisms and the mitochondrial response to physical training in a cohort of professional soccer players. For this purpose, the aim of the present study was to analyze the distribution of nuclear genotypes' and to assess mitochondrial genomes content as a marker of mitochondrial biogenesis.

MATERIALS AND METHODS

Subjects

A total of 43 male soccer players from a team of the 2nd Division of the Italian National League and 128 healthy non-athletic subjects were recruited between May and October 2015. The control group was recruited mainly among sedentary people or those who practiced an amateur sport with irregular attendance. One hundred and sixty-nine participants were of Caucasian origin and 2 of African ancestry (only in the group of soccer players). All the participants in the study provided informed written consent.

Genetic analysis

Total genomic DNA from peripheral blood samples was extracted by Wizard Genomic DNA Purification (Promega, USA). *rs1815739* in *ACTN3* exon 16 results in the replacement of an Arg (R) with a stop codon (X) at 577 amino acid that creates three different genotypes: 577RR homozygous wild-type, 577RX heterozygous and 577XX homozygous mutated. The *ACTN3* exon was PCR-amplified using the following primers: Forward 5'-CGTTGCCTGTGGTAAGTGGG-3' and Reverse 5'-TGGTCACAGTATGCAGGAGGG-3'. The 577 alleles were distinguished by the presence (577XX) or the absence (577RR) of a *DdeI* restriction site in exon 16. *rs4340* in *ACE* gene consists of an insertion of 287bp *Alu* sequence in intron 16 that creates three different genotypes: DD homozygous wild-type, ID heterozygous and II homozygous mutated. ID *ACE* polymorphism was PCR-amplified using the following primers: Forward 5'-GCCTGCAGGTGTCTGCGCATG-3' and Reverse 5'-GGATGGATATAAAAGAATTGTCTC-3'. Relative quantification of the mtDNA copy number was performed by real-time PCR using *MT-ND1* genes as the mtDNA target by the following primers: Forward 5'-ACGCCATAAACTCTTCACCAAAG-3' and Reverse 5'-GGGTTTCATAGTAGAAGAGCGATGG-3'; and β 2microglobulin gene, *β 2M* as a reference for nuclear DNA content by the following primers: Forward 5'-CACTGAAAAAGATGAGTATGCC-3' Reverse 5'-AACATTCCCTGACAATCCC-3' (18).

Statistical analysis

Data were reported in a database created by FileMaker Pro software and analyzed by STATA MP11. Continuous variables were described as means $\pm$ standard deviations; we evaluated the normal distribution by SK test, and then we compared means by the student's *t*-test for not paired groups. To investigate the agreement of the *ACTN3* and *ACE* genotypes, we used the z-score test. A multivariate logistic regression model was created to study the associations between *ACTN3* or *ACE* genotypes and groups (soccer players vs controls), sport practices, age, height, weight; OR was calculated and the z-score test was carried out. mtDNA content in the groups was analyzed by using the GraphPad Prism, applying the ANOVA test in conjunction with the Mann-Whitney non-parametric test. A multivariate linear regression model was designed to study the association of mtDNA content with *ACTN3* or *ACE* genotypes, age, gender, or absence or presence of physical activity, calculating coef and using student's *t*-test in the soccer players and control groups. Categorical variables were described as percentages, and percentages were compared by the *Chi*-squared test. Statistical significance was set at $p < 0.05$.

RESULTS

The sample was composed of 43 soccer players (25 $\pm$ 6 years) and 128 healthy non-athletic controls (40 $\pm$ 15 years; $t=6.2$; $p < 0.0001$). All of the soccer players were males; the proportion of males among the controls was 70/128 (*Chi*-squared=29.0; $p < 0.0001$). Mean weight was 74.1 $\pm$ 12.2, without differences between soccer players (76.2 $\pm$ 6.8) and controls (73.3 $\pm$ 13.7; $t=1.3$; $p=0.09$). Mean height was 173 $\pm$ 9.9, higher among the soccer players (181 $\pm$ 6.7) than among the controls (169.7 $\pm$ 9.1; $t=7.4$; $p < 0.0001$). All 43 of the players participated on average in $\sim$ 14 hours of combined soccer-specific training and competitive play per week (6–7 soccer training sessions; one strength-training session; 1–2 conditioning sessions; one domestic game per week and one national Club game per week). All players had a minimum of 10-year prior soccer-specific training. Among the 128 controls, 30 reported

a regular physical activity, and the others were sedentary or people who practiced an amateur sport with irregular attendance; this information was not available for 21 controls.

SNP genotyping

ACTN3 and *ACE* genotypes as analysed in the soccer players with respect to the healthy non-athletic controls are reported in Table I. For *ACTN3* gene, 577RR genotype tends to be more prevalent than 577RX and 577XX genotypes in soccer players whereas 577RX genotype is more prevalent in respect to both homozygous genotypes in the control group; when we consider the R allele, we obtain the same distribution between soccer players and controls. For *ACE* gene, DD genotype tends to be more frequent in respect to DI and II genotypes both in soccer players and in the control group, although all these differences did not reach statistical significance. We evaluated the co-occurrence of the two genotypes with the z-score test. This analysis resulted in the highest k values of agreement, and statistical significances ($P < 0.05$) were obtained both for *ACTN3*RR/*ACE*DD and for *ACTN3*XX/*ACE*II in the soccer players group; while no genotype combinations were significantly associated to the control group (Table II). Multivariate logistic regression models showed no associations for either *ACTN3* or *ACE* genotypes when stratified for age, height, and weight in both the soccer players and the non-athletic healthy controls groups (data not shown).

Analysis of mtDNA copy number

We evaluated the mitochondrial DNA copy number estimated as mtDNA/nDNA in peripheral blood samples from the 43 soccer players. With the aim of comparing the mtDNA copy number from soccer players in comparison with a homogeneous control group as per age, we selected 76 from the 128 total healthy non-athletic controls, based on an age range (37 $\pm$ 13 yrs) that was comparable to that of the athlete group (25 $\pm$ 6 yrs). We found a statistically significant higher mtDNA content in soccer players (242 $\pm$ 35) in comparison with the 76 controls (207 $\pm$ 56; $P=0.0018$) (Fig. 1). A multivariate

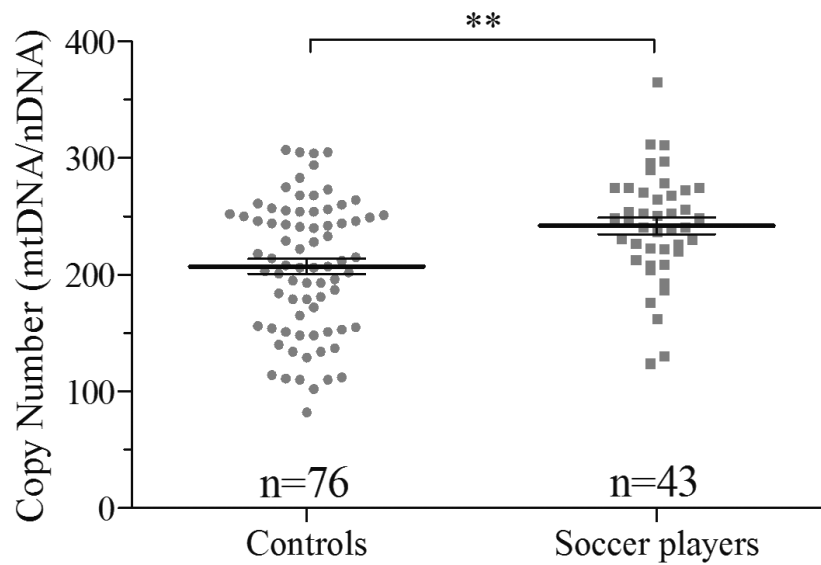


Fig. 1. *mtDNA content in soccer players versus non-athletic healthy controls. mtDNA copy number (mtDNA/nDNA) with mean $\pm$ SD was evaluated in peripheral blood samples of soccer players and age-matched non-athletic healthy controls. Experiments were performed in triplicates for all samples. To compare mtDNA levels among the cohorts of subjects, we used t-test after the Mann-Whitney correction for multiple comparisons. Asterisks indicate statistical significance: soccer players vs control group ** $p=0.0018$.*

Table I. *Genotype distribution of soccer and control populations.*

Gene	Genotype	Soccer players (n = 43)	Controls (n = 128)
ACTN3	RR	49% (n=21)	31% (n=40)
	RX	39% (n=17)	56% (n=72)
	XX	12% (n=5)	13% (n=16)
		$p=0.10$	$Chi-square=4.5$
ACE	DD	82% (n=35)	73% (n=94)
	DI	9% (n=4)	15% (n=19)
	II	9% (n=4)	12% (n=15)
		$p=0.56$	$Chi-square=1.2$

Percentage of ACTN3 and ACE genotype distribution in soccer players and non-athletic healthy control subjects. Statistical analysis was performed by SK test and then compared by student's t-test. Statistical significance was set at $p < 0.05$. n: number of subjects.

Table II. Agreement of the *ACTN3* and *ACE* genotypes in soccer players and controls.

<i>ACTN3/ACE</i>	<i>Soccer players</i>				<i>Controls</i>			
	<i>Agreement</i>	<i>k</i> (0-1)	<i>z</i>	<i>p-value</i>	<i>Agreement</i>	<i>k</i> (0-1)	<i>z</i>	<i>p-value</i>
RR/DD	62.8	0.26	2.28	0.01	43.8	0.04	0.70	0.24
RR/DI	46.5	0.09	-1.00	0.84	61.7	0.38	-0.50	0.69
RR/II	41.9	0.18	-2.05	0.97	63.3	0.03	-0.41	0.65
RX/DD	34.9	0.15	-1.47	0.92	48.4	0.09	-1.16	0.87
RX/DI	65.1	0.16	1.52	0.06	49.2	0.06	1.16	0.12
RX/II	60.5	0.04	0.45	0.32	46.0	0.02	0.31	0.37
XX/DD	20.9	0.06	-1.31	0.90	34.4	0.02	0.76	0.22
XX/DI	79.1	0.11	0.76	0.77	74.2	0.1	1.03	0.05
XX/II	88.4	0.38	2.51	0.006	78.9	0.10	0.10	0.45

The co-occurrence of the *ACTN3* and *ACE* genotypes in soccer players and non-athletic healthy control subjects was evaluated using the z-score test. The values that are statistically most significant are enhanced in italics. Statistical significance was set at $p < 0.05$. K = Cohen's coefficient; z = z-score value.

linear regression model did not show any correlation between mtDNA content and either *ACTN3* or *ACE* genotypes, when stratified by age, height, and weight both in soccer players and in healthy non-athletic controls (data not shown).

DISCUSSION

We examined the polymorphisms in *ACTN3* and *ACE*, both genes impinge on skeletal muscle structure and function to define power/sprint and endurance capacity, between adult professional soccer players and healthy non-athletic controls. Our results show that *ACTN3*RR/*ACTN3*RX is distributed in soccer players similarly to the control population.

Nevertheless, the predominance of the *ACTN3*R allele in our cohort of players, mostly of Caucasian origin, suggests that the presence of *ACTN3* protein, which is advantageous to the sarcomeric Z-disks, enables better power and strength during muscle contraction activity. In our cohort, *ACTN3*XX represented only 12% of the soccer players, the same as in the control population. It has been suggested that during human evolution, the null (X) allele has undergone strong, positive selection (14), and studies in *ACTN3* knockout (KO) mouse (19) have shown that KO mice use energy more efficiently whereas the wild-type prefer the anaerobic system. While this favors endurance activities, the trade-off is that the muscle cannot generate the rapid contractions

needed to excel in sprinting (20). As far as the *ACE* gene is concerned, it has been shown that a higher percentage of anaerobic-oriented, type II muscle fiber, is associated with the *ACEDD* genotype, whereas a higher percentage of aerobic-oriented type I muscle fiber is associated with the *ACEII* genotype (21). When we looked into the distribution of *ACE* genotypes in our cohort, we found that the *ACEDD* genotype was predominant in soccer players as well as in the control population, as also shown in previous studies (22). The predominance of the *ACEDD* genotype confirms that the higher level of circulating enzymes leads to an increase of blood pressure, and this may have a role in increasing the oxygen and nutrients available to the muscle fibres to regulate skeletal muscle performance and endurance. When we examined the possibility that in soccer athletes, a specific combination of *ACTN3* and *ACE* gene variants could be advantageous, we found a clear prevalence of the co-occurrence of *ACTN3RR* and *ACEDD* genotypes, both associated with fast-twitch fiber activity, suggesting that professional soccer players have a genotype that favours both speed and endurance. We also found a co-occurrence of *ACTN3XX* and *ACEII* genotypes in soccer players, although this finding cannot be taken into account as it is restricted to only two subjects. On the other hand, no correlation between the two SNPs in *ACTN3* and *ACE* with regard to age, height, and weight was found for either group, soccer players or controls. To assess how the skeletal muscle adapts in response to a metabolic request, we performed the evaluation of mtDNA content in soccer players and matched healthy non-athletic control subjects. Measuring mtDNA content in skeletal muscle tissues from athletes is not a simple task, but we bypassed this problem by considering that variations of mtDNA level in post-mitotic tissues, i.e. skeletal muscle, are reflected in the peripheral blood, as already reported in human disease conditions due to mitochondrial dysfunction (18, 23, 24). In this study, we report, for the first time, that mtDNA content was significantly higher in soccer players in respect to the control population, suggesting that skeletal muscle contractile activity due to intense training induces an enhancement of oxidative

metabolism that is sustained by a mitochondrial content increase (17). Thus, we provide evidence that for professional soccer players, the increase of mtDNA could be correlated with the sports load. This observation supports a mechanism in which performance is modulated by the ability to activate mitochondrial biogenesis efficiently in response to the elevated request for aerobic ATP generation. The greater propensity to aerobic metabolism found in soccer players confirms the importance of training in directing muscle metabolism. While we are still far from identifying the nuclear genetic profile that defines the perfect athlete, on the other hand, mitochondrial genetics could provide a valuable tool for monitoring metabolic and muscular activity demands.

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LETTER TO THE EDITOR

USE OF SUPERCHARGED COVER SCREW AS STATIC MAGNETIC FIELD GENERATOR FOR BONE HEALING, 1st PART: *IN VITRO* ENHANCEMENT OF OSTEOBLAST-LIKE CELL DIFFERENTIATION

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Since 1979, Pulsed electromagnetic fields (PEMFs) have been 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 the proliferation rate and gene expression profile of MG63 osteoblastic-like cells after a 24, 48 and 72-hour SMF stimulation, generated by a small, customized cover screw-shaped neodymium-iron-bore magnet placed in the inner cavity of a dental implant. As a result, we found that the application of a SMF to osteoblastic-like cells does slightly decrease cell proliferation rate while enhancing the expression of those genes correlated to differentiation and mineralization. Our findings represent, to our knowledge, the first clinical ready technique for dental implants showing the ability of SMF to promote the osteogenesis process *in vitro*.

To the Editor,

Pulsed electromagnetic fields (PEMFs) and static magnetic fields (SMFs) have been widely investigated in orthopaedic studies. Indeed, PEMF stimulation is an effective method in the treatment of non-unions (1), since it was approved by 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 have shown 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).

To date, none of the performed studies suggests

Key words: static magnetic fields, MG63, osseointegration, dental implant, cover screw, permanent magnets

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a stimulation method that could be easily applied to the 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 vitro* model. In particular, we compare the proliferation rate and gene expression profile of MG63 cells after a 24, 48 and 72-hour magnetic field stimulation, generated by Nd-Fe-B magnetic cover screws.

MATERIALS AND METHODS

Experimental design

In the present study, MG63 cells were cultured in 4-well plates for 24, 48 and 72 h incubation periods. Each plate included: (i) the implant with magnetic cover screw (A tested group); (ii) the implant without magnetic cover screw (KA control group); (iii) magnets free in the culture medium and cells in direct contact with the magnetic cover screw (M positive control); (iv) only cells (KM negative control).

The implants used were 11 mm in length and 4 mm in diameter. (Nanotite®, Biomet 3I, Palm Beach Gardens, FL, USA).

Generation of SMF

The magnetic field was generated by Nd-Fe-B magnetic screws (Supercharged Screw®, Supercharged Production, Senigallia, AN, Italy). These magnets belong to the natural magnet class with rare earth and, among these, 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 at: (i) direct contact with magnets; (ii) direct contact with implant surface; and (iii) 1 mm distance from the implant surface. For each, the measurements were taken at 4 different points.

Cell culture

MG63 osteoblast-like cells (ATCC, Rockville, MD, USA), a widely used cell model, were utilized for *in vitro* study. The cell line was cultured and maintained as monolayer in T25 cm² culture flask in Dulbecco's modified Eagle's medium (DMEM/ Ham's F-12), supplemented with 10% fetal bovine serum and 50 µg/ml gentamicin. Cultures were incubated in a 5% CO₂ and 95% O₂ humidified atmosphere at 37°C.

The cells were then trypsinized, centrifuged and collected, and the pellet was resuspended in the culture medium. Cells were counted and seeded at different concentrations: (i) 6x10⁵ cells/well for 24-h incubation time; (ii) 3x10⁵ cells/well for 48 hours incubation time; (iii) 9x10⁴ cells/well 72-h incubation time. All the plates were then placed into an incubator at 37°C in a humidified atmosphere consisting of 5% CO₂ and 95% O₂, and cultured for 24, 48, or 72 hours.

Cell proliferation assay

After 24, 48 and 72 hours of incubation, cell viability was evaluated by Trypan Blue exclusion test and MTT assay. Cells were plated and treated as described for 24, 48 and 72 hours. After each time point, the medium was removed; 200 µL of MTT (Sigma, Milan, Italy) solution (5 mg/mL in DMEM without phenol red) and 1.8 mL of DMEM were added to the cell monolayer; the 4-well plates were incubated at 37°C for a further 4 h. After discarding the supernatants, the dark blue formazan crystals were dissolved by adding 2 mL of solvent (10% HCl 1N in isopropanol, Sigma, Milan, Italy) and quantified spectrophotometrically (Secomam, Anthelie light, version 3.8, Contardi, Italy). The mean and the standard deviations were obtained from three different experiments of the same specimen.

RNA extraction, cDNA synthesis and real time-PCR array.

The cells were centrifuged for 6 min at 1200 rpm and washed twice with PBS. Then the supernatants were discarded and the resulting cellular pellets were frozen at -80°C . For each sample, the total RNA was isolated from a pellet of 500,000 cells, using the RNeasy Micro Kit (Qiagen Italia, Milano, Italy). RNA samples were tested by ultraviolet absorption at 260 nm to determine the concentration. The quality and concentration of the RNA samples were further confirmed by electrophoresis on denaturated 1% agarose gels.

The human Osteogenesis PCR array PAHS-026 supplied by SABiosciences Corporation (Frederick, MD, USA) was then used to profile the expression of 84 genes related to osteogenic differentiation.

The cDNA for each RNA sample was obtained using the Super Array RT² First Strand kit (SABiosciences Corporation, Frederick, MD, USA) according to the manufacturer's instructions. Briefly, after genomic DNA elimination, the reverse transcription reaction was performed at 42°C for 15 min and then heated at 95°C for 5 min to inactivate the enzyme. The cDNA was then mixed with RT² SYBR green/fluorescein qPCR master mix (SABiosciences Corporation, Frederick, MD, USA) and

25 μl aliquots were loaded into each well of the RT² Profiler PCR Array. PCR array experiments were performed on a MyiQ real-time PCR detection system (Bio-rad, USA). Conditions for amplification were as follows: 1 cycle of 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C . The PCR array data were analyzed by the $\Delta\Delta\text{Ct}$ method. Genes with Ct values ≥ 35 were considered as non-detectable (negative call) and assigned a value of 35. β -actin (ACTB) was used as housekeeping gene to obtain the ΔCt value for each gene of interest. The $\Delta\Delta\text{Ct}$ value for each gene was calculated by the difference between the ΔCt of the sample and the ΔCt of the control. The fold-change for each gene was calculated by $2^{-\Delta\Delta\text{Ct}}$, and the statistical analysis to determine differences between cellular samples was performed using the RT² Profiler PCR Array Data Analysis web-based software (SABiosciences Corporation).

RESULTS

SMF measurements

The intensity of magnetic field registered at the surface of implants with magnetic cover screws was lower than the intensity registered at direct contact

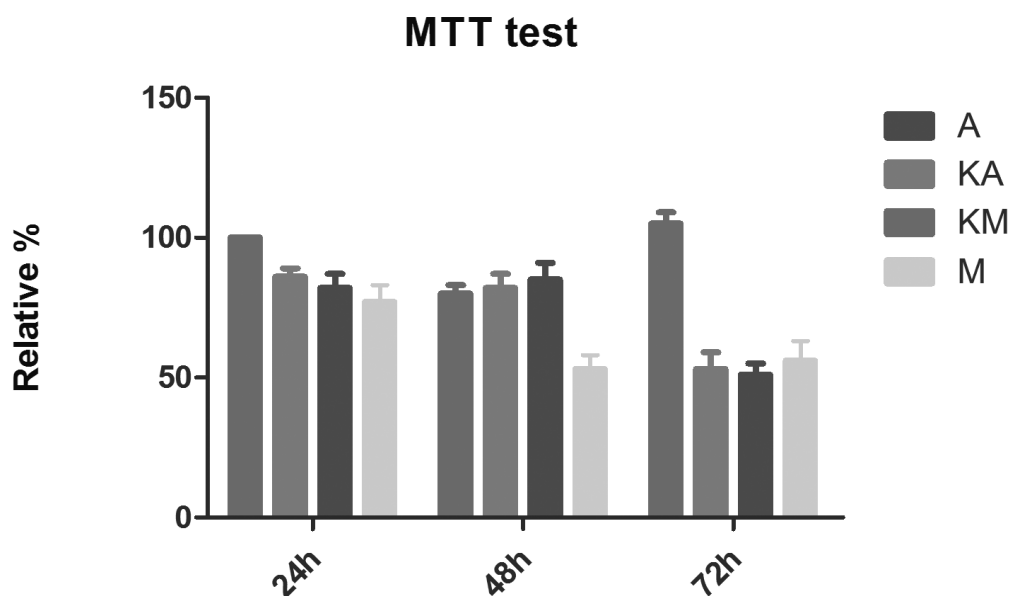


Fig. 1. MTT assay at 24, 48 and 72 hours for cases and controls. Data were presented as relative percentage assuming KM at 24h as 100%.

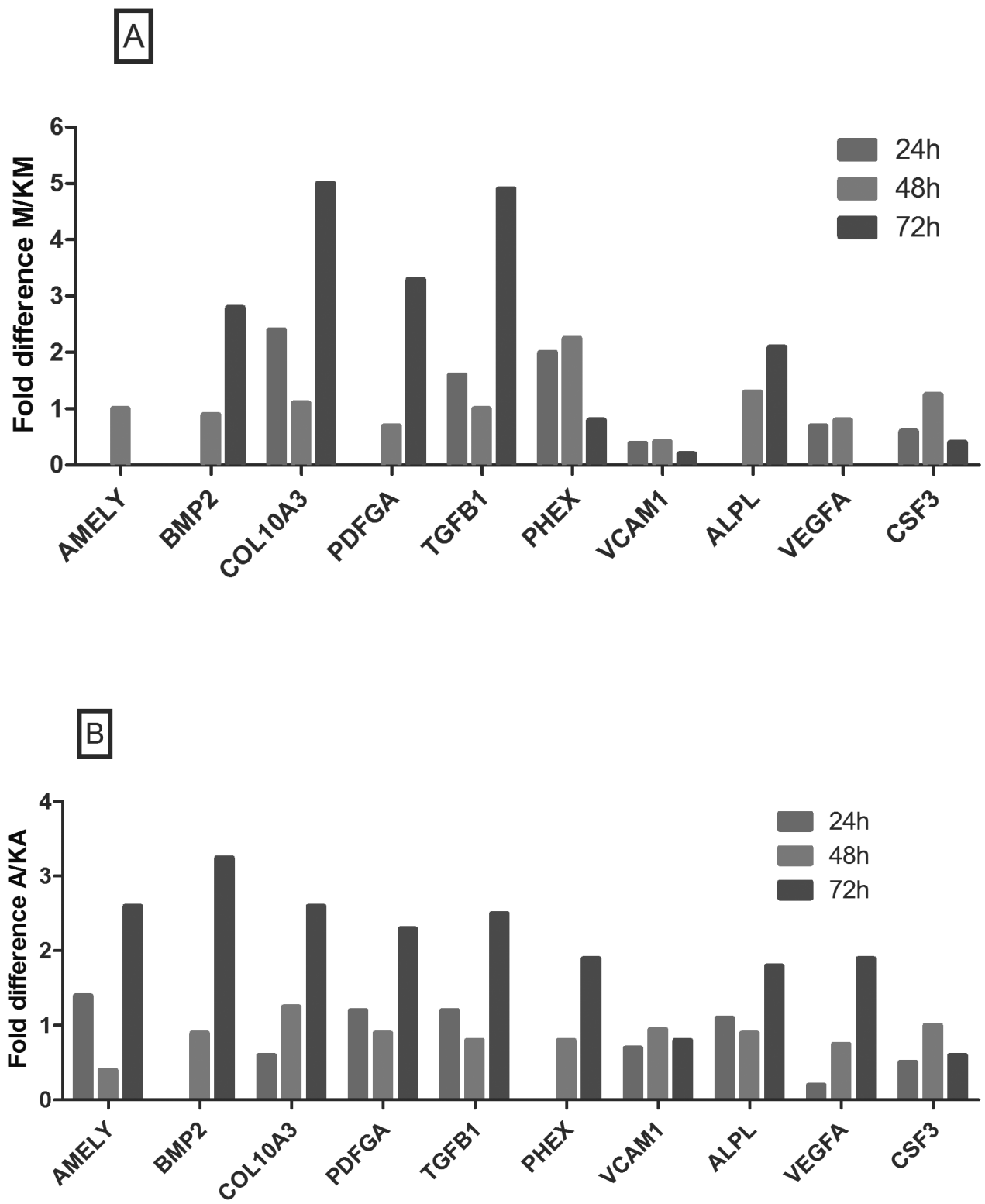


Fig. 2. Differential expression of selected genes in samples (A) at direct contact with magnets compared to controls (only cells) (B) with implants with magnetic cover screw compared to controls (implants without magnets) after 24, 48 and 72 hours.

with the magnets. According to Coulomb's law, the inferior intensity recorded at the implant surface can be explained by the increase of distance from the magnetic source. Indeed, the titanium of which implants are made is a paramagnetic material which does not influence the magnetic field line density and course. Positioning the probe at 1 mm from the implant surface, increasing the distance between the magnetic source and the probe, the flux density was further decreased.

Although the magnets were very small and there was the interposition of the implant wall, the magnets were able to produce a magnetic field extended to the peri-implant tissues, where the osteogenesis process took place.

Cell proliferation assay

Results of cell proliferation are shown in Fig. 1. The cells exposed to the highest magnetic flux density (M) showed a lower proliferation rate than the unexposed cells (KM), and of exposed (A) and unexposed cells (KA) of samples with implant at 24, 48 or 72 hours.

The comparison between exposed (A) and unexposed cells (KA) of samples with implant did not show significant differences in the proliferation rate at 24, 48 or 72 hours ($p > 0.05$; Mann Whitney test).

Gene expression profiling.

Towards a better data comprehension, among the 84 genes examined, a total of 10 genes involved in osteogenesis were selected for their prominent role in bone biology. The expression of the selected genes, considered the most important for osteoblast differentiation, is reported in Fig. 2.

Briefly, the expression of the following genes was increased in samples at direct contact with magnets compared to controls (only cells): PHEX, (phosphate regulating endopeptidase homolog X-linked) increased 2.3-fold after 48 hours of exposure; COL10A1 (collagen type X α 1), increased 10.97-fold; TGF β 1 (transforming growth factor β 1), increased 4.9-fold; VCAM-1, decreased by 82%; ALPL (alkaline phosphatase), increased 2.1-fold; and BMP-2 (bone morphogenetic protein 2), increased

2.9-fold after 72 hours of exposure.

Moreover, the expression of the following genes was increased in samples with implants with magnetic cover screws compared to controls (implants without magnets): CSF-3 (colony stimulating factor 3), decreased by 47%; IGF-1 (insulin-like growth factor 1), increased by 2.2-fold after 24 hours of exposure; COL10A1, increased 2.5-fold; TGF β -1, increased 2.5-fold; VEGF-A, increased 1.9-fold; PDGF-A (platelet derived growth factor α polypeptide), increased 2.3-fold; BMP-2, increased 3.3-fold; AMELY (amelogenin y-linked), increased 2.5-fold after 72 hours of exposure.

DISCUSSION

The application of a magnetic field in order to facilitate the dental implant osseointegration process had been proposed (8). The scientific studies in this field, though still few and extremely heterogeneous, have shown that PEMF and SMF stimulation may positively influence osseointegration both *in vitro* (9) and *in vivo* (10).

Cell viability data showed an inhibitory effect of magnetic field on cell proliferation in agreement with previous studies (6), which showed a down-regulation in MG-63 proliferation rate after 24, 48 and 72 hours of exposure to a magnetic field (flux density values between 100 and 618 mT).

The present findings were in agreement with previous studies. Indeed, the gene expression profiling showed an up-regulation of genes involved in the differentiation process in cells exposed to the magnetic field. Genes expressed in the early stage of osteoblast maturation and related to extracellular matrix production (TGF β 1 and COL10A1) (11) as well as growth factor promoting osteoblast differentiation (BMP-2) (12) were markedly increased in cells after the exposure period. On the contrary, genes involved in osteoclastogenesis (VCAM-1) (13) were decreased. Even when the magnet was placed into the implant, exposed cells showed the up-regulation of genes related to the differentiation process, especially after 72 hours.

The present findings showed the ability of SMF to decrease the cell proliferation rate whilst increasing

the expression of those genes related to osteoblast differentiation and mineralization, suggesting that the exposed cells are influenced by the magnetic field to express a more differentiated phenotype. This fact may be translated into an enhancement of the osteogenesis process.

Although further research is required to set optimal stimulation timing and flux density both *in vitro* and *in vivo*, this study may lay the basis for an adjuvant therapy to implantology, original and easy to realize.

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LETTER TO THE EDITOR

**MATERNAL OBESITY AND PERINATAL OXIDATIVE STRESS:
THE STRENGTH OF THE ASSOCIATION**

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Maternal obesity is a chronic inflammatory state, which has been shown to induce increased levels of free fatty acids, reactive oxygen species and inflammatory cells. Recent evidence reveals increased levels of lipid peroxidation products in the plasma of obese women during pregnancy. The aim of this study was to test the hypothesis that maternal overweight or obesity is associated with increased oxidative stress (OS) in offspring. Two hundred and forty-five pregnant women and their newborns were prospectively enrolled. Mothers were divided in two groups: lean control - LC (n=175, Group I); overweight or obese (n=70, Group II) according to BMI ≥ 25 before pregnancy. Cord blood F2-isoprostanes (F2-IsoPs), as reliable markers of OS, were measured in all newborns. Lower 1 minute APGAR score and higher weight at discharge were found in Group II neonates, compared to those of Group I ($p < 0.05$). Small for gestational age (SGA) newborns of both groups showed increased levels of F2-IsoPs than appropriate (AGA) or large (LGA) for gestational age (GA) ($p < 0.01$). SGA newborns of Group II had higher F2-IsoPs levels compared to SGA of Group I ($p < 0.01$), which were significantly correlated to maternal BMI at the end of pregnancy ($r = 0.451$, $p < 0.01$). Multivariate regression analysis corrected for confounding factors, showed that maternal overweight or obesity was significantly associated with high F2-IsoPs levels in SGA offspring ($p < 0.01$). Maternal overweight or obesity is associated with increased OS in their SGA newborns. Data suggest the need of antioxidant protection for both mothers during pregnancy and infants soon after birth.

To the Editor,

Overweight and obesity are serious public health challenges all over the world. The World Health Organization (WHO) estimates worldwide that there are a billion overweight and about three hundred millions obese people. The obstetrical population has not been spared from this pandemic. The number

of obese women of childbearing age is increasing at a similar rate, predominantly in the “developing countries”. The adverse effect of obesity stretches far beyond maternal health and has important repercussions for the developing fetus. Pre-pregnancy overweight/obesity has been shown to increase the risk of preterm delivery, low/high birth weight,

Key words: maternal obesity, oxidative stress, F2 isoprostanes, intrauterine growth restriction, newborn infant

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congenital anomalies, neonatal asphyxia, neonatal death, hypoglycemia, hyperbilirubinemia, increased requirement for neonatal intensive care and a longer duration of hospital stay (1). Adverse outcome to the offspring can extend beyond the neonatal period and includes neurodevelopmental disorders (cognitive problems, attention deficit hyperactivity and psychotic disorders), asthma, insulin resistance, diabetes mellitus, hypertension, coronary heart disease, stroke, and even death. In this context the placenta plays a leading role mediating transport of substances from the maternal to the fetal circulation. It also responds to a variety of inflammatory stimuli producing a number of inflammatory cytokines and free radicals (FRs) (2). Maternal obesity (MO) is a chronic inflammatory state, which has been shown to induce a lipotoxic placental environment that is associated with decreased regulators of angiogenesis and increased production of free fatty acids, FRs, inflammatory cells and markers of endothelial dysfunction (3). FRs play a central role in human placentation and in many signal transduction pathways. Deleterious effects of FRs include initiation of lipid peroxidation, oxidative damage of bio-molecules, cellular dysfunction, and may initiate maternal vascular endothelial dysfunction and leukocyte activation (4). Recently, Malti et al. reported that MO is associated with metabolic alterations and dysregulation of fetal redox balance (5).

Over the past decade, F2-Isoprostane (F2-IsoPs), which are prostaglandin-like products of the free radical-catalyzed peroxidation of arachidonic acid, have emerged as the 'gold standard' of *in vivo* assessment of oxidative stress (OS) (6). In adults, F2-IsoPs are associated with obesity, closely related to adipose mass and to leptin plasma concentrations (7). We previously found that F2-IsoPs levels in amniotic fluid are reliable markers for early identification of intrauterine OS conditions, such as preterm premature rupture of membranes, fetal growth restriction (FGR) or small for gestational age (SGA) offspring (8). Newborns are particularly exposed to OS due to the rapid environmental changes from the poor oxygen concentrations in the womb to the relative rich oxygen environment

postpartum, but also due to their relative antioxidant system deficiency. Reactions involving FR toxicity have a high damage potential for the newborn's tissues and organs. Since FR toxicity is a common event in the compromised fetus and newborn, the aim of the present study was to test the hypothesis that newborns from overweight or obese women have increased OS compared with newborns from lean control (LC) pregnant women.

MATERIALS AND METHODS

Pregnant women enrollment

A total of 273 pregnant women and their infants born between July 2009 and July 2013 in two main centers, the University Hospital of Siena, Italy, and the Aretaieion University Hospital of Athens, Greece, were enrolled in this prospective observational study. The newborns' gestational age (GA) ranged from 36 to 41 weeks. Data on maternal age, weight, height, body mass index (BMI), smoking, alcohol abuse and parity were recorded when attending the antenatal clinic at 10-12 weeks of gestation. Twenty-eight women were affected by gestational diabetes and were excluded from the study. The remaining 245 mothers of the final population were divided into two groups: LC (n=175, Group I, BMI<25); overweight or obese (n=70, Group II, BMI≥25), according to the BMI before pregnancy. BMI was calculated according to the standard definition [weight in kilograms divided by height in square meters (kg/m²)]. We chose to consider together overweight and obesity because in both conditions the abnormal or excessive fat accumulation presents a risk to health. Mode of delivery, BMI at the end of pregnancy and any other medical complications such as hypertension, FGR or chorioamnionitis were collected soon after the delivery, as well as clinical data of the neonates: GA, birth weight, length, head circumference, APGAR score at 1 and 5 minutes, length of stay in the neonatology department (postnatal days at the time of discharge), and weight at discharge. Newborns were respectively subdivided into large for GA (LGA), appropriate for GA (AGA) and small for GA (SGA) according to respective national growth charts.

Sample collection and F2-IsoPs analysis

Cord blood (CB) F2-IsoPs, as markers of lipid

peroxidation, were measured in all newborns, according to the LC-MS/MS methodology described in a recent paper by our OS team. Briefly, F2-IsoPs were extracted from 1 ml of sample collected in a tube containing EDTA. Five μL of the internal standard solution at 4 ng/mL and 50 μL of methanol were added to 50 μL of plasma. After gentle mixing and settling for 5 min, the mixture was vortex-mixed for 30 s. Proteins were separated by centrifugation and the supernatant was diluted with 80 μL of an aqueous solution of acetic acid at 0.6%. A clear extract was obtained by a further centrifugation and the final injection of 100 μL represented 25 μL of the original specimen. External calibration points were prepared by adding to 200 μL -aliquot of water 10 μL of the working solutions of F2-IsoPs with the concentrations ranging from 0.25 up to 25.0 ng/mL in order to obtain results in aqueous solutions from 12.5 pg/mL up to 1250 pg/mL. For measurements, the tandem mass spectrometer (AB Sciex, Toronto, Canada) was run in multiple reaction monitoring (MRM) with the electrospray source operating in negative ion mode and by exploiting the transitions m/z 353.3 > 193.2 for F2-IsoPs and 357.3 > 197.2 for the internal standard d4-8-iso PGF2 α .

The study was approved by the ethics committee of the two respective University hospitals. A written informed consent was obtained from all recruited parturients.

Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD), minimum and maximum for descriptive analysis of continuous variables, whereas for categorical variables the absolute frequencies are reported. A natural logarithmic transformation was used for the variables right skewed (F2-IsoPs and BMI at the end of pregnancy) and the geometric mean, as the anti-logged mean, was therefore used for the summary statistics. Box-plots and Dot-plots were used for graphic presentation of results.

The independent t -test and the χ^2 were used to make comparisons between groups for all patient characteristics. The ANOVA analysis with Bonferroni correction was used to compare F2-IsoPs values in the newborns' groups (AGA/LGA/SGA), while the independent t -test was used to compare F2-IsoPs in the mothers' group (LC vs overweight or obese mothers), for each group of newborns. Pearson correlation was also used to examine the relationship between maternal BMI at the end of pregnancy and F2-

IsoPs cord blood values in the newborns. The association between maternal overweight or obesity and F2-IsoPs was assessed through a multivariable regression model using maternal age, parity (one - four or more), maternal smoking (no smoking, less than 10 cigarettes/d, 10 or more cigarettes/d) and alcohol consumption [no consumption, occasional (once or twice a week, rare (once or twice a month) alcohol consumption] as confounding factors. A p value less than 0.05 was considered significant. Statistical analyses were performed using the SPSS V.20 for Windows statistical package (SPSS Inc., Chicago, IL, USA).

RESULTS

Baseline description of the population

According to the BMI definition, among the Group II mothers, 20 out of 70 had BMI ≥ 30 , while 50 out of 70 had BMI ranging from 25 to 29.9 before pregnancy [BMI before pregnancy mean (SD) of Group II: 28.06 (3.03); Group I: 21.20 (1.93); $p < 0.01$]. Maternal age at delivery and the percentage of hypertension, smoking, alcohol consumption and parity were similar in both mothers' groups ($p > 0.05$). Mothers of Group II had a higher cesarean section rate than Group I mothers, although not statistically significant (57.1% vs 44.6%, $p = 0.074$).

Babies of Group II presented with lower APGAR score at 1 minute and higher weight at discharge as compared to Group I babies [APGAR 1 mean (SD): Group II 8.97 (0.79) vs Group I 9.16 (0.58), $p = 0.042$; weight at discharge (g) mean (SD): Group II 3362.19 (525.74) vs Group I 3156.53 (527.86), $p = 0.036$]. No differences between the two Groups were found concerning the length of hospital stay (measured as postnatal days at discharge), auxological parameters at birth and the percentage of SGA/LGA/AGA infants ($p > 0.05$).

Relation between OS biomarkers and maternal overweight or obesity

SGA newborns showed higher levels of F2-IsoPs than AGA or LGA (respectively: 149.68 ± 95.86 vs 115.98 ± 89.42 vs 76.72 ± 57.92 , $p \leq 0.0001$) in the whole population (Fig. 1). F2-IsoPs levels were higher in SGA newborns of Group II than of Group

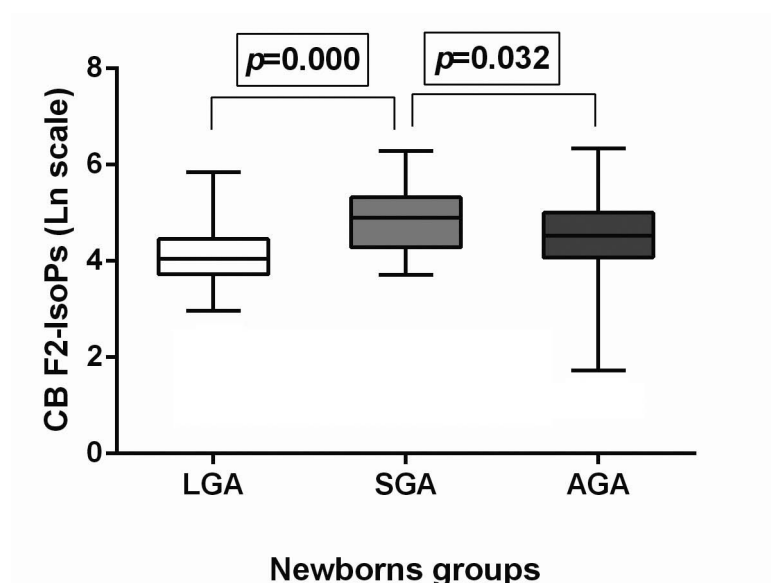


Fig. 1. Cord blood (CB) levels of F2-Isoprostanes (F2-IsoPs) in large (LGA), small (SGA) or appropriate for gestational age (AGA) newborns in a combined group (LC and overweight or obese women). CB F2-IsoPs are expressed on a logarithmic scale. The *p* values are representative of differences between groups, using the Bonferroni correction post-hoc test.

I (247.21 ± 148.66 vs 126.93 ± 63.27 , $p=0.009$; Fig. 2). No significant differences were found in F2-IsoPs levels of LGA and AGA newborns between Group II and Group I (respectively, LGA: 60.81 ± 25.01 vs 82.55 ± 65.43 , $p=0.468$; AGA: 97.97 ± 63.77 vs 124.73 ± 98.62 , $p=0.053$). Considering the SGA newborns and their respective mothers, cord blood F2-IsoPs levels significantly correlated with maternal BMI at the end of pregnancy ($r=0.451$, $p=0.006$, Fig. 3). In the multivariable regression model the overweight or obesity group showed a significantly independent association with the increase of F2-IsoPs in the SGA newborns ($b=0.654$, 95% CI 0.176–1.132, $p=0.009$).

DISCUSSION

Obese women are considered “high risk” obstetrical patients. Studies have shown that women suffering from obesity have an increased risk of a dystocic labour resulting in an operative delivery and other obstetrical complications. Neonates delivered by an obese mother will be more often transferred to

neonatal intensive care units (2).

In the present study, overweight or obese women were more commonly delivered by cesarean section and their newborns had a lower performance at birth. Only few neonates required intensive care and no differences were found in the length of hospital stay. These results are in agreement with the literature and show that our population included relatively healthy infants, without any differences in auxological parameters or in the frequency of SGA/LGA/AGA births between the two groups. The finding of increased F2-IsoPs levels in SGA newborns, and particularly in those born from overweight or obese mothers, may imply that a bad intrauterine environment could represent a risk factor during the sensitive and critical period of development permanently changing the structure, physiology and metabolism of the fetus. These observations led to the fetal programming hypothesis also known as “developmental origins of health and disease”. This theory suggests that the fetus will adapt to adverse changes in the intrauterine environment such as maternal overnutrition (9).

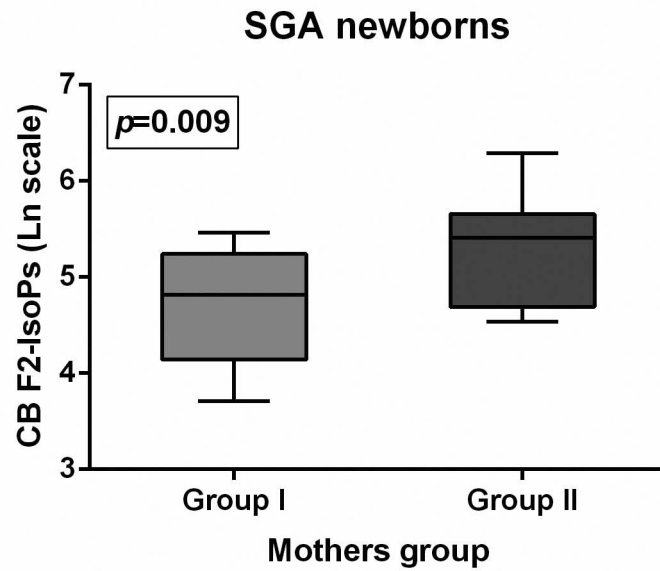


Fig. 2. Cord blood (CB) levels of F2-Isoprostanes (F2-IsoPs) in small for gestational age (SGA) newborns in LC (group I) and overweight or obese (group II) mothers. CB F2-IsoPs are expressed on a logarithmic scale.

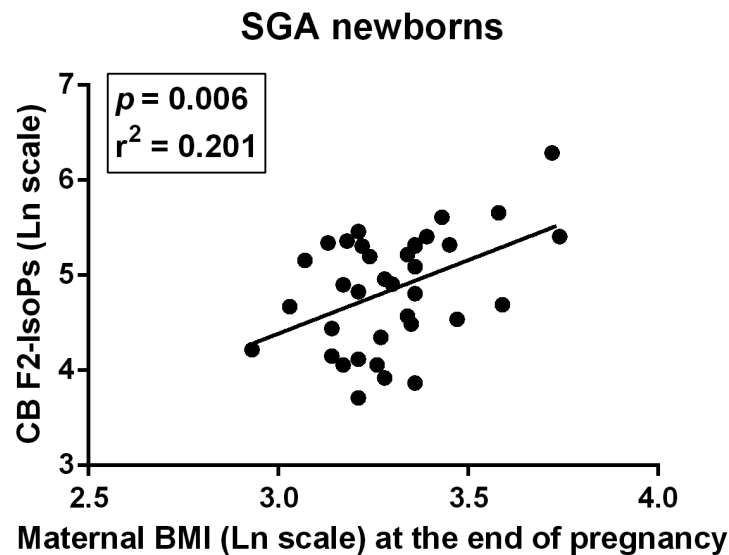


Fig. 3. Linear correlation between cord blood (CB) F2-Isoprostanes (F2-IsoPs) in small for gestational age (SGA) newborns and maternal body mass index (BMI) at the end of pregnancy of their respective mothers, considering all the subjects of the two groups in a combined group. Data regarding CB F2-IsoPs and BMI are expressed on a logarithmic scale.

The importance of maternal obesity (MO) and its influence on the in utero environment was recently shown in SGA newborns, in whom the dysregulation of leptin expression, a pro-inflammatory cytokine of the adipose tissue, interferes with the control of metabolism and the maturation of fetal tissues (10). It is common knowledge that MO causes chronic inflammation and is accompanied by elevated plasma levels of cytokines, resulting in capillary damage and leading to a state of ischemia with production of FRs (5, 7, 11). Capillary damage often represents the first step to placental diseases, exposing endothelial cells to a deleterious inflammatory process, ultimately leading to hypoxic environment and to FGR. In obese humans, inflammation may be derived from the accumulation of activated macrophages surrounding enlarged adipocytes or can be directly triggered by circulating free fatty acids through the activation of toll like receptors (TLR)-4 or nuclear factor kappa b (NF- κ B) (12). Furthermore, accumulating pyruvate in mitochondria may favour FR generation and inflammation during glucose metabolism (13). Taken together, these studies suggest that inflammation can possibly occur after every high calorie meal and that obesity is not a prerequisite. Consequent to inflammation, elevated production of reactive oxygen and nitrogen species by many cell types - including monocytes/macrophages - occurs. Moreover adipocytokines, such as leptin, regulate energy homeostasis, but also stimulate cell growth and may increase OS through multiple mechanisms. In a recent study by Ferretti et al., a higher leptin concentration together with lower levels of antioxidant enzymes, were found in the cord blood of children born to obese mothers, suggesting that these babies are more sensitive to OS (14). In addition to the large FR production, the lack of antioxidant power characterizing the perinatal period leads to a high risk of OS injury to fetuses and newborns. F2-IsoPs are widely considered as the most accurate markers for OS *in vivo* and more recently have been associated with obesity and other measures of fat distribution in cross-sectional studies (15).

In conclusion, a chain of events influences the in utero environment and, consequently, fetal growth and development. Obesity-related pathophysiological changes during pregnancy are associated to several

factors, including OS.

Our study demonstrates, for the first time, increased levels of F2-IsoPs in SGA newborns born to overweight or obese mothers. The understanding of how environmental stimuli, such as nutrition, can induce oxidative stress-mediated modifications in fetus is far from complete and further study is required in this area. However, given that there is evidence that these modifications are reversible, these results pave the way to intervention strategies, such as diet supplemented with antioxidants, that could combat the rise of growth developmental deviations.

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LETTER TO THE EDITOR

**EXHALED BREATH TEMPERATURE MEASUREMENT:
INFLUENCE OF CIRCADIAN RHYTHM**

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Exhaled breath temperature (EBT) is an expression of airway inflammation, an event that drives several lung diseases. The measurement of the exhaled breath temperature has recently been proposed as a popular tool in the diagnosis and monitoring of inflammatory lung diseases due to the fact that it is a non-invasive method. The influence of external factors on EBT, its reproducibility, and its sensitivity to treatment have already been explored. However, to reach clinical practice, EBT requires a complete validation that is still lacking. The aim of this study was to analyse the possible influence of an important internal variable, i.e. the circadian rhythm on EBT values in a group of 24 healthy adult volunteers. We repeated measurement of EBT at different hours of the day: 8.00 AM, 12.00 AM, 4.00 PM, 8.00 PM and analysed the correlation with axillary temperature measurement at these times. The EBT resulted significantly different during daily measurements (8.00 AM vs 12.00 AM vs 4.00 PM vs 8.00 PM: $28.01 \pm 1.64^\circ\text{C}$ vs $28.8 \pm 1.82^\circ\text{C}$ vs $29.34 \pm 1.79^\circ\text{C}$ vs $28.06 \pm 1.34^\circ\text{C}$). The highest EBT was reported at 4.00 PM and the lowest at 8.00 AM. For the first time we found an influence of the circadian rhythm on EBT. These data support the validation of the EBT necessary for its promotion in clinical practice.

To the Editor,

The exhaled breath temperature (EBT) is a new popular non-invasive tool for the analysis of airway inflammation in lung diseases. It has been used in the study of several inflammatory respiratory diseases such as asthma (1-11), chronic obstructive pulmonary disease (COPD) (12-14), lung cancer (15) and cystic fibrosis (16).

A very simple, non invasive, online, inexpensive, third generation thermometer, the X-halo (Delmedica), is available for the measurement

of EBT. However, notwithstanding the clinical potentiality of EBT, this tool is still confined to the research setting as the validation process has not been completed.

Several aspects have already been investigated to move EBT in the clinical setting through its validation: factors influencing EBT, such as room temperature and external temperature (unpublished data), atmospheric pressure and relative humidity have been identified (17). The reproducibility of EBT measurements has been demonstrated (17-19)

Key words: EBT, airway inflammation, non-invasive method, circadian rhythm

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and recently our group also reported the normal EBT values (unpublished). Also no adverse events have ever been reported during EBT measurements, confirming that it is a simple and safe tool. However, in order to validate a technique, more aspects need to be explored, such as the influence of an internal variable like the circadian rhythm of body temperature.

The aim of this study was to investigate, for the first time to our knowledge, the possible influence of circadian rhythm on EBT measurement.

MATERIALS AND METHODS

Population

We enrolled 24 healthy adult volunteers from the out-patient clinic of the Institute of Respiratory Diseases of the University of Foggia, Italy. All subjects were informed of the purpose of breath temperature measurement and after signing an informed consent form, anthropometric, physiological and clinical data were collected and axillary and EBT measured (Tables I, II). All measurements were performed in a clinical setting. We excluded from the population enrolled, all subjects with any respiratory, cardiac or other diseases to obtain a population of healthy subjects.

In order to fill in the blanks in the validation of the EBT,

for the first time, we measured EBT in a group of 24 healthy subjects, four times on the same days at 8.00 AM, 12.00 AM, 4.00 PM and 8.00 PM, correlating the EBT with paired axillary temperature.

Exhaled breath temperature measurement

EBT was measured with an X-halo device (Delmedica Investments, Singapore) according to previously validated methods (17). Briefly, patients were requested to inhale freely through the nose and to exhale into the device at a rate and depth typical of their normal tidal-breathing rhythm. Room temperature was tested and registered for each measurement with an external thermometer. The EBT was measured at 8.00 AM, 12.00 AM, 4.00 PM, 8.00 PM in each subject enrolled.

Statistical analysis

The studied variables were reported as means and standard deviation (SD) when appropriate. Means and Standard deviations (SD) of EBT (°C) at different hours of the day (8.00 AM, 12.00 AM, 4.00 PM, 8.00 PM) were reported. *t*-test for independent groups was used to assess differences at the different hours in terms of: gender, age (less than vs more than median age), BMI (<24.99 – normal weight vs >25 – overweight/obese) and smoking habits (non-smokers vs smokers/ex-smokers).

Table I. *Sample description.*

		N.	%
Gender			
	Male	15	62.5
	Female	9	37.5
Age			
	>36.5 yr (median age)	12	50
BMI			
	Normal weight	14	58.3
	Overweight, obese (BMI>24.99)	10	41.7
Smoking habits			
	Non smokers	15	62.5
	Smokers/ex-smokers	9	37.5

Table II. EBT at 8:00 AM, 12:00 AM, 4:00 PM, 8:00 PM.

Time	mean	SD	<i>t</i> -test	<i>p</i>				
8:00 AM	28.01	±1.64	-2.634	<u><0.05</u>	<i>t</i> -test	<i>p</i>		
12:00 AM	28.80	±1.82			-1.491	>0.05	<i>t</i> -test	<i>p</i>
4:00 PM	29.34	±1.79					4.488	<u><0.05</u>
8:00 PM	28.06	±1.34						

Table III. EBT differences at 8:00 AM, 12:00 AM, 4:00 PM, 8:00 PM by gender.

Male								
Time	Mean	SD	<i>t</i> -test	<i>p</i>				
08:00 AM	28.20	±1.75	-2.497	<u>≤0.05</u>	<i>t</i> -test	<i>p</i>		
12:00 AM	29.14	±2.05			-0.642	>0.05	<i>t</i> -test	<i>p</i>
4:00 PM	29.20	±1.66					2.8289	<u>≤0.05</u>
8:00 PM	28.32	±1.35						
Female								
Time	Mean	SD	<i>t</i> -test	<i>p</i>				
08:00 AM	27.70	±1.47	-1.051	>0.05	<i>t</i> -test	<i>p</i>		
12:00 AM	28.24	±1.27			-1.540	>0.05	<i>t</i> -test	<i>p</i>
4:00 PM	29.34	±1.79					3.896	<u>≤0.05</u>
8:00 PM	27.63	±1.34						

To evaluate differences of EBT (°C) at the different time points, a *t*-test for paired groups was used; the analysis was stratified by: gender, age, BMI and smoking habit. The significance level was fixed at $p < 0.05$ ($p < 0.05$ was considered significant). Data were analysed by using STATA 12 MP for Mac OS X.

RESULTS

There were no significant EBT differences by gender, age, BMI and smoking habit ($p > 0.05$) at any time point considered. The EBT resulted significantly higher at 12.00 AM compared to 8.00

AM (28.01 ± 1.64 vs $28.8 \pm 1.82^\circ\text{C}$, $p < 0.05$) and at 4.00 PM compared to 8.00 PM (29.34 ± 1.79 vs $28.06 \pm 1.34^\circ\text{C}$, $p < 0.05$). The highest EBT was reported at 4.00 PM and lowest at 8.00 AM (Fig. 1, Table II).

These differences were confirmed among males, in young patients (< 36.5 years), normal weight, and non-smokers ($p < 0.05$); among females, in subjects aged > 36.5 years, among the overweight/obese and smokers/ex-smokers only the EBT difference between 4.00 and 8.00 PM ($p < 0.05$; Tables III, IV) was confirmed. However, when the EBT were compared with other variables, there was no significant correlation.

DISCUSSION

In this study, for the first time, we analysed the effect of circadian rhythms on EBT measurements reporting

differences between 08.00 and 12.00 AM and between 4.00 and 8.00 PM. Females, older subjects, smokers and overweight/obese subjects showed significant EBT differences between 4.00 and 8.00 PM.

Currently, great interest is being shown toward non-invasive methods. The latest non-invasive tool in the study of airway inflammation is EBT, that already reached the threshold of 200 scientific publications on Pubmed. Most of these contributions regard the measurement of EBT in respiratory inflammatory diseases such as asthma (1-11), COPD (12-14), lung cancer (15) and cystic fibrosis (16). However, few authors have directed their efforts to the validation of this tool, such as Popov et al. who analysed the influence of external temperature, atmospheric pressure and relative humidity on EBT (17, 18) and demonstrated that EBT is reproducible (17-19).

Our data, for the first time showed that the EBT has a daily variability, being influenced by an internal

Table IV. EBT differences at 8:00 AM, 12:00 AM, 4:00 PM, 8:00 PM by smoking habit.

Non smokers								
Time	Mean	SD	<i>t</i> -test	<i>p</i>				
08:00 AM	27.93	±1.88	-2.245	<u><0.05</u>	<i>t</i> -test	<i>p</i>		
12:00 AM	28.75	±1.98			-1.562	>0.05	<i>t</i> -test	<i>p</i>
4:00 PM	29.48	±2.15					4.300	<u><0.05</u>
8:00 PM	28.01	±1.48						
Smokers/ex-smokers								
Time	Mean	SD	<i>t</i> -test	<i>p</i>				
08:00 AM	28.75	±1.98	-1.562	<u><0.05</u>	<i>t</i> -test	<i>p</i>		
12:00 AM	29.48	±2.15			-0.373	>0.05	<i>t</i> -test	<i>p</i>
4:00 PM	29.10	±1.04					1.875	<u><0.05</u>
8:00 PM	28.16	±1.15						

variable such as the circadian rhythm. We know that body temperature is a complex, non-linear variable that is subject to many sources of internal and external variation (19). Changes in body temperature are the final effects of several physiological processes that result in the production of heat and successive dispersion through the skin or breath (20). Among internal sources of temperature variability, time of the day is predictable and is driven by a circadian pacemaker. The lowest point of body temperature is reached in the morning hours and the highest in the afternoon (19). There are daily variations that can be as much as 0.25 to 0.5°C (19). All these concepts are applicable to the body temperature measured at all possible sites: oral, rectal, axillary and also by exhaled breath.

We found that EBT measurement changes during the various hours of the day. It is highest at 4.00 PM and lowest at 8.00 AM. The EBT coherently follows the circadian rhythm of body temperature and therefore it also positively correlates with the corresponding axillary temperature. In view of the circadian variability observed in this study, it would be appropriate to always

carry out EBT measurements in the morning.

Although daily differences in EBT measurement were slight, they merit great consideration in clinical practice especially in subjects with respiratory diseases, such as asthma, where the EBT is considered expression of airway inflammation, and might influence, when validated, clinical and therapeutic approaches. A new study on circadian variation of the EBT also in asthmatic subjects could therefore be very important. However, the lack of validation of the EBT method makes it a controversial tool in clinical practice (especially in asthma and COPD) and this work points out problems related to the hourly variability on the day of execution, offering a contribution at this regard (21).

The EBT follows the circadian rhythm linearly in males, in young patients (<36.5 years), normal weight, and non-smoker subjects. Slight differences in the oscillations, however, have been observed in the morning among females, older subjects, overweight/obese and smokers/ex-smoker subjects although the main difference between 4.00 PM and 8.00 PM has been confirmed also in these groups. Differences in

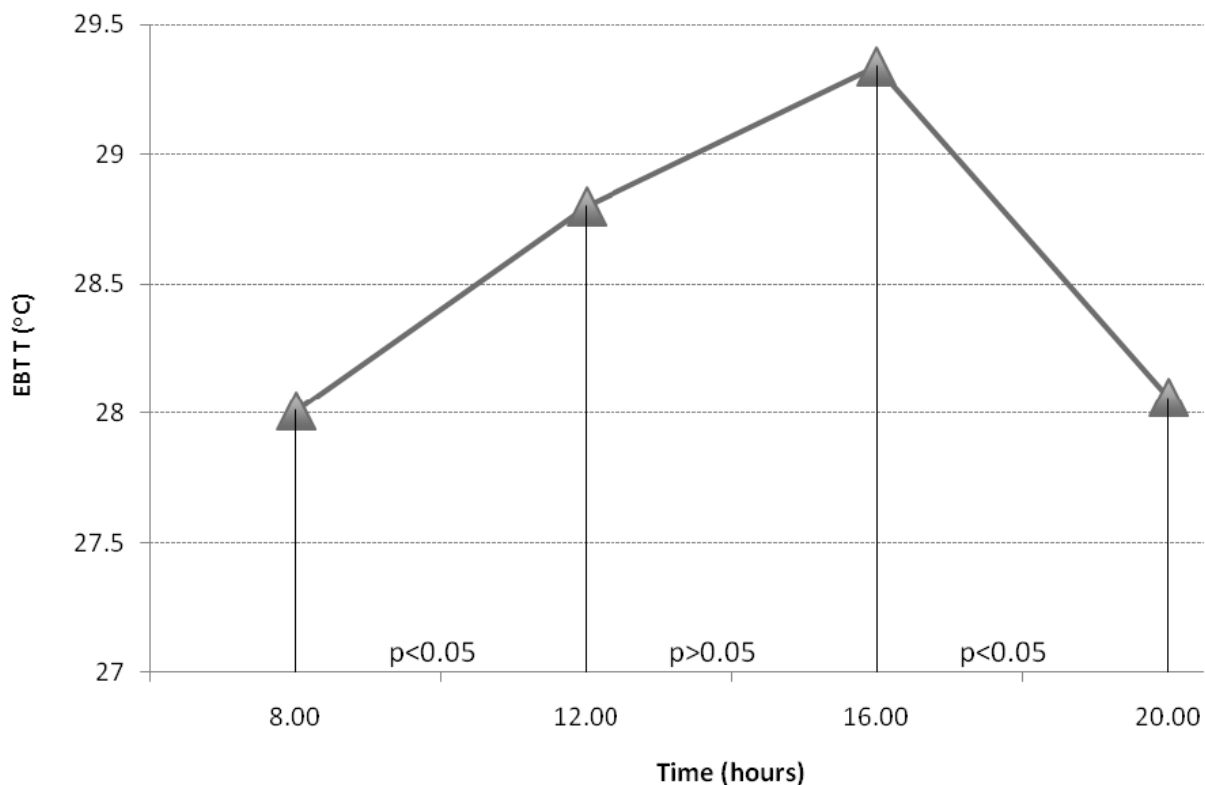


Fig. 1. Daily variability of the EBT (8:00 AM, 12:00 AM, 4:00 PM and 8:00 PM).

circadian rhythms of EBT in these categories of subjects are not surprising as inflammatory effects of hormones in females and the obese subjects and of cigarettes in smokers might influence EBT during the day.

The limit of this study is surely the low number of subjects enrolled. Furthermore, the cross sectional design of the study is limiting. Repeated circadian measures on different days might give important information. However, this is a preliminary validation study that might be confirmed on a larger population of healthy subjects and subjects affected by respiratory diseases and with repeated measurements during the week.

In conclusion, for the first time, we report different values of EBT during the day in healthy subjects that are influenced by the body's circadian rhythm changes in temperature, giving a contribution to the validation of this new non-invasive tool. We believe in the clinical potentialities of EBT and support further studies directed toward the completion of the validation process of this inflammatory marker toward its promotion to the bedside.

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LETTER TO THE EDITOR

MELANIN OF THE NIPPLE AREOLA COMPLEX

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Biological pigments or biochromes are ubiquitous in animals, plants, and simpler organisms such as fungi and bacteria. They serve a wide spectrum of functions from photosynthesis, camouflage, mimicry, photo protection from the environment to attracting mates. The human female nipple areola complex (NAC) is a highly-pigmented area. Currently, the prevailing theory as to the evolution of the pigmented human NAC is based on infant recognition of breast feeding latching zone; however, due to the protruding shape of the nipple and surrounding breast, the authors of this letter believe that the evolutionary advantage of the pigmented NAC has a direct physiological function, namely the initiation of involution at the end of the infant lactation period.

To the Editor,

Life on Earth depends on energy from the sun. The highest solar radiation found on the surface of Earth is ultraviolet radiation (UVR). UVR is divided into several subtypes based on wavelength range: UVA (315nm-400nm), UVB (280nm-315nm) and UVC (100nm-280nm). UVA and UVB radiation affect living tissue, while UVC being more energetic and potentially more harmful is almost entirely absorbed by the ozone layer. While UVR is essential to life on earth, it also exerts damage to living tissue. Humans evolved melanin, a biological pigment that absorbs up to 99% of UVB and reduces reactive oxygen species (ROS) formation in skin and other organs (1, 2).

Melanin is produced in melanocytes, neural crest-derived cells, by oxidation and polymerization of tyrosine. The three types of melanin are pheomelanin,

eumelanin, and neuromelanin. Various combinations of the different melanins are often present in the same organism. Besides in the skin, melanocytes are also present in the uveal tract of the eye (iris, choroid and ciliary body), inner ear and leptomeninges due to embryonic migration along the dorsolateral pathway via mesenchymes (3). In the skin, melanocytes located at the basal layer of the epidermis produce melanin and pack it for transport into melanosomes, specific membrane-bound organelles located within its cytoplasm, the function of which is synthesis, deposition and transport of melanin.

In healthy human skin, 1000-2000 melanocytes are present per square millimeter. In the epidermal basal layer, the ratio of melanocytes to keratinocytes is approximately 1 to 10. The ratio of melanocytes to keratinocytes can change depending on the body localization; for example, the ratio of melanocytes to

*Key words: melanin, melanogenesis, melanocytes, nipple, areola**Mailing address:*

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keratinocytes in the human areola is far greater than the surrounding breast tissue (1:9.7 vs 1:14.7) (4). In living organisms, melanin has many functions; for example, environmental photo-protection, camouflage to avoid predators, and sexual and reproductive behavior. Even though the evolutionary advantage of increased melanocyte keratinocyte ratio in the human nipple areola complex (NAC) remains unknown, NAC pigment expression is influenced by hormones as is evidenced in the higher incidence of melasma among pregnant women (5).

CONCLUSION

The influence of hormones on melanogenesis was demonstrated in animal studies. Snell et al. demonstrated that surgical removal of ovaries in guinea pigs resulted in reduction of melanin and shrinkage of melanocytes in the NAC, while subsequent treatment with estrogen increased melanin expression of the NAC (6). One explanation put forward by our group to the possible evolutionary advantage for melanin expression in the NAC is related to involution at the end of breastfeeding. ROS are potentially involved in the initiation of involution; hence, increased melanin during lactation (and darker NAC) serves as a negative feedback mechanism to halt involution (7, 8). After cessation of lactation, change in hormonal expression may contribute to reduced melanin in the NAC and subsequently increase in mammary ROS leading to involution (9, 10). In fact, this concept led us to hypothesize that women with darker NAC pigment are less likely to develop breast cancer. Our group plans to conduct a survey to investigate this effect which may elucidate novel targets for the treatment of breast cancer.

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LETTER TO THE EDITOR

SUBSTANCE P EXPRESSION IN THE GINGIVAL TISSUE AFTER UPPER THIRD MOLAR EXTRACTION: EFFECT OF KETOPROFEN, A PRELIMINARY STUDY

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The aim of this study was to evaluate substance P (SP) levels and the effect of a non-steroidal anti-inflammatory drug (NSAID), ketoprofen, on SP in the pericoronal gingival tissue after extraction of upper third molars. A sample of 20 young non-smoking systemically healthy adults of both sexes, with a healthy upper third molar to extract for orthodontic purposes, was selected. After extraction, a sample of the gingival tissue of the pericoronal region was collected with a sterile scalpel, placed into test tubes and kept frozen at -20°C until the SP determination. SP levels were determined by using a commercially available enzyme immunoassay (ELISA) kit. The subjects were randomly divided into two groups: group 1 received a single dose of ketoprofen 30 minutes prior to the experimental procedure. The subjects of group 2 did not receive any kind of drug administration before extraction. The patients were asked to complete a diary on the postoperative pain. A relevant amount of SP was measured in all the gingival samples. No statistically significant difference could be detected in SP expression between the two groups. In group 1 pain appearance was significantly delayed (6.2 ± 0.13 hours) in comparison with group 2 (3.95 ± 0.2 hours). In this small selected group of subjects and limited study design, preventive administration of ketoprofen did not significantly affect the gingival levels of SP, the clinical recommendation emerging is that of NSAID administration postoperatively but before pain appearance in order to optimize the management of pain of the patient.

To the Editor,

Findings suggest a potential role for substance P (SP) in the gingival tissues, and in particular in the resident fibroblastic cells and a relationship between the neuropeptide release and neurogenic inflammation (1). SP is produced in a subset of capsaicin-sensitive sensory peripheral neuron cell bodies localized in the dorsal root and trigeminal ganglia (2), and plays an

essential role in the transmission of noxious stimuli in the spinal cord. The stimulation of capsaicin-sensitive sensory peripheral terminal of the neurons results in the peripheral release of several neuropeptides including SP (3, 4, 5), and evidence supports in particular a role of SP in the development and maintenance of dental pain and inflammation (6). Previous findings have demonstrated how SP

Key words: substance P, gingival tissue, tooth extraction, Ketoprofen

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represents a key neuropeptide in the generation of neurogenic inflammation as in presence of caries (7), occlusal trauma (8), after cavity preparation (9), dentin-bonding agent application (10), after the application of tooth bleaching products (11) and in orthodontic movements (12). Several studies have shown that SP expression is significantly increased in the inflamed pulp, suggesting that SP plays a key role in the pulpal inflammatory process (13).

Non-steroidal anti-inflammatory drugs (NSAIDs) are medications used frequently all over the world. NSAIDs may adequately control postoperative symptoms after different dental procedures such as the removal of third molars. Effective analgesia provides the patient with a better quality of life in the postoperative period, allowing faster recovery and earlier return to their daily activities. Common forms of postoperative pain prevention include the preoperative administration of NSAIDs (14, 15). The degree of nociception of patients seems to be related in part to the concentration of histamine, kinines, and prostaglandins at the site of inflammation. The maximum concentration of prostaglandins in acute tissue injuries occurs simultaneously with the peak of postoperative pain intensity (3 to 4 hours after injury). NSAIDs are capable of limiting peripheral sensitization by reducing prostaglandin synthesis at the site of surgery, however, few studies have evaluated the role of NSAID administration on neurogenic inflammation (13). This study aimed to measure SP expression in the gingival tissue after third molar extraction and to evaluate the effect of preoperative ketoprofen administration, a non-steroidal anti-inflammatory drug (NSAID), on the quantity of SP in this tissue and on the degree of nociception of the patients.

MATERIALS AND METHODS

A sample of 20 (19-30 years old) systemically non-smoking healthy adults of both sexes was selected among the patients of the Department of Orthodontics of the University of Insubria, Varese, Italy. Inclusion criteria comprehended the presence of a healthy upper third molar programmed for extraction for orthodontic purposes. Written informed consent was obtained from

each patient. The study was performed according to the recommendations regarding ethical issues in research with human tissues of the University of Insubria of Varese, Italy, and was conducted in accordance with the Declaration of Helsinki. The subjects were randomly divided into two groups: group 1 received a single dose of 80 mg ketoprofen lysine salt (Oki® manufactured by Dompe CPA, Italy) in the form of sachet to dissolve in water, 30 min prior to the experimental procedure. The subjects of group 2 did not receive any kind of drug administration. Prior to extraction the teeth were anaesthetized (1.8 mL of 4% articaine with 1:100.000 epinephrine by infiltration injection); 15 min later a sample of the gingival tissue from the pericoronal region of the extraction site was collected with a sterile scalpel and placed into a test tube and kept at -20°C until the SP expression. The wound was then sutured and the patient was asked to complete a diary on the postoperative pain until the control appointment seven days later, in which time of appearance and recurrence of pain, necessity of use ketoprofen, and efficacy of the drug in controlling pain were analysed. A verbal pain intensity scale was used, where pain intensity was indicated as: no pain, slight, moderate, intense, severe (16). Before surgery, the patients were carefully instructed on the compilation of the diary, and were further interviewed at the control appointment about the modalities of compilation in order to check the effectiveness of pain records. For SP determination, gingival samples were weighed and 0.5 ml of Acetic Acid 0.5N, containing a protease inhibitor cocktail (Roche, Milano, Italy), was added to all samples which were then boiled for 10 min. Samples were homogenized and centrifuged at 10,000 x g for 10 min at 4°C. Supernatants were collected, dried with a Speed Vacuum Concentrator and resuspended in 0.3 ml of assay buffer. SP levels were determined by using a commercially available enzyme immunoassay (ELISA) kit according to the manufacturer's instructions (R&D system, Milano, Italy). Sensitivity of the kit was 15 pg/ml. Non-parametric Mann-Whitney U test was used to analyse differences in pain duration and in SP levels since normality test failed.

RESULTS

A relevant amount of SP was measured in all the gingival samples. SP concentrations, expressed as pg/

mg of tissue of each patient are reported in Tables I and II. As reported in Table III, no statistically significant difference was present in SP levels between the two groups. In Tables I and II also appearance of pain after extraction (expressed in hours), intensity of pain (classified by patients according to: no pain; slight; moderate; intense), time of reduction of pain after ketoprofen intake (expressed in minutes) for the patients who felt the necessity to take it, and the duration of pain relief (expressed in hours) are reported. Generally, patients experienced only slight-to-moderate pain, and only one patient described pain as intense.

In group 1, appearance of pain was significantly delayed (6.2 ± 0.13 hours) in comparison to patients who did not receive ketoprofen before the experimental procedure (3.95 ± 0.2 hours). This difference resulted statistically significant ($p < 0.0001$, Mann-Whitney U test), as reported in Table III. Half of the patients in group 1 felt the necessity to take a second ketoprofen dose after tooth extraction, while in group 2 all the patients decided to take the drug at the appearance of pain after the surgical procedure.

DISCUSSION

This study investigated the expression of SP and the effect of ketoprofen administration, a commonly used non-steroidal anti-inflammatory drug (NSAID), on the quantity of SP in the gingival tissue after upper third molar extraction. In the patients of group 1 a single dose of ketoprofen lysine salt ("Oki", Dompe CPA, Italy) was administered 30 minutes prior to extraction while in group 2 no drug was administered before the procedure. No statistically significant difference in SP expression could be detected between the two groups.

Caviedes and coworkers (17) reported an SP concentration of 0.453 pmol per mg of periodontal ligament of intact teeth; SP levels have also been investigated in the pulp and in the gingival crevicular fluid, however, no data is available on the pericoronal gingival tissues. The presence of increased SP levels could open new directions for treating inflammation and pain occurring after oral surgical procedures. In this study, considered preliminary due to the

Table I. Substance P and pain evaluation after tooth extraction of upper third molars, in patients with ketoprofen administration 30 minutes before the procedure.

Patients group 1	SP pg/mg gingiva	Appearance of pain (hours after extraction)	Intensity of pain	Onset to pain relief (minutes after second ketoprofen intake)	Duration of pain relief (hours)
1	14.03	7	slight	no drug	
2	147.17	6	moderate	30	> 5
3	39.1	6	moderate	30	> 5
4	18.61	6	slight	no drug	
5	77.72	7	slight	30	> 5
6	42.8	6	slight	no drug	
7	32.87	6	moderate	no drug	
8	15.24	6	moderate	30	4 - 5
9	43.62	6	slight	no drug	
10	21.99	6	moderate	30	3 - 4

Table II. Substance P and pain evaluation after tooth extraction of upper third molars in patients with no drug administration before the procedure.

Patients group 2	SP pg/mg gingiva	Appearance of pain (hours after extraction)	Intensity of pain	Onset to pain relief (minutes after ketoprofen intake)	Duration of pain relief (hours)
1	40.99	3	moderate	30	> 5
2	43	4.5	slight	30	> 5
3	36	4	moderate	30	3 - 4
4	89.67	5	moderate	30	4 - 5
5	14.26	5	slight	30	> 5
6	33.80	3	slight	30	3 - 4
7	46.44	4	moderate	30	> 5
8	10.69	3.5	slight	30	> 5
9	19.35	3.5	slight	30	3 - 4
10	52.0	4	intense	30	4 - 5

Table III. Pericoronal Substance P levels and time to appearance of pain.

	Group 1 (ketoprofen)	Group 2 (no drug)	Mann-Whitney U test
Gingival SP (pg/mg tissue) (mean±SEM)	45.31±12.81	38.62±7.18	p= 0.9
Appearance of pain (hours) (mean±SEM)	6.2±0.13	3.95±0.23	p< 0.0001

limited number of patients, Ketoprofen did not seem to interfere significantly on the SP expression in the pericoronal tissues after third molar extraction. Considering the efficacy of this molecule in the modulation of pain and inflammation in third molar surgery previously reported (18), this result may appear rather disappointing and could be interpreted as inability of the drug to prevent SP increase during surgical procedures even if the capacity to produce analgesia in the postoperative period is widely reported. These data are in accordance with a previous study evaluating SP levels in the pulp after induced lesion (13).

The data of the present study are in accordance with evidence (14) suggesting that there seem to be no basis for pre-emptive analgesia with various NSAIDs in the third-molar surgical model. No

prevention of postoperative pain was here reported, the only modification was a significantly postponed appearance of pain after tooth extraction with preventive ketoprofen administration (6.2±0.13 hours vs 3.95±0.2 hours). According to Kaczmarzyk and coworkers (19) ketoprofen was most effective when administered in the postoperative period. Liporaci Junior (20) showed no significant difference using ketoprofen for pre-emptive analgesia in surgical extraction of third molars. According to the data of this preliminary study, 5 patients of 10 of group 1 used the drug also postoperatively, 5 patients of 10 of group 2 took more than one dose of ketoprofen, so pre-emptive administration did not seem to result in reduction of pain and of quantity of painkillers used in the postoperative period.

In conclusion, ketoprofen does not seem to

interfere significantly on the SP expression in the pericoronal tissues after third molar extraction. Considering the efficacy of this molecule in the modulation of pain and inflammation in third molar surgery this result could be interpreted as inability of the drug to modulate SP during surgical procedures even if the capacity to produce analgesia in the postoperative period is widely reported. More studies with other molecules and performed on larger samples are necessary to evaluate the effect of NSAIDs on SP expression in the gingival tissues. In this small, selected group of subjects and limited study design the clinical recommendation that seems to emerge is that of NSAID administration postoperatively but before pain appearance in order to optimize the management of the postoperative pain of the patient.

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LETTER TO THE EDITOR

THE FINGERPRINT OF THE HUMAN GASTROINTESTINAL TRACT MICROBIOTA: A HYPOTHESIS OF MOLECULAR MAPPING

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The precise etiology of Inflammatory Bowel Disease (IBD) remains unclear and several factors are believed to play a role in its development and progression, including the composition of microbial communities resident in the gastrointestinal tract. Human intestinal microbiota are extensive with at least 15,000-36,000 bacterial species. However, thanks to the new development in sequencing and molecular taxonomic methodologies, our understanding of the microbiota population composition, dynamics, and ecology has greatly increased. Intestinal microbiota play a critical role in the maintenance of the host intestinal barrier homeostasis, while dysbiosis, which involves reduction in the microbiome diversity, can lead to progression of inflammatory disorders, such as IBD and colorectal cancer. It is hypothesized that fingerprinting characterization of the microbiota community composition is the first step in the study of this complex bacterial ecosystem and a crucial step in the targeted therapy. Molecular fingerprinting of human gastrointestinal tract microbiota could be performed by different techniques including the semi quantitation, 16SrRNA, the DNA- microarray as well as other relatively new methods which were developed to study many complex bacterial ecosystems. These techniques provide individual data and profiles, using fast and sensitive tools for the high taxonomic level fingerprint of the human intestinal microbiota and provide estimation of the relative presence of the microbial target groups within each individual. Such personalized information serves as a remarkable and unprecedented opportunity to improve targeted medical treatment and probably develop strategies to prevent disease.

To the Editor,

The human normal flora, or microbiota, is extensive, both in its absolute quantitative mass and in its qualitative diversity. The human intestinal tract hosts about 100 trillion microorganisms with at least

15,000-36,000 bacterial species. The microbiome is now considered to be a functional organ involved with multiple physiological functions. On the other hand, many pathological conditions arise in dysbiosis, when the microbiome is drastically altered. IBD

Key words: microbiota, dysbiosis, inflammatory bowel diseases, fingerprint microbiota

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is one of those conditions which clearly involves a breakdown in the healthy interactions between the host and the microbiome. A dramatic reduction occurs in the phylum Firmicutes and concomitant increase in Proteobacteria (1).

The vast majority of bacteria in the human intestinal microbiota (>99%) belongs to the six bacterial phyla: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria and Verrucomicrobia. However, the two dominant divisions are Firmicutes (65%) and Bacteroidetes (25%) of total microbiota. The intestinal microbiota of each individual have a specific complement of hundreds of genera and thousands of bacterial species (1). Conventional microbiological techniques fail to give a detailed inventory of the normal microbiota, but the development of recent high-throughput sequencing and high level molecular taxonomic methodologies have greatly increased our understanding of the microbiota population composition, dynamics, and ecology (2).

The composition of intestinal flora is remarkably stable at different anatomic locations along the gut, but absolute numbers vary greatly, ranging from 10^{11} cells/g in the ascending colon to 10^7 - 10^8 in the distal ileum, and 10^2 to 10^3 in the proximal ileum and jejunum (1, 3).

It is also well documented that intestinal microbiota play a critical role in the maintenance of host intestinal barrier homeostasis by exerting beneficial effects on diverse physiological intestinal functions while dysbiosis can lead to progression of inflammatory disorders such as inflammatory bowel disease (IBD) and colorectal cancer (CRC) (4, 5).

A well balanced gut microbiota promotes the health of colocytes through the production of a variety of important compounds and the correct modulation of the immune system (2).

Along this line, probiotics (PBs) have been used, and some studies have suggested three mechanisms for their action in IBD therapy they may: i) block the pathogenic bacterial effect and compete with pathogenic toxins for adherence to the intestinal epithelium; ii) enhance innate immunity and modulate inflammation through their regulated signaling pathways via their interaction with Toll-like

receptors, and iii) enhance intestinal barrier function and thus regulate intestinal epithelial homeostasis. Moreover, PBs modulate different host cell signaling pathways such as Akt, mitogen-activated protein kinases and nuclear-kappa β that could potentially mediate intestinal epithelial functions (6).

Some bacteria, such as *Lactobacillus delbrueckii* and *Lactobacillus fermentum* have been documented to ameliorate the inflammatory background. In fact, clinical studies on ulcerative colitis patients showed significant amelioration of the inflammatory state by decreasing the colonic concentrations of multiple pro-inflammatory mediators and reducing myeloperoxidase activity compared to controls. Furthermore, re-establishment of microbial homeostasis and manipulation of microbiota in IBD treatments, through fecal microbiota transplantation of selective bacteria, have been practiced and are gaining great interest (7).

Based on this background and the authors experimental and clinical experiences, we propose the following working hypothesis: the characterization of the microbiota community composition is the first step in the study of a complex bacterial ecosystem. Fingerprinting of IBD patients' microbiota and its mapping, using various molecular techniques, could serve as a very crucial and essential step in the targeted therapies of IBDs. The metagenomic sequencing data provide new insight into the importance of the microbial network and its interaction with the host genome, which could be therapeutically important, as early targeted intervention can restore normal flora (8).

MATERIALS AND METHODS

Gut microbiota have been extensively investigated by anaerobic culture techniques, but it is now well known that culture-based methods provide only an incomplete picture of the overall diversity of microbiota (9).

However, further techniques have been successively developed to explore microbial biodiversity. In fact, the composition of the adult intestinal microbiota has been determined in 3 large scale 16SrRNA sequence surveys. Other methods, such as the High Taxonomic Fingerprint (HTF-Microbi.Array) and the Ligase Detection Reaction-

Universal Array (LDR-UA) approach have been recently extensively used. The HTF Microbi-Array enables specific detection and approximate relative quantification of 16S rRNA from 30 phylogenetically-related groups of human intestinal microbiota. This technique was applied to adult human subjects with high reproducibility of the high level taxonomic microbiota fingerprint for each individual (8, 10-12). Other techniques are also being employed such as:

i. Denaturing/temperature gradient gel electrophoresis (D/TGGE) which is used to separate proteins or nucleic acids (DNA and RNA) across a temperature gradient. TGGE is based on the principles that the structure of proteins and DNA changes with temperature and that these changes can be characterized by their movement through a gel. It is often associated with Polymerase Chain Reaction (PCR) analysis.

ii. The strand conformation polymorphism (SSCP) analysis is a simple and sensitive technique for mutation detection and genotyping. The principle of SSCP analysis is based on the fact that single stranded DNA has a defined conformation. Altered conformation due to a single base change in the sequence can cause Single-Stranded DNA to migrate differently under a non-denaturing electrophoresis conditions.

iii. Fluorescent *in situ* hybridization (FISH) is a cytogenetic technique which uses fluorescent probes binding parts of the chromosomes to show a high degree of sequence complementarity. This technique provides a novel way for researchers to visualize and map the genetic material in an individual cell or bacteria, including specific genes or portions of genes.

iv. Terminal restriction fragment length polymorphism (T-RFLP) analysis is used to study complex microbial communities based on variation in the 16SrRNA gene. This technique can be used to examine microbial community structure and community dynamics in response to changes in different environmental parameters or to study bacterial populations in natural habitats. It has been applied to the study of complex microbial communities in diverse environments, such as soil, marine, oral bacterial flora in saliva in healthy subjects versus patients. RFLP is culture independent, rapid, sensitive and reproducible without the need for any genomic sequence information.

v. Real time polymerase chain reaction (PCR) is a powerful method for amplifying particular segments of DNA, distinct from cloning and propagation within the

host cell; this technique uses the oligonucleotide as a primer and elongates its 3' end to generate an extended region of double stranded DNA.

vi. Human Intestinal Tract Chip (HIT chip) is a 16SrRNA gene-based diversity microarray which was developed and validated for the characterisation of human gut microbiota. It is an oligonucleotide microarray commonly used for phylogenetic profiling (13, 14).

RESULTS

Thanks to such advances of the 16S ribosomal RNA genomic sequencing, a decrease in Bacteroides and Firmicutes numbers in the colon and an increase in Enterobacteriaceae, such as adherent-invasive *E. coli* and other Proteobacteria, have been detected in Crohn's disease (5, 12).

Moreover, the HIT Chip technology, which is a 16SrRNA gene based diversity for phylogenetic profiling of human intestinal tract communities (15), and the pyrosequencing have been developed to serve as a more economic sequencing approach than high-coverage amplicon sequencing, to study many complex bacterial ecosystems.

The data emanating from many techniques intersect, and could sometimes be complementary in determining microbial community profiling. Using the HIT Chip hybridization method has shown strong concordance with the pyrosequencing-based profiling data and sequencing of 16S rRNA gene clone libraries (4, 16).

In brief, microbiota fingerprinting in IBDs is a feasible and useful cost effective approach that could lead to a targeted therapy or even prevention through supplying missing bacterial species through probiotics or other.

DISCUSSION

It is well established that inflammatory pathologies affecting the gastrointestinal tract are narrowly correlated to dysmicrobism (2). However, the question frequently asked for more than a decade is whether dysbiosis is a cause of IBDs or just a secondary phenomenon. In this context, could a molecular mapping or fingerprinting of intestinal

microbiota provide convincing evidence regarding its role in causing, maintaining and/or determining the severity of IBDs?

Earlier studies, whether experimental in animals or clinical in patients, are in support of such an approach. Saitoh et al. in 2002, as well as others, performed mucosal bacterial isolation in IBD patients and showed increased concentrations of *Bacteroides vulgatus* and *Enterobacteriaceae*, especially *E.coli*, and decreased concentrations of Bifidobacteria species (17).

In addition, Bull et al. in 2003 demonstrated, in mucosal specimens of CD patients, a highly decreased amount of *Faecalibacterium prausnitzii*, thought to predict a high risk for early reactivation of CD (18).

However, data also proved that administration of *Lactobacillus casei* and *Bifidobacterium lactis* in mice with TNBS induced colitis, resulting in a significant reduction of inflammation in the colonic mucosa (19). Thus, they reversed malignant changes and exerted a potential role in colorectal cancer management and prevention.

In addition, IBD treatments based on microbiota manipulation are gaining great interest in clinical practice. In this context, IBD management is being carried out by fecal microbiota transplantation from a healthy donor to IBD patients; by infusion of liquid stool suspension, re-establishment of microbial homeostasis has been demonstrated by significant increases in bacteroidetes and clostridium as well as decreases in proteobacteria according to healthy donor profiles. Such profiles are best determined by fingerprinting. More than 100 trillion microbes, which we host in the human microbiota to a great extent, can identify us as individuals much like fingerprints. Using publicly available data produced through the Human Microbiome Project (HMP), researchers examined microbes from 242 individuals over a 9-month period. Then they applied an adapted version of a classic computer science algorithm to scan the microbial genetic code and search through different sequence features for patterns that are unique to each individual. To do so, they combined stable and distinguishing sequence features from the individual's initial microbiome samples and

compared them both to samples from the same individuals collected at follow-up visits and samples collected from control group individuals. Researchers found that the "codes" were unique among hundreds of participants and that also a number of them (more than 80%) remained stable over the sampling period of one year. In some ways, it could be said that they were able to identify "microbiome fingerprints". In brief, there are many aspects that are the same for many people but the existing differences are enough to characterise each individual.

Nowadays, it is possible to perform a comprehensive microbiota assessment of an individual, including DNA and RNA sequence, and at least some characterization of proteome, microbiome and epigenome. It has become abundantly clear that each of us actually has a one-of-a-kind biological content. Analysing the large volume of microbial gut, based on the whole genome sequencing (WGS) and preparing high quality representative RNAs for sequencing to generate metatranscriptome along with the establishment of the gut microbial interaction network model, is a major challenge (12).

Well beyond the allure of the matchless fingerprint or snowflake concept, these singular individual data and information set up a remarkable and unprecedented opportunity to improve medical treatment and develop preventive strategies for disease (13).

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LETTER TO THE EDITOR

**CARDIOPATHY AND OSTEOPOROSIS:
THE EPIDEMIOLOGY IN A REGION OF ITALY**A. NOTARNICOLA¹, G. MACCAGNANO¹, L. MORETTI¹, S. TAFURI² and B. MORETTI¹¹*Orthopaedics Unit, Department of Basic Medical Science, Neuroscience and Sensory Organs, Faculty of Medicine and Surgery, University of Study of Bari, General Hospital, Bari, Italy;*²*Department of Biomedical Sciences and Human Oncology, Faculty of Medicine and Surgery, University of Study of Bari, General Hospital, Bari, Italy**Received June 25, 2016 – Accepted November 28, 2016*

Cardiopathies and osteoporosis are inter-related due to pathogenetic, hormonal, genetic features as well as an increased risk of fragility. An important feature is attributed to the process of atherosclerosis, which is responsible for an osteopenia effect and degeneration of vascular walls. To date the study populations have been limited. To verify the incidence of cardio-vascular disease in a larger osteoporotic population, we designed a retrospective clinical study analyzing the “Hospital Discharge Data” (HDD) in Apulia between 2006 and 2010. All patients over 55 years with a hospitalization for a fragility fracture and/or drugs prescription for osteoporosis were crossed with the diagnosis and/or drugs prescription for cardiovascular disease. We observed that between 2006 and 2010, in Apulia, 177,639 patients were hospitalized and diagnosed as having fragility fractures, 66.3% had a diagnosis of cardiopathy, with a higher prevalence in males and in patients over 80 years. The incidence of fractures were as follows: femur (51.9%), spine (20.2%), humerus (10.6%), forearm (9%), tibial pilon (7.2%) and tarsus and metatarsus (1.1%). Cerebrovascular diseases were the most frequent, followed by arrhythmias, heart failure and cardiomyopathies. In these patients, the most prescribed drugs were anti-coagulants, ACE inhibitors and diuretics. In patients affected by cerebral circulation disorder there is a greater propensity to fall and thus have a fragility fracture, particularly of the femur. The vertebral fracture, misdiagnosed in 60-70% of patients, may compromise the cardio-respiratory function of these patients. We verified a higher incidence of fragility fractures in patients who were prescribed certain categories of drugs for the treatment of cardio-vascular disease. This hypothesis is not supported by the literature, where contradictory results on the potential effects of these drugs on bone have been published. The high incidence of heart disease found in patients with fragility fractures supports the need for specific screening for osteoporosis in the population with cardio-circulatory pathology.

To the Editor,

Heart disease and fragility fractures are two important and frequent health problems (1). In Italy the prevalence of people affected by cardiovascular

disability is equal to 0.4% (ISTAT). In the last ISTAT report, the osteoporosis subjects represented 4.7% of the total population and 17.5% of people over sixty-five; these percentages seem underestimates

Key words: heart disease, vascular disease, fragility, fracture, osteoporosis, drug, epidemiology

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considering the 465,000 fragility fractures/year. Osteoporosis and cardiopathy have common risk factors, such as age, gender, smoking, diabetes mellitus type 2 (2), justifying the coexistence of these two diseases. Cardiopathies have usually been seen as a cause of osteoporosis. However, we hypothesize an inverse correlation between osteoporosis and heart disease. Each reduction of one standard deviation of bone mineral density is associated with an increase of 1.3-2.3 times of cardiovascular mortality (3).

Pathophysiology, genetics and increased risk of falls for patients with a heart condition may warrant an acceleration of the mass loss and the increased incidence of fragility fractures. Studies in the literature regarding these are limited due to the small sample size. With regard to pathophysiology, in a case series of 101 patients affected by heart disease a high incidence was found of low levels of vitamin D (26%), high levels of parathyroid hormone (30%) and low densitometric values (respectively 42% and 43% at the femoral and vertebral level) (4). In another series of 18 patients with heart disease, a high level of aldosterone was identified, which may explain an increased excretion of calcium and high PTH levels (5).

Furthermore, genetic features may have a role. Bone and vascular systems are regulated by common factors, in which the calcification of the vascular walls reproduces the process of bone formation (6). A study of 31,936 Swedish twins supports the hypothesis that the same genes are involved in common cellular mechanisms of the vascular system and bone which predisposes the individual to heart disease and osteoporosis (7).

The increase of fall risk is frequent in hemiplegic patients with sarcopenia, and for long periods of immobilization. After hospitalization for stroke, the risk for fracture increases 7-fold within the first year (8). To our knowledge, there are no studies on large populations in the same geographic area that analyze the prevalence of heart disease in patients with fragility fractures.

MATERIAL AND METHODS

Patients

The Local Ethics Committee gave approval to carry out a clinical retrospective observational study. A survey was carried out of patients admitted to the Orthopedics departments of hospitals in a southern region of Italy: Apulia. Apulia has a population of 4,090,266 inhabitants (Istat Data of 31 December 2013). The HDD (Hospital Discharge Data) database was checked for the period 2006–2010 to identify patients with a fragility fracture diagnosis that required hospitalization. For this retrospective study male patients were enrolled who were over 65 years of age and female patients over 50 years whose HDD reported domestic accidents in the “trauma field” and who did not die in hospital.

The absence of specific codes for fragility (mainly osteoporotic) fractures results in a lack of perception of fracture burden, leading to problems in the exact evaluation of osteoporotic fractures in the elderly.

The database of the drug prescriptions was also checked to identify those subjects who took at least one of the drugs for osteoporosis and calcium and vitamin D integrator.

The database of patients with fragility fracture or osteoporosis drugs prescription were cross-checked by using the individual health care code to eliminate the same patients being reported twice. The resulting database provides the prevalent population for the study. The proportion of subjects with osteoporosis and cardio-circulatory disease was the main study outcome. This was ascertained by performing a data-linkage between the population and the HDD database and drug prescriptions.

The following subjects were classified as a diabetic cardio-circulatory disease patient:

- every subject of the prevailing population who was admitted to hospital for a main cause correlated with cardio-circulatory disease;

Table I. *Frequency of fragility fracture.*

year	Freq.	Percent
2006	61,566	34.66
2007	30,945	17.42
2008	29,262	16.47
2009	28,369	15.97
2010	27,497	15.48
Total	177,639	100

- every subject with at least one prescription of cardio-circulatory disease drug ascertained through data-linkage with the regional archive of pharmaceutical prescriptions.

Data were stratified by decade of chronological age (50-59 years, 60-69 years, 70-79 years, ≥ 80 years) and gender (male and female).

RESULTS

In the period 2006 to 2010 in Apulia 177,639 patients had been diagnosed with fragility fractures with the highest number in 2006 (34.66%); in the following years the percentage progressively decreased, as can be seen in Table I. The analysis of fragility fracture data according to decade of age showed the greatest incidence to be in the 70-79 age group (64,917) and the lowest in the 50-59 age group (17,260) (Table III). Women were vastly more affected by fragility fracture compared to men (41,943 vs 1,867) (Table II).

In the prevalent population, 66.3% were found to be affected with cardio-circulatory disease, of whom 112,632 were taking drugs (table III). This condition tended to increase with age passing from 37.6% for the 50-59 years age group to 82.6% for the over-80 years group (Table II). The data demonstrated a greater prevalence of cardio-circulatory disease in males with fragility fracture (80.7% males vs 64.8% females) (Table II). The sites most affected were femur, spine and humerus.

The most frequent diagnosis of cardio-circulatory disease was circle cerebrovascular disorder (4,023),

followed by arrhythmias (1,380), heart failure (1,071) and cardiomyopathies (117). Data show that drugs for cardio-circulatory disease mostly assumed in osteoporotic and/or fragility fracture patients were anti-coagulants, ACE inhibitors and diuretics (Table III).

DISCUSSION

In the present study, in the cohort of Apulia patients over 55 years of age affected by fragility fracture, a mean of 66.3% also complained of heart disease. The prevalence was higher in the male gender and in the over 80s. For years, osteoporosis and Cardio-Vascular Disease (CVD) were thought to be independent chronic diseases that increased markedly with advancing age. However, increasing evidence now supports a direct association between these chronic conditions. In respect to CVD, men are more prone to atherosclerosis (approximately

Table III. *Cardiologic drugs assumed by patients with fragility fractures.*

Cardiologic drugs	Patients affected by fragility fractures
Anti-coagulants	29,981
Regulator of renin angiotensin system	24,196
Diuretics	18,556
Cardiologic therapy	10,683
Beta-blocker	12,623
Calcium-antagonist	12,623
Anti-hypertensive drugs	3,958
Vasodilatory drugs	12
Total	112,632

Table II. *Frequency of fragility fracture according to age and gender.*

Age group	M	F	Total
50-59	-	17,260	17,260
60-69	1,867	41,943	43,810
70-79	7,923	56,994	64,917
≥ 80	6,903	44,747	51,650
Total	16,693	160,946	177,639

3–4 fold greater) than women (9). We verified that the incidence of fractures in relation to anatomic sites of fragility fracture. This incidence is higher for femur (51.9%) followed by spine (20.2%), humerus (10.6%), forearm (9%), tibial pilon (7.2%) and tarsus and metatarsus (1.1%). The proximal part of femur is still the main bone involved in terms of incidence, as in the general population. Vertebral fractures deserve special attention, as they are asymptomatic and underestimated in 60–70% of cases (10).

A higher incidence was found of osteoporotic fractures in patients with cerebrovascular disease, followed by diagnosis of arrhythmias, heart failure and cardiomyopathies. In the literature, Authors report that the osteoporotic patient has a high risk of other associated co-morbidities, such as diabetes, hypertension, hyperlipidemia, chronic kidney failure, COPD, asthma (11). The higher incidence of fragility fractures in patients with vascular circulation disorder suggests that the etiopathogenesis is due to a common process of atherosclerosis (12). Low densitometric values are associated to greater calcium deposits in coronary artery and calcific aortic atherosclerosis (13).

Furthermore, a greater distribution was verified of fragility fractures in patients taking anti-coagulant drugs, ACE inhibitors and diuretics. Less frequent are fragility fractures in patients taking beta blockers or calcium channel blockers. In the literature, the data analysis is inconsistent. On the one hand the treatment with thiazide diuretics, statins, digoxin, angiotensin-converting enzyme (ACE) -inhibitors, and organic nitrates have not been associated with harmful effects on bone. Indeed, treatment with these drugs may improve bone strength (14). On the other hand, some Authors show continuous use of ACE inhibitors is associated with a significant increase in the average rate of BMD loss at the total hip and trochanter over 4 years (15). Other articles report increased risk of osteoporotic fractures by taking furosemide and vitamin K antagonists, ACE inhibitors, and a reduced risk for beta-blockers and statins (16–19).

The main strengths of the present study are its large cohort size and the possibility of collecting data of both hospitalization for fragility fracture and

prescription of medicaments throughout the region. The weaknesses include potential selection bias (e.g. misclassification of fragility fracture due to the absence of a specific code). Some coding errors probably occurred.

In accordance with findings from literature and our study of a correlation between cardio-circulatory disease and osteoporosis, we suggest a close monitoring of risk in osteoporotic patients with heart disease. The prevention of a fragility fracture in the cardiac patient can guarantee an improvement in expectation and quality of life. Prospective clinical studies will verify the results of specific treatment strategies.

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LETTER TO THE EDITOR

**HELICOBACTER PYLORI IN PERIODONTAL POCKETS AND SALIVA:
A POSSIBLE ROLE IN GASTRIC INFECTION RELAPSES**

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It has been a long time since the scientific community started to speculate upon the presence of *Helicobacter pylori* (HP) in periodontal pockets as an extra-gastric reservoir responsible for gastric relapses after eradication therapy. The aim of this study is to evaluate the presence of oral HP in a group of patients who underwent examination for gastric infection. Sixty patients were enrolled in the current study, subdivided into two groups: 30 patients with a positive result for HP gastric infection with C-Breath Test Urea examination, and 30 patients with a negative result for HP gastric infection. Crevicular fluid and salivary samples were collected in a sterile tube and then sent to the laboratory for evaluation. Specimens were processed to quantify the levels of HP and bacterial load by real time PCR technique. Even though there was no statistically significant difference among the two groups (A vs B) with regard to the total amount of HP in saliva or in periodontal tissues, this study demonstrates that the oral cavity is an extra-gastric reservoir of HP when it is affected by periodontal disease, and that periodontal disease is correlated to gastric HP infection.

To the Editor,

Helicobacter pylori (HP) is a widespread bacterium, which is found in water and in some animal biological fluids, and is responsible for gastric diseases, such as chronic gastritis, acute gastritis, peptic ulcer and gastric cancer (1). In 1979, Warren described the presence of a bacterium in the superficial epithelium from several gastric biopsies (2). This discovery was sensational, since, until then, the possibility of a bacterial proliferation in the stomach had always

been thought to be impossible, and the gastric lumen was dogmatically considered as fully sterilized by gastric acids. Warren was opposed by the medical community, but he was so strongly convinced that his own theory would be effective that he infected himself by ingesting bacterial cultures, developing a well-documented gastritis that was successfully treated. In 1989 Goldwin called the bacterium *Helicobacter pylori* (4).

HP is a Gram-negative, microaerophilic bacterium,

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which shows a helicoidal shape in its active form, while, on the contrary, it exhibits a coccoid shape during latency. It is 2.5-5 x 0.5 mm long and has 5-6 unipolar flagellae, and shows intense catalase, oxydase and urease activities. The prevalence of HP infection has changed in recent years and has diminished with peptic ulcers and gastric cancer. However, about half of the world population is still infected, with approximately one-third in Western countries and over 50% in Southern and Eastern Europe, South America and Asia (5). In developing countries, the infection rate is between 70 and 90% and is generally acquired during infancy, while in Western countries it is between 25 and 50% during infancy (6). In developed countries, upper-class subjects with higher economical standards of living are less affected by this infection, while it seems that individuals who emigrated from countries in which the infection is diffuse are more affected. This is due to the fact that one main way of transmission seems to be oral and/or oro-foecal, thus the lack of primary hygienic services and clean water play an important causal role. The second way of transmission seems to be linked to the use of infected endoscopes or instruments. Geographic factors, ethnic background and age greatly influence the epidemiology of the infection, but a low social and economical status represents the key factor in many cases (7).

HP infection is typically associated with active chronic gastritis, non-ulcer dyspepsia, multifocal gastric atrophy and intestinal metaplasia, gastric adenoma and gastric lymphoma.

In 1994, the WHO classified HP as a cancerogenic agent, and the IARC defined it as a first-class carcinogenic agent (8). Nowadays, diagnosis is performed through non-invasive procedures, like C-Breath Test Urea (C-BTU), which measures urease activity in breath (9). Relapses after eradication therapy are not uncommon. This leads to the hypothesis of a possible extra-gastric reservoir of infection. Apart from the stomach, HP has also been found in the distal esophagus, proximal duodenum, colonic contents, and the oral cavity, including tonsil and adenoid tissue (10). The presence of HP in the oral cavity was discovered for the first time in 1989 (11). Following studies diagnosed the presence of HP

in dental plaque, saliva, tongue, tonsil tissue and root canals (12). Some evidence-based studies suggested that the oral cavity may be a potential reservoir for HP (13, 14). A meta-analysis published in 2011 reported a strong association between the presence of HP in the oral cavity and in the stomach (15). However, triple eradication therapy has no or little effect on oral HP elimination; studies have recorded eradication rates for oral infection below 40% (16). The presence of oral HP might diminish the effect of eradication therapy and acts as a causal element in the recurrence of HP infection.

It has been a long time since the scientific community started to speculate on the presence of HP in periodontal pockets as an extra-gastric reservoir responsible for gastric relapses after eradication therapy. The international literature reports are very controversial.

Some papers highlight the presence of HP especially in patients with low oral hygienic conditions (17), while other authors have pointed out the presence of the bacterium in the oral cavity in association with dyspeptic symptoms (18). On the contrary, other authors have rejected the correlation between oral cavity and gastric relapses, claiming that HP in the oral cavity is due to gastro-esophageal reflux disease or contaminated food intake (19). Unsuccessful eradication of HP from the oral cavity has been shown because of the presence of dental plaque biofilm, which is a very effective barrier against antibiotics and drugs. Only periodontal therapy, through dental debridement can eliminate resident bacteria, including HP (20). Consequently, periodontal therapy plus systemic therapy could prevent gastric relapses more than systemic therapy alone (21).

The aim of this study is to evaluate the presence of HP in a group of patients who underwent examination for gastric infection. Firstly, the presence of oral HP in patients with negative diagnosis of gastric infection was compared with patients with a positive diagnosis. Then, the role of periodontal health as a predisposing factor for oral HP infection was thoroughly investigated, comparing the HP loads in a group of patients affected by periodontal disease with those in a group of patients periodontally

healthy. Finally, the main purpose of this research is to ascertain whether the presence of HP within oral tissues can represent an extra-gastric reservoir able to contribute to gastric relapses after systemic therapy in a significant way. This paper contributes new research on the role of the oral cavity as an extra-gastric reservoir of HP which could be a determining factor in gastric HP infection relapses. The study was carried out at the Department of Medicine and Surgery, University of Insubria, ASST dei Sette Laghi, Unit of Gastroenterology, Varese, Italy. The experimental protocol was reviewed and approved by the local ethics committee. The experimental protocol was evaluated by the Institutional review Board.

A total of 60 patients, 36 females and 24 males, were enrolled in the study. Ages ranged between 16 and 78, with a mean value of 52.89 ± 14.67 . The patients were divided into two groups: 30 with a positive result for HP gastric infection with C-UBT examination (group A), and 30 patients with a negative result for HP gastric infection (group B). Inclusion criteria were the presence of compliance and general good health, age over than 16 years, and the absence of gastric tumours or resection; exclusion criteria concerned the intake of antibiotics or proton-pump inhibitors for 60 days prior to the breath test. An informed consent was read, understood and signed by all patients. The patients swallowed urea labelled with an uncommon isotope, radioactive carbon-14, and 30 min after ingestion the detection of isotope-labelled carbon dioxide in exhaled breath indicated that the urea was split and whether the urease was present in the stomach, and hence if HP bacteria are present. A dental clinician performed periodontal examination by dental probing, recording the deepest probing for every quadrant and classifying patients as periodontally affected when the mean probing value was ≥ 3 mm, or as periodontally healthy when < 3 mm. Afterwards, a paper tip was inserted for 30 s into the gingival sulcus of the dental element with the deepest probing value registered for each quadrant and then sent in a sterile tube to the laboratory for evaluation. Saliva was collected through the spitting method for 1 min in a sterile tube. Specimens were processed to extract and purify DNA by using a method that

includes two consecutive phases of incubation with lysozyme and proteinase K. Once extracted, DNA was purified through a silica spin-column (Sigma-Aldrich, St. Louis, MO, USA). Quantitative PCR of 16S rRNA genes was performed with the hydrolysis probes method to identify and evaluate both the amount of HP and the total bacterial load. Reference sequences of 16S rRNA gene were from the Human Oral Microbiome Database (HOMD 16S rRNA RefSeq Version 10.1). All sequences were aligned to find either a consensus sequence or less preserved spots, useful to optimize the specificity of primers and dual labelled hydrolysis probes. Absolute quantification assays were performed using a 7500 Sequence Detection System (Applied Biosystems). The thermal cycle included 10 min incubation at 95°C to activate polymerase, followed by a two-step amplification of 15 s at 95°C and 60 s at 57°C for 40 cycles. Each experiment included non-template controls to exclude reagents contamination and serial dilutions of the specific synthetic template (Eurofin MWG Operon, Ebersberg, Germany). To obtain standard curves, plasmids containing bacterial target sequences were used (Eurofin MWG Operon, Ebersberg Germany). The plasmid concentration was calculated by using the Thermo Scientific Nanodrop spectrophotometer. Standard curves were created with serial dilutions between 10^7 and 10^1 plasmids copies. The total quantification of absolute bacterial charges determined the relative amount of HP. Under 10 U the results were considered as negative. Comparative statistics among the different groups was performed by using the IBM SPSS version 20.0, SPSS Inc, Chicago, IL, USA.

Firstly, a Student's *t*-test was performed to compare oral HP load in saliva and periodontal pockets between group A and group B. Afterwards, a Student's *t*-test was used to compare periodontal HP load in patients affected by periodontal disease with those who looked like periodontally healthy. Finally, a Student's *t*-analysis was performed to verify whether salivary HP loads were associated to periodontal scores, therefore demonstrating whether a pathway for gastric infection from gums through swallowing was present.

Of the 24 male patients, 10 tested positive and

14 negative for Breath test, while among females, 20 tested positive and 16 negative. Ages ranged between 21 and 76 among negative patients (mean age 53.07 ± 15.72) and between 16 and 78 among positive patients (mean age 52.7 ± 13.81). Salivary samples values ranged from 0 to 103 units, with a mean value of 11.73 ± 20.08 . Of these, 38 tested negative because they showed a value lower than 10 U, while 22 tested positive. Student's *t*-test for independent variables was used to compare the absolute values of HP detected in saliva within the two groups of positive and negative Breath test. There were not statistically significant differences ($p=0.860$). The same test was conducted for every quadrant in comparison with group variables of the Breath Test (group A vs group B). There was no statistically significant difference regarding the presence of HP in saliva among the two groups of patients ($p=0.967$; $p=0.123$; $p=0.293$ and $p=0.545$). Afterwards, the statistical analysis focused on the presence of HP in saliva and periodontal pockets following a new grouping based on the periodontal health conditions. The mean probing depth (PD) was calculated for every patient, and a patient was considered to be healthy from a periodontal point of view when their PD was under 3, while he/she was affected by periodontal disease when his/her PD was ≥ 3 . Thirty-one patients tested negative for periodontal disease (healthy), and 29 tested positive (unhealthy). This grouping procedure did not take into account the results from Breath Test. The mean load of HP registered among the periodontally healthy patients was 10.52 with SD 16.51, while it was 22.78 with SD 55.96 among affected patients. A Student's *t*-test for independent variables was performed to compare the mean load of HP in each patient with the presence or absence of periodontal disease. The result was statistically significant ($p=0.24$), demonstrating a correlation between periodontal disease (mean probing depth probing ≥ 3 mm) and the presence of HP in periodontal tissues. Another statistical examination was conducted in order to verify whether there was any difference within the group of periodontal affected patients subdivided into positive and negative to Breath test. There was no statistical significant difference ($p=0.102$).

The last analysis regarding HP load in the saliva was conducted between periodontal patients and healthy patients. There was a significant difference between the two groups: periodontal patients showed an increased level of HP in saliva compared with healthy patients ($p=0.05$).

The current study represents a preliminary approach to investigate the role of oral HP in the relapses of gastric infection.

Sixty patients were examined, subdivided into two groups of 30 individuals each according to the Breath test results: 30 were positive (group A) and 30 were negative (group B). Every patient underwent a thorough periodontal examination by a dental clinician. A paper tip was inserted for at least half a minute into the gingival sulcus near the dental element with the deepest probing depth. In addition, a salivary sample was collected with the spitting method.

The quantitative real time PCR technique allowed to evaluate the levels of HP in the oral cavity. Even though there was no statistically significant difference among the two groups (A vs B) regarding the total amount of HP in saliva ($p=0.860$) or in periodontal tissues ($p=0.967$; $p=0.123$; $p=0.293$; $p=0.463$), it should be considered that Breath test and PCR-RT in the oral cavity are two completely different methods of bacterial detection. The first one measures urease activity and distinguishes between positive and negative gastric infection, while the second one is quantitatively more accurate in counting bacteria but requires a sample of gastric mucosa which is a more invasive procedure since it needs esophagogastroduodenoscopy. Besides, performing Breath test and PCR-RT for gastric and oral mucosa evaluation, respectively, does not allow to determine a causal factor, since these tests provide a picture of two anatomical areas at the same moment with two different methods. This was also confirmed by the comparison between group A and group B patients within the group of the only periodontal unhealthy patients ($p=0.102$). On the contrary, there was an interesting result as to the behaviour of HP in the oral cavity in relation to periodontal condition. Patients were subdivided into another two groups according to their periodontal health: if the PD was below 3

mm they were considered to be periodontal healthy, while if it was equal or greater than 3 mm they were classified as affected by periodontal disease. Comparing the mean amount of HP in periodontal pockets within the two groups, affected patients showed higher values, confirming that HP is present in the oral cavity and that it is not related to gastro-esophageal reflux or stomach load, as some authors have claimed, but it has an active role in periodontal tissues. HP in the periodontal tissues is not confined to periodontal pockets, but migrates from the gingival sulcus into saliva, as was demonstrated by comparing the presence of the bacterium in the salivary fluid between periodontally healthy and unhealthy patients (Student's *t*-test $p=0.05$; *Chi-squared* $p=0.046$). Therefore, periodontal disease provides a suitable environment for HP. Periodontal pockets are the favourable site where the bacterium can thrive in synergy with other bacteria. Furthermore, once the bacterium has gained enough load, it migrates from the sulcus into the oral cavity and enters the saliva. Since the saliva is continuously swallowed and descends into the stomach, the oral cavity represents an extra-gastric reservoir of HP for the stomach.

This preliminary study demonstrated that the oral cavity is an extra-gastric reservoir of HP when it is affected by periodontal disease.

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