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EDITORIAL

INTERLEUKIN-1 FAMILY CYTOKINES AND MAST CELLS:
ACTIVATION AND INHIBITION

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This editorial focuses on recent progress reports on mast cells (MCs) and IL-1-family cytokines in the dynamics of inflammation. MCs are cells of haematopoietic derivation that mature and reside in almost all vascularized tissues. MCs are a potential source of cytokines and chemokines which participate in allergic reactions and inflammation. Activated MCs secrete a number of compounds including pro-inflammatory cytokines. MCs can be activated by IgE through its receptor FcεRI, but also by Toll-like receptors. They can be a target for both pro-inflammatory and anti-inflammatory cytokines. IL-1 activates MCs to release inflammatory chemical mediators, and cytokines/chemokines, an effect which can be potentially inhibited by IL-37. In addition, IL-36 is also a powerful cytokine with a pro-inflammatory activity. IL-38 binds IL-36R and inhibits the pro-inflammatory activity of IL-36, thus performing a therapeutic action. Here, in this article we review the role of MCs in relation to pro-inflammatory and anti-inflammatory IL-1 family member cytokines and a possible therapeutic effect in inflammatory disorders.

Mast cells (MCs) are ubiquitous bone marrow immune cells that derive from hematopoietic stem cells (CD34⁺) and reside in all the vascularized tissues and serous cavities where they reach the maturity (1).

MCs are immune cells with a potential source of a number of cytokines/chemokines, and growth factors that mediate allergic disease and participate in both innate and adaptive immune responses (2). They are effector cells that participate in IgE-mediated

responses, but can also mediate inflammation after activation by various molecules through the Toll-like receptors, interleukin (IL)-1 receptors, and neurotransmitter receptors, without the intervention of IgE. Therefore, the binding of the antigen to these receptors can activate MCs without degranulation (3).

FcεRI receptor expressed on the cell surface of the MCs binds the high affinity antigen and stimulates the MCs with the initiation of phosphorylation. FcεRI receptor is made up of 2 alpha sub-units, 4 beta and

Key words: interleukin; mast cell; inhibition; inflammation; cytokine

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two gamma units which start the reaction amplified by the 4 beta sub-units (1). Phosphorylation begins with activation of tyrosine kinase and recruitment of the γ phospholipase ($\text{PLC}\gamma$) and protein kinase C (PKC) (1).

In this pathway, intracellular calcium (Ca^{2+}) is regulated by $\text{PLC}\gamma$, and PKC results in the formation of IP3 and diacylglycerol (DAG). MAPK, ERK, JNK and p38 participate in the transcription of cytokines and subsequently the generation of proteins. Activation of the phospholipase A2 (PLA2) leads to the formation of prostaglandins (PGs), leukotrienes (LTs) and lipoxins (LXs). All of these molecules, and others, including cytokines of the IL-1 family, are intensely involved in the inflammatory process (1).

Members of the IL-1 family cytokines: IL-1, IL-18, IL-33 and IL-36, play an important role in immune response and inflammation. Cytokines such as TNF and IL-1 are produced by MCs and also have the ability to activate MCs (4).

In this article, we discuss how MCs can be an important source of pro-inflammatory cytokines that may be inhibited by IL-37, a member of the IL-1 family (5) (Fig. 1.).

IL-1

IL-1 is one of the most important regulators of innate immune responses and participates in numerous inflammatory processes (6-7).

It has been reported that MCs also contribute to innate immunity and generate IL-1 upon stimulation with LPS, bacteria and parasites. IL-1 generated by MCs participates in the inflammatory process in many disorders including autoimmune diseases, allergies, atherosclerosis, psoriasis, endocrinopathies and neuropathies (8). MC-derived proteases are also able to activate IL-1, in fact, serine protease chymase has been shown to convert pro-IL-1 β into a biologically active form. Therefore, MC-derived proteases might act

MAST CELL ACTIVATION

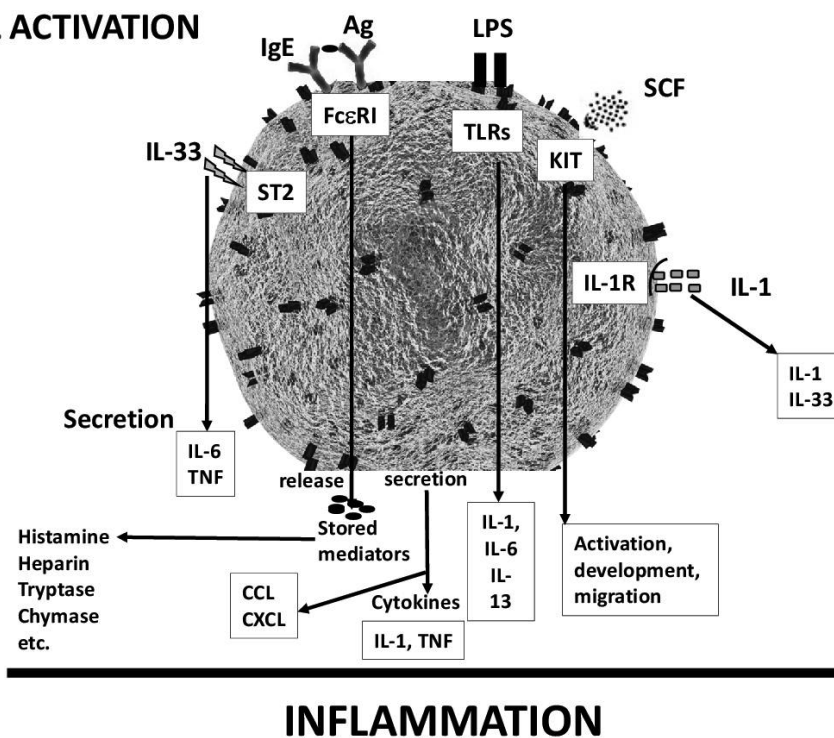


Fig. 1. Mast cell antigen-mediated signal events leading to degranulation and production of inflammatory cytokines.

secondary to caspase-1 activation and pro-IL-1 β formation (9).

IL-1 is strongly involved in innate immunity, and the cytoplasmic domain of the IL-1 receptor type I, which binds IL-1, is similar to the cytoplasmic domains of all Toll-like receptors (TLRs). IL-1 β is a defense cytokine produced by the body, especially against infections, and is the most studied member of the IL-1 family. The IL-1 family is made up of 11 members including IL-33 which binds the ST2 receptor and performs various functions on the MCs. IL-1Ra binds to the IL-1RI receptor and inhibits IL-1 α and IL-1 β , performing anti-inflammatory activity. In the generation of IL-1, caspase-1 causes cleavage of the IL-1 β precursor with formation and secretion of mature IL-1 β , a process that can be inhibited by IL-1Ra and IL-37. In inflammatory disease, by inhibiting IL-1 β there is a reduction in the symptoms and the inflammatory molecules involved in the disease. In addition, IL-1 also

plays an indirect role on lymphocyte-mediated immunity; while its direct participation is in fever, pain, vasodilatation, hypotension, and in all those diseases where there is inflammation. IL-1 β is also involved in the generation of adhesion molecules and chemokines with infiltration of inflammatory immune cells. It also plays a role in cancer and metastasis, promoting angiogenesis and stem cell differentiation of myeloid cells, including MCs (10). IL-1 β participates in the production of IL-6, another important pro-inflammatory cytokine, and in many cases, by blocking IL-1 β , IL-6 is also inhibited. IL-1, together with a mitogen, participates in the stimulation and amplification of T cells, and acts on thymocytes as a growth factor, contributing to TH2 cell polarization. Also in the formation of antibodies by plasma cells, IL-1 β plays an important role and is a growth factor for B cells, probably through the production of IL-6. It has been previously reported that members of the IL-1 family are: IL-1 α , IL-1 β ,

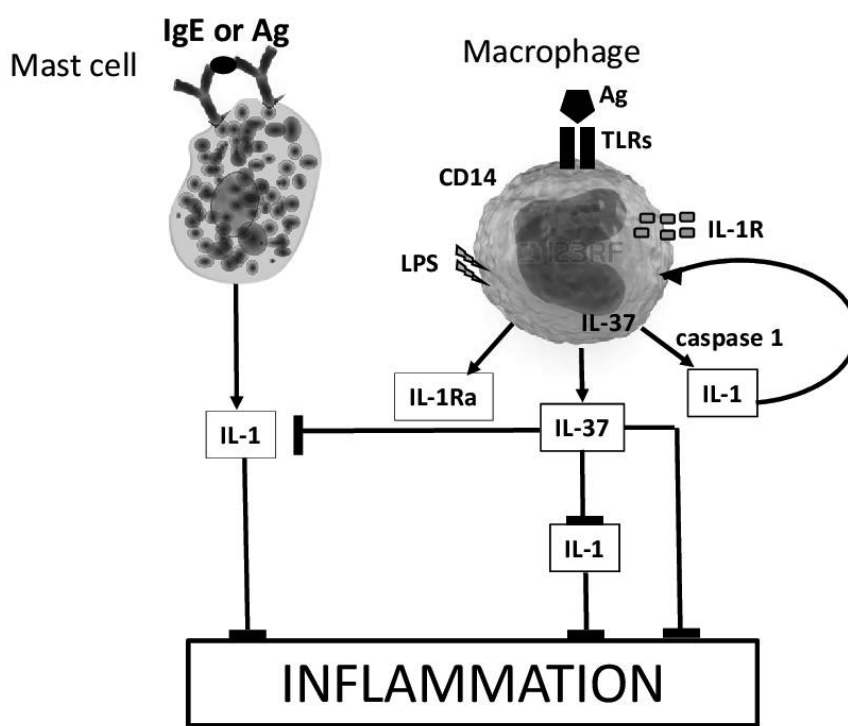


Fig. 2. Activated macrophage releases IL-1 family members including IL-37 which inhibits IL-1, and therefore inflammation. In addition, IL-1 released by mast cell inducing inflammation is also inhibited by IL-37.

IL-1Ra, IL-18, IL-33, IL-36 α , IL-36 β , IL-36 γ , IL-36Ra, IL-37, IL-38 (11). Blocking IL-1 (e.g. with IL-37 or IL-1Ra) or deficient IL-1 animals, show a reduction in the severity of inflammatory disease and an increased vulnerability to infection.

IL-33

IL-33 (also known as IL-1F11), which is an intracellular cytokine linked to the nucleus, controls inflammation and is involved in the T helper 2 (Th2) responses (12-13). ST2, receptor of IL-33, is composed of three extracellular Ig domains and an intracellular Toll domain, and is similar to IL-1RI and IL-18R α receptors. IL-33 binds ST2 receptor membrane and interacts with IL-1RAcP complex and induces MyD88 for signal transduction. The structure of IL-33 is more similar to IL-18 than to IL1 β and it induces the production of IL-6, IL-1 β , and PGE from MCs. IL-33 plays a crucial role in polynomial diseases by causing infiltration of eosinophils and MCs (14). IL-33 induces IL-5 and IL-13 production, initiates NF- κ B-mediated signal transduction and causes changes in clinical parameters in experimental animals. IL-1 induces IL-33 in MCs, and blocking IL-1 results in a noticeable improvement in inflammatory response.

IL-37

The proteins of the IL-1 family are important for the defense of the host against external insults, such as bacteria, parasites, fungi and virus, but also against tumor and autoimmune diseases. Several IL-1 family cytokines are pro-inflammatory, but some are anti-inflammatory and take part in the regulation of inflammatory balance.

Anti-inflammatory cytokines in the IL-1 family include IL-37 and IL-38 proteins and two receptor antagonists, IL-1Ra and IL-36Ra (15).

IL-37 is a cytokine member of the IL-1 family with immunosuppressive and anti-inflammatory properties. IL-37 indeed plays a crucial role in the regulation of inflammatory response by lowering the levels of proinflammatory cytokines related to IL-1. IL-37 acts as an inhibitor of inflammation and influences the action of Treg cells by suppressing the lymphocyte response. However, it is not clear how

IL-37 can expand Treg cells (16).

Inhibition of inflammation and immune response occurs when IL-37 binds to the IL-18R α ligand receptor by blocking the entire cascade in Fig. 2.

IL-37 comprises 5 variants a, b, c, d, and e, and may be induced in different tissues and cells, mainly in peripheral blood mononuclear cells. Similarly to IL-1, this cytokine, requires the cleavage of caspase-1 to be activated (17).

IL-37 binds to the cell nucleus and acts as a transcriptionist carrying out an anti-inflammatory and immunosuppressive effects.

As IL-1 stimulates MCs and is produced by MCs performing an important inflammatory action, blocking IL-1 may exercise a new therapeutic approach in inflammatory diseases and therefore should be taken into consideration (18-20).

IL-36

There are 3 subfamilies of IL-36 cytokine which comprise IL-36 α , β , γ and one receptor antagonist IL-36Ra (21). IL-36 plays an important role in psoriasis where MCs play a crucial role. In fact, transgenic IL-36 animals show a severe inflammatory skin pathology. IL-36 is therefore expressed mainly in the skin and acts on fibroblasts and keratinocytes. This cytokine is part of the IL-1 family and the maturation of IL-36 is unclear.

When IL-38 binds to IL-36 receptor (IL-36R), the biological activity of IL-36 is inhibited. IL-36 is expressed in various organs, such as bone marrow and skin, and in cells including CD4⁺ lymphocytes and dendritic cells. IL-36 is involved in the production of pro-inflammatory cytokines and promotes the polarization of T lymphocytes.

MCs release various pro-inflammatory cytokines including IL-36, which can take part in allergic inflammation. Inhibiting IL-36 with IL-38 could be one of the solutions to alleviate the allergic inflammatory phenomenon.

IL-38

When IL-38 was first discovered, it was uncertain whether it was an agonist or an antagonist of inflammation (22). Today, we know that IL-38 binds to the IL-36R receptor and

inhibits IL-36 pro-inflammatory action, although in some cases IL-38 manifests itself as an agonist of the inflammatory process. The reason for this dual activity of IL-38 is not yet known.

In addition, IL-38 is homologous to IL-36Ra and IL-1Ra, suggesting a similar antagonist function of inflammation. Moreover, IL-38 is part of the IL-1 family and belongs to the IL-36 cytokine group. IL-38 is expressed in many tissues including skin, spleen, tonsil, thymus, heart, fetal liver and placenta.

Some proteases, but not caspase-1, have been seen to split the cytokine IL-36; while proteases that split IL-36Ra and IL-38 are unknown. IL-38 acts on the IL-36R receptor, similar to IL-36Ra, inhibiting the biological effects of IL-36. To date it seems that IL-38 performs mainly anti-inflammatory activity by inhibiting IL-36. In some fungal infections, it seems that IL-38 is very important in inflammatory balance. The inhibitory effect of IL-38 may play a role in rheumatic diseases where MCs play a crucial role. High levels of IL-38 are often associated with C-reactive protein (CRP) levels, and it could be a marker of inflammation. The suppression of IL-38 in macrophages *in vitro* leads to an increase in some pro-inflammatory cytokines, such as IL-6 which is also produced by activated MCs. The processed and truncated IL-38 has a great inhibitory action on IL-6; while the whole form can have a non-effect or even a stimulatory effect. This allows us to understand the importance of IL-38 processing.

In addition, IL-38 inhibits IL-1, IL-36 and IL-17 which play an important role in skin diseases including psoriasis, a disease in which MCs give a clear contribution to the inflammatory process. Therefore, overexpression of IL-38 leads to the inhibition of IL-17 and some related inflammatory diseases.

The study of pro-inflammatory and anti-inflammatory cytokines allows us to better understand the network of inflammation that is found in almost all diseases, helping us to achieve new and effective therapeutic compounds.

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PROTECTIVE EFFECT OF *LYCIUM BARBARUM* POLYSACCHARIDES AGAINST HIGH-FAT DIET-INDUCED RENAL INJURY AND LIPID DEPOSITION IN RAT KIDNEYS

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This study aimed to explore the protective effect of *Lyceum barbarum* polysaccharides (LBPs) against hyperlipidemia and lipid-induced renal injury in a rat model. Male Sprague-Dawley rats (n = 30) were randomly divided into three equal groups: a control group (fed a regular diet) and two experimental groups (fed a high-fat diet). By feeding rats a high-fat diet for 12 weeks, an animal model of hyperlipidemia was established, after which one experimental group received oral LBPs at a dose of 300 mg/kg per day. Blood lipids, renal function, and urinary proteins were measured after 12 weeks. Changes in renal pathology and expression levels of sterol regulatory element binding transcription factor 1 (SREBP-1), interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), AMP-activated protein kinase (AMPK) were determined. Rats with hyperlipidemia induced by a high-fat diet showed increases in blood lipids and blood urea nitrogen, serum creatinine, and urinary protein, as well as increases in renal levels of SREBP-1, TNF- α , and IL-6 and decreases in renal levels of adiponectin and AMPK. Administration of LBPs restored blood lipid, blood urea nitrogen, serum creatinine, and urinary protein levels, downregulated renal levels of SREBP-1, TNF- α , and IL-6, and upregulated renal levels of adiponectin and AMPK. These results indicate that LBPs may mediate lipid metabolism, enhance anti-inflammatory responses, and ameliorate renal injury caused by lipid metabolism disorders in a rat model of hyperlipidemia.

Obesity, characterized by the accumulation of adipose tissue, is considered a primary risk factor for many chronic disorders, including dyslipidemia, hypertension, and diabetes (1, 2). Long term, these conditions may further lead to additional complications, such as chronic kidney diseases (CKDs) (3, 4). Epidemiological studies have revealed that there may be an important link between hyperlipidemia and CKDs. Studies on animal models have shown that hyperlipidemia is a risk factor for the development

of renal dysfunction, and renal injury pathogenesis is characterized by the deposition of lipids in the glomerulus, mesangial cell proliferation, accumulation of extracellular matrix, glomerulosclerosis, epithelial injury, and renal interstitial injury (5, 6).

Obesity with excessive fat accumulation is associated with inflammatory stress (7, 8). Although inflammatory factors are products of normal cellular metabolism, the overproduction of inflammatory factors induces inflammatory and oxidative stress

Key words: hyperlipidemia; lipid metabolism; lipid-induced kidney injury

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by damaging proteins and DNA molecules (9). Inflammatory and oxidative stress play crucial roles in the pathogenesis of a number of diseases, including CKDs (10, 11). AMPK is important in lipid metabolism and oxidation, and AMPK activation can reduce lipid synthesis and oxidative stress (12). Animal studies have shown that hyperlipidemia and inflammatory stress are involved in the progression of lipid-induced renal injury (13, 14). Hyperlipidemia, especially that induced in animals fed with high-fat diet, could induce inflammatory and oxidative pathways in multiple organs (15). Thus, drugs that can decrease lipids and reduce inflammatory stress and oxidative damage have received increasing attention.

LBP is a natural compound that possesses wide-ranging pharmacological effects, including reductions in lipids and inflammatory stress, as well as antioxidant, hepatoprotective, anti-tumor, anti-aging, and immunoregulatory effects (16, 17). Reports have shown that LBPs may protect mouse livers from carbon tetrachloride-induced oxidative stress and necroinflammation (18, 19). In addition, LBPs may suppress SREBP-1 expression and prevent diet-induced fatty liver through AMPK activation (20). However, whether LBPs can alleviate hyperlipidemia and lipid-induced renal injury remains unknown. Therefore, in the present study, we aimed to determine whether LBPs exert a protective effect against hyperlipidemia and lipid-induced renal injury in a rat model, and to uncover the underlying mechanisms of this effect.

MATERIALS AND METHODS

Materials

LBPs for clinical application with an endotoxin content < 0.1 EU/mg were purchased from Pharmagenesis Inc. (Newtown, PA, USA). LBPs mainly consist of mannose, arabinose, galactose, rhamnose, glucose, and xylose. All reagents were of the highest purity and analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse polyclonal antibodies against (SREBP-1) and adiponectin and rabbit polyclonal antibody against AMPK were ordered from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Animals

Thirty healthy adult male Sprague-Dawley (SD) rats (age, 6 weeks; body weight, 160–180 g) were purchased from Xiangya School of Medicine, Central South University, Changsha, China. Animal experiments were performed in compliance with the requirements of the Animal Management Rules of the Ministry of Health of China and the guidelines of Central South University. The rats were acclimatized for 1 week before the experiment and were maintained in a 12:12-h light-dark cycle. The body weights of the rats were measured weekly, and the diet, appearance, and behavior of the rats were recorded daily. The control group (ND), consisting of 10 rats, was fed a normal chow diet (21% crude protein, 5% lipids, 4% crude fiber, 8% ash, 1% calcium, 0.6% phosphorus, 3.4% glucose, 2% vitamins, and 55% carbohydrates). The other 20 rats were fed a high-fat diet (73.8% standard diet, 1% cholesterol, 10% yolk powder, 15% lard, 0.2% sodium deoxycholate) for 12 weeks. Subsequently, the high-fat diet-fed rats were randomly divided into two groups consisting of 10 animals each. One group continued to be fed the high-fat diet (HFD group), while the other received the high-fat diet and was orally treated with LBPs at a dose of 300 mg/kg/day (HFD + LBP group) for 12 weeks. The rats in the control and HFD groups were treated with saline solution daily for 12 weeks. The rats in ND group were still fed with normal diet in the second 12 weeks. Rats were sacrificed by an overdose of anesthesia (150 mg/kg pentobarbital, intraperitoneal injection) according to protocols approved by the Committee of Animal Use for Research and Teaching at Central South University (NO.20162010). Blood and kidneys were harvested for biochemical and histological analysis, and 24-hour urine was collected for urine protein tests. Kidney tissues were frozen at -80°C for Western blot or embedded in optical cutting temperature cryostat sectioning medium containing 30% sucrose for immunohistochemistry. Other samples were fixed in 4% paraformaldehyde for paraffin sectioning and ultrastructural examination by electron microscopy.

Analysis of blood biochemistry

After overnight fasting, blood samples were collected by cutting the tip of the tail. Serum was collected by centrifugation from whole blood at 3500 rpm and 4°C for 10 min. The serum glucose concentration was measured

using the immobilized glucose oxidase method. Serum triglycerides (TGs), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), serum creatinine (Scr), and blood urea nitrogen (BUN) levels were assessed using an H7600-020 automatic analyzer (Hitachi, Tokyo, Japan). The urinary albumin level was determined with an Albuwell MELISA kit (Exocell Inc., Philadelphia, PA, USA) according to the manufacturer's instructions.

Hematoxylin-eosin and Oil Red O staining

Kidneys were fixed in 4% paraformaldehyde for 48 h and embedded in paraffin. Paraffin sections were prepared and stained with hematoxylin and eosin (H&E). To investigate histological damage, slides were observed under a light microscope (9400, Nikon, Tokyo, Japan). Frozen sections were stained with 0.3 % Oil Red O solution to analyze the neutral lipids in renal tissues, and the relative density was quantified by Image.

Electron microscopy

Renal tissue specimens were fixed in 4% paraformaldehyde and then in 1% osmium tetroxide before being dehydrated in graded alcohols and embedded in Epon. Ultrathin sections (200–400 Å) were cut on a nickel grid, stained with uranyl acetate and lead citrate, and examined using a digital electron microscope (JEM-1400, JEOL USA, Peabody, MA, USA). All glomeruli in a grid were examined at 6,000× for identification of podocytes, endothelial cells, and mesangial cells.

Immunohistochemistry

The immunoreactivities of SREBP-1 and adiponectin were quantified in the kidney cortex using the avidin-biotin-peroxidase complex (ABC) and peroxidase substrate kit (Abcam, Cambridge, UK). Briefly, tissue slices were heated (95–100°C) in 10 mM citrate buffer (pH 6.0) for epitope retrieval. Endogenous tissue peroxidase activity was blocked by incubation in

Table I. Primers used for RT-PCR.

Primer	Forward (5'→3')	Reverse (5'→3')
SREBP-1	CCTGGTGGTGGGCACTGAAGC	GCGTCTGAAGGGTGGAGGGGT
Adiponectin	AATCCTGCCCAGTCATGAAG	ATCCCACTGGGTCTCTCTAC
TNF-α	TGATCCGCGACGTGGAA	ACCGCCTGGAGTTCTGGAA
IL-6	GAGGATACCACTCCCAACAGACC	AAGTGATCATCGTTGTTTCATACA
GAPDH	ATGACCCCTTCATTGACCTCA	GAGATGATGACCCTTTTGGCT

Table II. Renal function parameters and serum lipid levels in rats from control and experimental groups.

	ND	HFD	HFD+LBP
BUN (mmol/L)	3.92 ± 0.37	16.02 ± 1.31 [*]	12.21 ± 1.14 ^{*#}
Scr (μmol/L)	42.34 ± 5.45	93.45 ± 6.98 [*]	74.86 ± 7.02 ^{*#}
Urea protein (mg/d)	15.05 ± 1.14	104.43 ± 12.32 [*]	73.32 ± 7.54 ^{*#}
Urea M-Alb (μg/d)	27 ± 9	467 ± 51 [*]	280 ± 60 ^{*#}
TC (mmol/L)	1.48 ± 0.22	2.64 ± 0.26 [*]	1.61 ± 0.13 ^{*#}
TG (mmol/L)	0.43 ± 0.23	1.23 ± 0.25 [*]	0.61 ± 0.18 ^{*#}
HDL-C (mmol/L)	0.82 ± 0.04	0.53 ± 0.09 [*]	0.76 ± 0.05 ^{*#}
LDL-C (mmol/L)	0.53 ± 0.1	1.02 ± 0.08 [*]	0.78 ± 0.19 ^{*#}
Body weight at 24w (mg)	395.5 ± 14.2	515.7 ± 19.1 [*]	490.2 ± 18.3 [*]

Results are expressed as mean ± SEM (n = 10). ^{*}p < 0.05 compared with the ND group, [#]p < 0.05 compared with the HFD group.

methanol containing 0.3% H_2O_2 for 30 min. The normal horse serum blocking solution was incubated for 1 h in a humidified chamber to block non-specific binding of immunoglobulin. Endogenous avidin and biotin were blocked by avidin/biotin blocking reagent (Dako, Carpinteria, CA, USA). All sections were incubated with the anti-SREBP-1 antibody (1:100) and the anti-adiponectin antibody overnight at 4°C in a humidified chamber, followed by incubation at room temperature

with the appropriate biotinylated secondary antibodies for 1 h and the ABC reagent. Finally, tissue sections were incubated with peroxidase substrate for 3 min, and immunoreactive areas (five slides for each group) were quantified by IOD (intensity of density) with an automated image analyzer (Image Pro Plus 6.0).

RNA extraction and real-time PCR

Total RNA was extracted from renal tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and

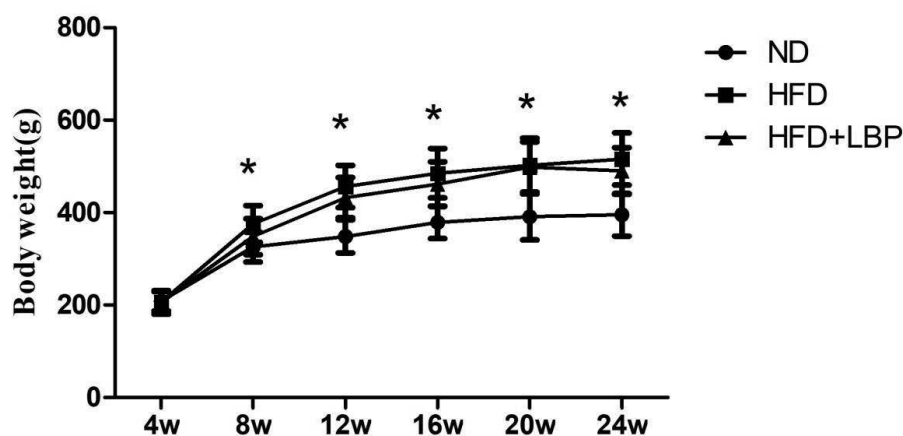


Fig. 1. Body weights of rats from different treatment groups. Results are expressed as means $\pm$ SEM ($n = 10$). * $p < 0.05$, HFD group vs ND group.

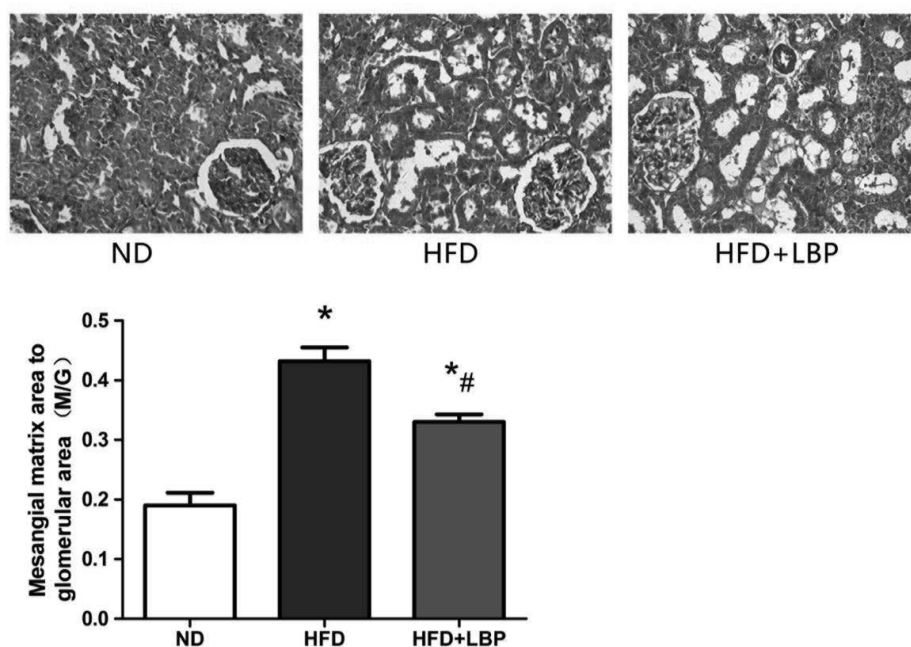


Fig. 2. H&E staining of renal tissues from rats in the ND, HFD, and HFD + LBP groups (magnification, $\times 200$). Ratio of mesangial matrix area to glomerular area (M/G) in the three groups, * $p < 0.05$ compared with the ND group, # $p < 0.05$ compared with the HFD group.

RNA purity and concentration were assessed with a spectrophotometer Nano Drop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). RNA was transcribed with Super Script III Reverse Transcriptase (Invitrogen) using an oligo dT primer. Real time-PCR was performed on a Step One Plus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) using Fast SYBR Green master mix (Applied Biosystems). The mRNA expression levels of *SREBP1*, adiponectin, *TNF- α* , and *IL6* were normalized to that of *GAPDH* in the same cDNA sample. Relative quantification of gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method. A list of primer sequences used in this study can be found in Table □.

Western blot analysis

The other halves of the frozen renal tissues were

homogenized in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1 mM PMSF) with a tissue homogenizer (IKA-Werke GmbH & Co. KG, Staufen, Germany). The protein homogenates were centrifuged twice at $10,000 \times g$ for 20 min at 4°C. The supernatants were obtained, and the protein concentrations were assessed with Bicinchoninic Acid Kits (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein (40 μ g/lane) were separated on 10–12% SDS polyacrylamide gels (PAGE) and transferred to nitrocellulose membranes (GE Healthcare Biosciences, Pittsburgh, PA, USA). Membranes were blocked by incubation in 5% skim milk for 1 h. Following incubation with the indicated primary antibodies overnight, the blots were washed with TBST and incubated with appropriate

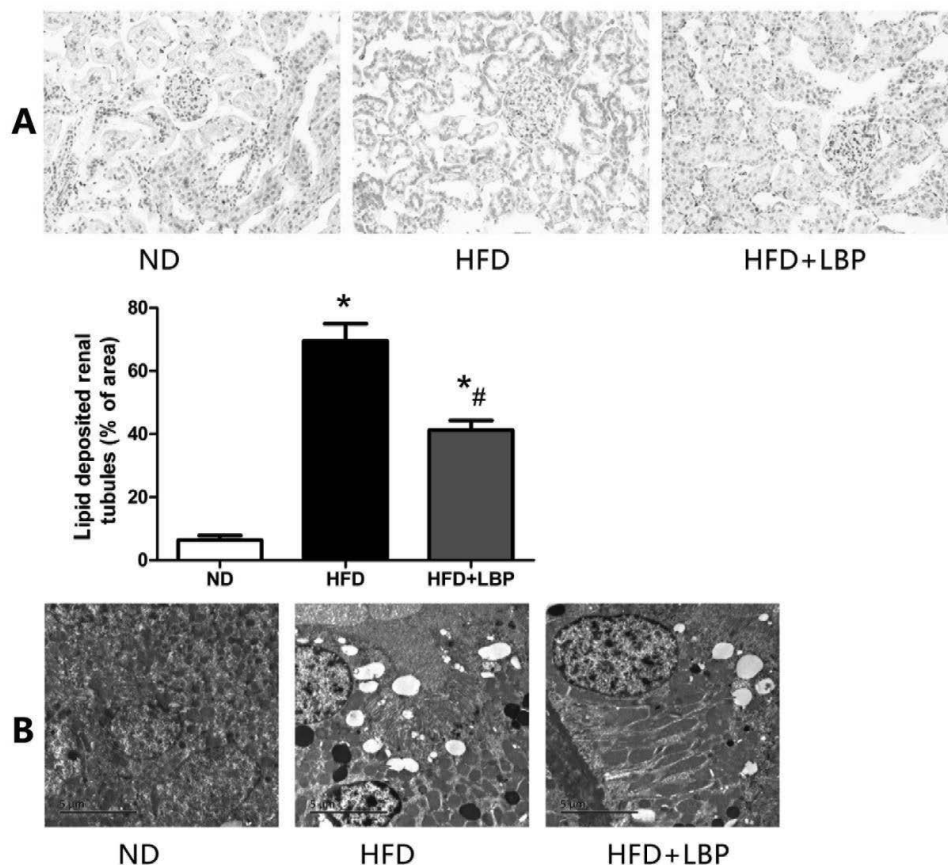


Fig. 3. *A*) Staining of the kidneys from control and experimental groups (magnification, 200 $\times$). Graph shows lipids deposited in renal tubules (% of area) in the three groups. *B*) Representative electron microscopy images of lipid droplets (arrows) in the kidneys from the ND, HFD, and HFD + LBP groups (magnification, 6000 $\times$).

secondary antibody for 1 h. Chemiluminescent images were captured with an ImageQuant LAS 4000 Mini (GE Healthcare Biosciences).

Statistical analysis

SPSS 17.0 software was used for statistical analysis. Quantitative data are expressed as means $\pm$ SEM. One-way ANOVA was used for comparisons between groups, and the least significant difference (LSD) test was conducted for homogeneity of variance. Dunnett's T3 test was conducted for heterogeneity of variance. $p < 0.05$ indicates a statistically significant difference.

RESULTS

LBP treatment protected against renal dysfunction and dyslipidemia

We analyzed renal functional parameters, including BUN, Scr, urea protein, and urea microalbumin (M-Alb) levels, in the control and experimental rats. While the HFD group exhibited increased levels of BUN, Scr, urea protein, and

M-Alb ($p < 0.001$), LBP treatment significantly decreased these levels ($p < 0.005$), suggesting that LBPs may protect renal function. At the end of the treatment period, the TC and TG levels of rats in the HFD group were higher than those in the ND group ($p < 0.001$), indicating that the dyslipidemia model was successfully established. Rats receiving LBPs, however, exhibited lower TC, TG, and LDL-C levels and increased HDL-C levels ($p < 0.05$) compared with levels in the HFD group (Table 1). LBPs administration had little effect on body weight ($p > 0.05$) (Fig. 1).

LBPs alleviated renal structural changes

H&E staining of kidney sections in the ND group showed a typical architecture of the kidney glomerulus and tubules, with a clear boundary between the renal capsule and the glomus (Fig. 2A). Tubular epithelial cells were clearly defined, well arranged, and similar in size. An increased number of glomerular cells were observed in the kidneys of the HFD group, accompanied by glomerular swelling, an

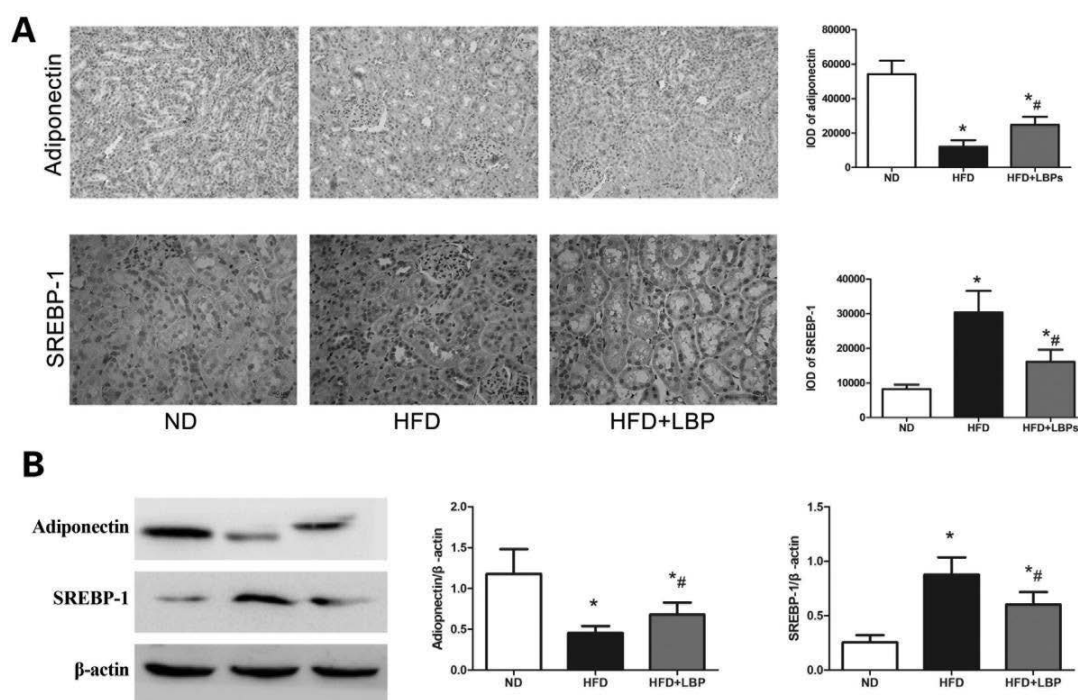


Fig. 4. A) Immunohistochemical staining of adiponectin and SREBP-1 in the renal cortex of rats in the control and experimental groups (magnification, 400 $\times$). B) Western blot analysis of the relative expression of adiponectin and SREBP-1 in the kidney. * $p < 0.05$ compared with the ND group, # $p < 0.05$ compared with the HFD group.

irregular morphology of some glomeruli, lobulated capillary loops, and a significantly increased renal capsular space. The glomerular mesangial matrix was increased, and obvious epithelial proliferation of kidney tubules was observed ($p < 0.005$). Hyperplasia of mesangial cells and the matrix were ameliorated in the HFD + LBP group (Fig. 2B).

LBP attenuated HFD-induced renal lipid deposition

Renal lipotoxicity has adverse effects on normal cellular state, mainly contributing to the accumulation of TGs. Oil Red O staining is a useful method for analyzing the neutral lipid content of tissues. As shown in Fig. 3, renal tissues from rats in the HFD group contained large lipid droplets stained red, but LBP treatment effectively decreased lipid accumulation. According to electron microscopy images, lipid droplets were present in the cytoplasm of podocytes, mesangial cells, and tubular epithelial cells in the HFD group kidneys, while LBP administration reduced lipid droplet deposition.

LBP downregulated SREBP-1 and upregulated adiponectin in renal tissue

To assess the potential contribution of LBPs to the regulation of lipid metabolism, we measured the expression levels of SREBP-1 and adiponectin in renal tissues via immunohistochemistry (Fig. 4A) and Western blot (Fig. 4B). The results revealed that expression of SREBP-1 was higher in the HFD than in the ND group ($p < 0.005$), while expression of adiponectin was lower in the HFD than in the ND group ($p < 0.005$). LBP administration effectively attenuated HFD-induced accumulation of SREBP-1 and HFD-induced reduction of adiponectin in glomeruli and tubules.

LBP downregulated SREBP1, TNF- α , and IL-6 and upregulated adiponectin mRNA expression

SREBP1 mRNA levels were significantly ($p = 0.0018$) higher in the HFD group than in the ND group but significantly ($p = 0.0043$) lower in the HFD + LBP group than in the HFD group. Similarly, after LBP treatment, the expression of adiponectin

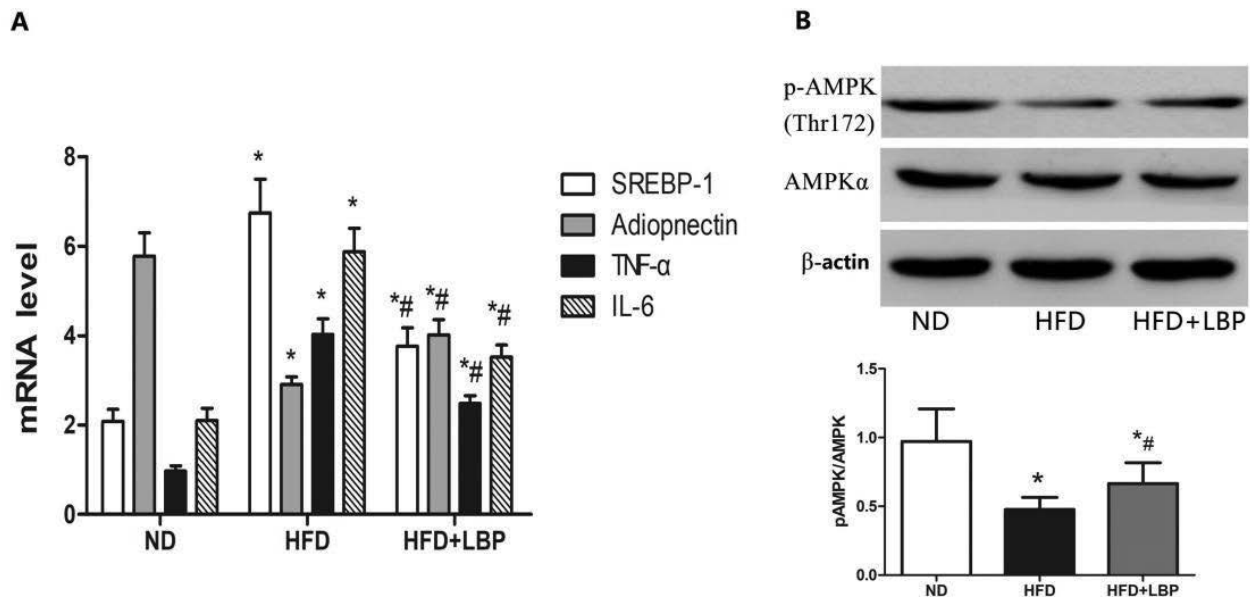


Fig. 5. A) LBP downregulated mRNA levels of SREBP1, TNF- α , and IL6, while upregulating levels of adiponectin. * $p < 0.05$ compared with the ND group, # $p < 0.05$ compared with the HFD group. B) Representative Western blot and densitometric analysis of the ratio of phospho-AMP kinase (pAMPK) to AMPK expression in renal cortical tissues from control and experimental groups. * $p < 0.05$ compared with the ND group, # $p < 0.05$ compared with the HFD group.

mRNA was significantly decreased ($p=0.0079$). In addition, the expression levels of TNF- α and IL-6 mRNA were higher in the HFD group than in the ND group ($p=0.0021$, $p=0.0056$ respective) and were downregulated following LBP treatment ($p=0.0037$, $p=0.0096$ respective, Fig. 5A).

LBP reduced the effect of HFD on renal AMPK activity

The AMPK pathway is a key regulator of intracellular lipid metabolism in the kidney and other tissues. We observed a significant decrease in the phospho-AMPK/AMPK ratio in the renal cortical tissues of HFD rats ($p=0.032$), and this was ameliorated by LBP treatment ($p=0.041$) (Fig. 5B). These results suggest the important role of the lipolysis process, especially p-AMPK targeting, in renal lipid metabolism.

DISCUSSION

To our knowledge, this study is the first to indicate that LBPs can improve lipid profiles and alleviate renal injury in a rat model of hyperlipidemia. This may be because LBPs mitigate abnormal lipid metabolism and regulate certain adipokines secreted by adipocytes.

Metabolic syndrome can induce chronic renal injury in humans as well as rats. Too much lipid deposition in the renal tubules can lead the ratio of the glomus areas to the glomerulus area and the glomerular density inside the renal cortex both decreased. LBPs has a long history of use in Chinese and indian medicine. In the past years, the main research focused on the therapeutic properties of LBPs as an antioxidative and immunomodulatory drug against diabetes and atherosclerosis. Recently, LBPs pharmacological effects on reductions in lipids have received more and more attention. Considering the beneficial effects of LBPs, it holds great potential as a lipid-lowering drug in the prevention of obesity and other diseases related to metabolic syndrome with oxidative stress and dysfunction of the immune system as the major underlying risk factors. In our study, LBPs was found to protect against renal dysfunction and dyslipidemia and

alleviate renal structural changes. For example, an increased number of glomerular cells were observed in the kidneys of the HFD group, accompanied by glomerular swelling, an irregular morphology of some glomeruli, lobulated capillary loops, and a significantly increased renal capsular space.

In previous studies, a rat model correlating obesity-related renal disease and lipid metabolism was successfully established and characterized by hypertrophy, proteinuria, and increased serum lipids (21, 22). In this study, we fed rats a high-fat diet for 12 weeks to establish a model of hyperlipidemia and renal damage. Increased serum BUN and Scr, as well as renal tissue pathological changes, further demonstrated that the high-fat diet induced a classical histological and functional model. Moreover, in this study, we found that the administration of LBPs reduced serum TC, TG, and LDL levels in a dose-dependent manner and alleviated lipid-induced renal damage, suggesting that LBPs are promising compounds for addressing abnormal lipid metabolism and obesity-related renal injury.

Adipokines, synthesized and secreted by adipocytes, are involved in the regulation of appetite, blood pressure, and inflammatory and immune processes and play a role in the metabolism of carbohydrates and fats (23, 24). Adiponectin, a classical adipokine, has been identified as an anti-inflammatory, anti-atherosclerotic, and endothelium-protective factor (25, 26). TNF- α is a pro-inflammatory cytokine the expression of which is upregulated in adipose tissue and the sera of patients with obesity (27). Similarly, IL-6 is a pro-inflammatory factor that has been implicated as an independent factor related to obesity and insulin resistance, and it could also play a dual role in inflammatory pathways, acting as a downregulator factor of TNF- α gene expression (28, 29). Furthermore, it has been shown that TNF- α and IL-6 are associated with the severity of CKD. Using RT-PCR and immunochemistry, our study showed that adiponectin expression was restored in the high-fat kidney, while TNF- α and IL-6 were downregulated in renal tissue following LBP administration. These results indicate that LBPs exert anti-inflammatory and anti-atherosclerotic effects. These cytokine modulations may contribute

to the renal protective effect of LBPs against high lipids.

In addition to adipokines, some kinases also play a key role in fat metabolism. AMPK, a critical kinase in energy metabolism, inhibits lipolysis and lipogenesis; promotes fatty acid oxidation in adipocytes; and controls cholesterol synthesis, lipogenesis, and TG synthesis, once phosphorylated (30), thus lowering blood lipids and boosting energy metabolism. SREBP-1, a membrane-bound transcription factor, regulates lipid homeostasis by controlling the expression of lipid metabolism genes. Previous studies (19, 31) showed that LBPs protect the liver from fatty deposition through AMPK activation and SREBP-1c-mediated suppression of liver injury in an NAFLD model. In this study, we found that LBPs activate AMPK and modify SREBP-1 expression in kidney tissues, which may be the underlying mechanism of LBP-mediated renal protection.

It has been reported that increased adipose mass leads to decreased levels of adiponectin and reduced activation of the adiponectin receptor on podocytes, reduced AMPK activation, and increased inflammation (32, 33). The binding of adiponectin to both AdipoR1 and AdipoR2 increases the activation of the downstream signaling mediator AMPK. Adiponectin also activates the downstream signaling mediator peroxisome proliferator-activated receptor, as well as the AMPK pathway *in vitro* (34, 35). In this study, we found that LBPs activated adiponectin as well as AMPK in the kidney tissue, reducing the damage induced by high lipids.

A high-fat diet leads to fatty acid metabolic disorders and induces oxidative stress as well as renal injury. Superoxide anion plays a key role in the induction of oxidative stress, which leads to oxidative damage to lipids, proteins, and DNA. While LBPs may modulate lipid metabolism and exert a strong antioxidant effect, its underlying mechanism merits further investigation.

High lipid levels represent an important risk factor for renal injury. The mechanisms underlying this association involve systemic effects such as persistent inflammation, as well as oxidative stress. In this study, we found that LBPs reduce high-fat diet-induced renal injury, indicating that LBPs

mediate lipid metabolism and regulate TNF- α , IL-6, SREBP-1, adiponectin, and AMPK. This regulation may be the underlying mechanism by which LBPs ameliorate renal injury in a rat model of hyperlipidemia.

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EFFECT OF ZINC, COPPER AND RESVERATROL SUPPLEMENTATION ON THE URINARY LEVEL OF 15-HYDROXYEICOSATETRAENOIC ACID AT THE EARLY STAGE OF BREAST CANCER IN RATS

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The aim of the present study was to assess the effect of zinc and copper, separately or in combination with resveratrol, on the concentration of 15-hydroxyeicosatetraenoic acid (15-HETE) in urine of rats with mammary cancer (adenocarcinoma) induced with 7,12- dimethyl-1,2-benz[a]anthracene (+ DMBA). The research focused on the kinetics of alterations in urinary 15-HETE at the early stage of carcinogenesis, as well as on the influence of dietary factors on the process. The content of 15-hydroxyeicosatetraenoic acid in the rats' urine was determined by enzyme-linked immunosorbent assay (ELISA). The 15-HETE level was standardized by conversion to the creatinine level. Regardless of the diet (standard, Zn, Zn+resveratrol, Cu, Cu+resveratrol), DMBA-induced breast carcinogenesis was not inhibited. On the contrary, in the Zn+resveratrol supplemented group, tumorigenesis developed at a considerably faster rate. It was found that the supplementation of the rats' diet with zinc only or zinc plus resveratrol resulted in a reduction of the 15-HETE level in urine of rats with mammary cancer (+ DMBA) as compared with control rats (- DMBA). A statistically lower concentration of 15-HETE was found in urine of rats supplemented with zinc or copper with resveratrol when compared with the animals that received only zinc or copper ($p=0.02$; $p=0.0001$). Summing up the obtained results, it can be concluded that the level of 15-HETE in urine of the examined animals depended on the applied supplementation.

Mounting evidence suggests that lipoxygenase (LOX) catalyzed products have a profound influence on the development and progression of human cancers (1). Hydroxyeicosatetraenoic acids (HETEs) are monohydroxylated derivatives of arachidonic acid (AA), mainly produced via the action of stereospecific lipoxygenases. The initial products are unstable hydroperoxides which are either spontaneously or peroxidatively reduced to the corresponding monohydroxylated products (2). Due

to the different LOX isoforms that are involved in arachidonic acid metabolism, three main isomers of HETE are generated: 5-, 12- and 15-HETE (1). The elevated levels of 5-HETE and 12-HETE together with prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) might promote tumor progression, whereas others, such as 15-HETE, might interfere with the progression to malignancy (3). It was found that 5-LOX and 12-LOX were over-expressed in the breast cancer of patients with a poor survival rate,

Key words: 15-HETE; cancer; zinc; copper; resveratrol.

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while the level of 15-LOX moved in the opposite direction (4). 5-HETE and 12-HETE might stimulate breast cancer cells in the culture to proliferate and might thereby mediate the effect of 5-LOX and 12-LOX as related to survival (5). In human breast cancer cells, the specific inhibition of both 5-LOX and 12-LOX resulted in significant apoptosis of cells associated with induced cytochrome release, and the activation of many caspase family members (6). Nony et al. (7) observed that the addition of exogenous 15-HETE to breast cancer cells stimulated cell adhesion to type IV collagen and activated the p38 MAPK (mitogen-activated protein kinases) pathway. 15-Hydroxyeicosatetraenoic acid is an endogenous ligand of peroxisome proliferator-activated receptor gamma (PPAR- γ) (8-10). The activation of PPAR- γ can inhibit the growth of cancer cells (8-10). Gonzalez et al. (8) found that the reduced 15-HETE value in lung cancer was inversely proportional to the stage of tumor growth and intensification of cell proliferation. Yuan et al. (9) found that 15-LOX, 15-HETE and 13-HODE (13-hydroxyoctadecadienoic acid) levels were significantly reduced in human lung cancer tissue compared with non-tumor lung tissue. Their findings indicate that the reduction in 15-LOX, 15-HETE and 13-HODE results in the decreased PPAR- γ activity seen in lung tumors and contributes to the development of lung tumors induced by tobacco smoking (9). Such a negative correlated relationship between 15-LOX and PPAR- γ expression has also been found in other epithelial cancers (10). Given the strong associations between the level of 15-HETE and cancer risk, there is considerable scientific interest in determining whether dietary factors would stimulate its production.

The aim of the present study was to assess the effect of zinc and copper, separately or in combination with resveratrol, on the concentration of 15-hydroxyeicosatetraenoic acid in urine of rats with mammary cancer (*adenocarcinoma*) induced with 7,12-dimethyl-1,2-benz[a]anthracene (DMBA). The research focused on the kinetics of alterations in urinary 15-HETE at the early stage of carcinogenesis, as well as on the influence of dietary factors on the process.

MATERIALS AND METHODS

Female Sprague-Dawley rats (n=83) were obtained from the Animal Laboratory, Department of General and Experimental Pathology, Medical University of Warsaw. The study was approved by the Ethics Committee, Medical University of Warsaw. After a 10-day adaptation period the animals were divided into two experimental groups. In group 1 (+ DMBA, study group n = 49), the rats were treated twice with 80 mg/kg body weight DMBA (7,12-dimethyl-1,2-benz[a]anthracene; Sigma-Aldrich, St. Louis, MO, USA) in rapeseed oil (via gavage) to induce mammary cancer (adenocarcinoma); the first treatment was given at 50 days, followed by the same dose (a repeated dose of 80 mg/kg body weight) at 80 days of rat age, and in group 2 (- DMBA, control group n = 34), the rats were accommodated under the same conditions as those in Group 1; fed the same diet, but without DMBA treatment.

All rats were provided Labofeed H standard diet (standard diet: Labofeed H, Żurawia 19, 89-240 Kcynia, Poland) and water ad libitum and housed in an environmentally-controlled room maintained at 22°C with a 12-h light-dark cycle. The animals from both groups were also fed diets different in biocompounds. The diets were supplemented with:

- zinc 6.9 mg/mL (231 mg Zn(II)/kg diet) (as ZnSO₄ 7H₂O);
- copper 1.3 mg/mL (42.6 mg Cu(II)/kg diet) (as CuSO₄ 5H₂O);
- zinc 6.9 mg/mL (231 mg Zn(II)/kg diet) and resveratrol 0.1 mg/mL (0.2 mg/kg bw);
- copper 1.3 mg/mL (42.6 mg Cu(II)/kg diet) and resveratrol 0.1 mg/mL (0.2 mg/kg bw).

The rats were fed extra supplements suspended in water (0.4 mL daily via gavage), from 40 days until 20 weeks of age (sacrifice time by decapitation). The dose of Zn and Cu was established based on the values used in the Labofeed H diet (standard diet). The Zn-exposed group received zinc in an amount exceeding three times its fodder level (standard diet – 76.9 mg/kg diet), the Cu-exposed group received copper in the amount exceeding twice its fodder level (standard diet – 21.3 mg/kg). The resveratrol dose level was selected based on human average daily consumption (extrapolating on the rats' body weight) (11). Identifying the results of trace

elements and resveratrol activity in selected doses may allow application of these dietary compounds in human cancer prevention or for improvement of pharmacological treatment.

The animals were examined by palpation during the study to characterize the time course of tumor development. It was performed by a person with experience in this field. In order to obtain urine samples, each animal was individually placed in a metabolic cage for 24 h. Urine samples were collected once a week during the 10th, 14th and 19th weeks of rodent's age. The biomaterial samples were stored at a temperature of -70°C until the test time.

The concentration of 15-HETE in urine was determined by a competitive enzyme-linked immunosorbent assay (ELISA) (15-HETE EIA Kit, Cayman Chemical Company Ann Arbor, MI, USA).

The 15-HETE level was standardized by conversion to the creatinine level. The latter was determined in urine samples with a creatinine test (Hydrex, Warsaw, Poland)

based on Jaffe's reaction (in an alkaline environment, creatinine forms a colored complex with picric acid).

Histopathology

The tissues were recorded in a buffered formalin solution, then they were dehydrated, sealed in paraffin and cut into scraps 4 μm thick. The hematoxylin and eosin staining of tissue and cell sections was applied and the sections were evaluated using a BX43 Olympus research microscope.

Statistical analysis

Statistica 12.0 software (StatSoft, USA) was used for statistical analysis. The data normality was tested with Shapiro-Wilk method. For the normal data, the Student's *t*-test and ANOVA, followed by Newman-Keuls test were used for analysis. The non-normal data was analyzed with Mann-Whitney U nonparametric test. In order to verify the results of tumor incidence, in the DMBA treated rats, the relative risk (RR) calculation was used.

Table I. Tumor induction in 7,12-dimethylbenz[a]anthracene treated groups in relation to diet.

Diet	The week when first tumor occurred	Tumor incidence ¹ (%)		
		10 week	14 week	19 week
Standard	15	-	0/9 (0)	9/9 (100)
Zn (6.9 mg/mL)	15	-	0/9 (0)	9/9 (100)
Zn (6.9 mg/mL) and resveratrol (0.1 mg/mL)	13	-	2/10 (20)	10/10 (100)
Cu (1.3 mg/mL)	14	-	1/9 (11)	9/9 (100)
Cu (1.3 mg/mL) and resveratrol (0.1 mg/mL)	14	-	2/12 (17)	12/12 (100)

¹Tumor incidence is expressed as the number of tumor-bearing rats over total number of rats in the group.

RESULTS

The rats were treated with DMBA at week 7 of their life (on day 50 of life – it is considered that the animals become sexually mature). The first urine samples were collected at week 10. No tumors were found by palpation at that time (week 10) in any of the examined rats. The maintenance dose of DMBA was applied to the examined group of rats during week 11 of their life. The first palpable tumor was found in one out of 10 rats in the group of animals treated with Zn with resveratrol in the 13th week of their life (Table I). At week 14, tumors were observed in 11% of the cases of the rats supplemented with copper as well as in 17% of the cases of the rats supplemented with copper and resveratrol. At week 19 of life the incidence of carcinogenesis was 100% for all the examined groups (12). Tumors were histopathologically evaluated to

confirm their malignancy and to prove that they were adenocarcinomas (degrees II and III) (Table II) (Fig. 1). Spontaneous cancers were not found in the non-DMBA groups.

As a result of the performed investigation, it was found that the animals with mammary cancer (+ DMBA), supplemented with zinc and zinc plus resveratrol were characterized by a statistically significant lower biomarker level in urine from week 10 and week 14 (early stage of carcinogenesis), as compared with the non-DMBA-treated rats (-DMBA) that received the analogous supplementation ($p \leq 0.001$; $p \leq 0.01$) (Fig. 2). An increase of the 15-HETE level was observed in urine of the rats from the study group (+ DMBA) supplemented with copper plus resveratrol ($p \leq 0.05$), as compared with the respective control group (DMBA-) in week 19 of the investigation (Fig. 2).

Table II. Number of tumors and maximum tumor weight in one rat group (g) in relation to diet.

Diet	Number of tumors in one rat	Maximum tumor weight in one rat group (g)
standard	1-5	12
Zn (6.9 mg/mL)	1-5	20
Zn (6.9 mg/mL) and resveratrol (0.1 mg/mL)	2-10	30
Cu (1.3 mg/mL)	1-5	7
Cu (1.3 mg/mL) and resveratrol (0.1 mg/mL)	2-6	11

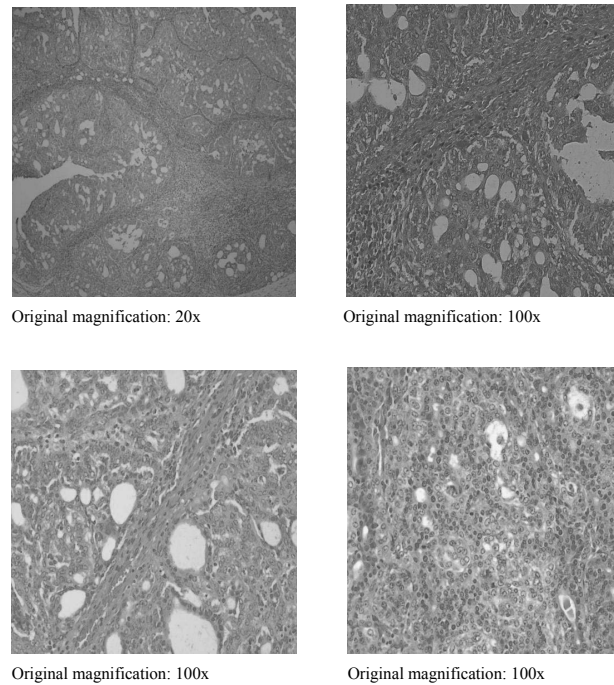


Fig. 1. Hematoxylin and eosin staining of rat mammary tissue sections (adenocarcinoma).

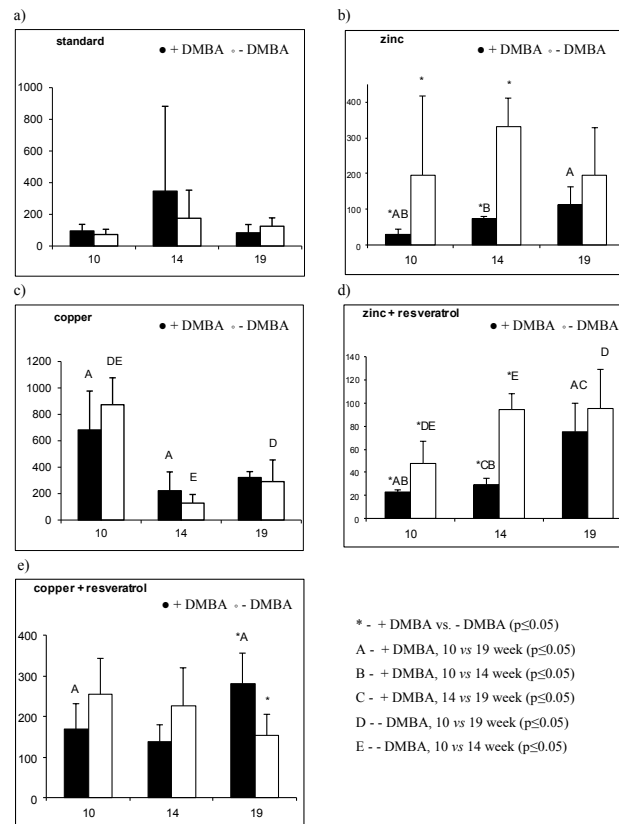


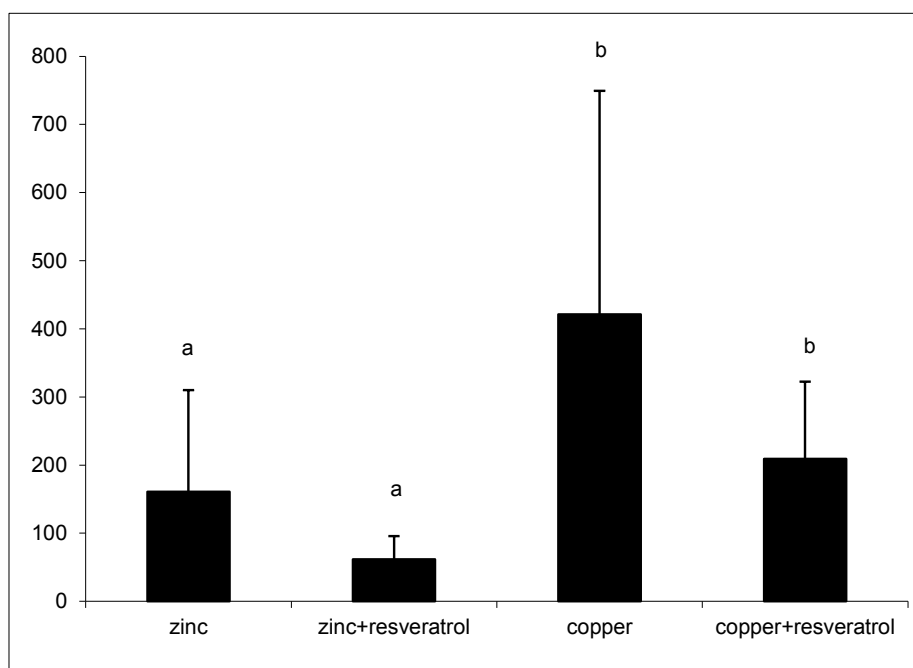
Fig. 2. Urinary 15-hydroxyeicosatetraenoic acid (15-HETE) (pg/mg creatinine) (mean±standard deviation) in DMBA-exposed and nonexposed rats fed different diets (weeks 10-14-19).

A significant increase of the 15-HETE level was observed between weeks 10 and 19 of the investigations in the case of rats supplemented with zinc ($p \leq 0.005$) and also with the rats supplemented with zinc plus resveratrol ($p \leq 0.005$) and copper with resveratrol ($p \leq 0.05$) (groups + DMBA). In the groups supplemented with zinc and zinc plus resveratrol a significant increase of 15-HETE was also found between weeks 10 and 14 of the investigations ($p \leq 0.001$; $p \leq 0.01$) (groups + DMBA). In the groups supplemented with copper: both in the rats from the study groups and the control ones, a significantly reduced 15-HETE level was observed between week 10 and week 14 ($p \leq 0.02$; $p \leq 0.001$), and in the control groups between weeks 10 and 19 ($p \leq 0.005$) of the investigations.

A statistically lower concentration of 15-HETE was found in urine of rats supplemented with zinc or copper with resveratrol when compared with the animals that received only zinc or copper ($p = 0.02$; $p = 0.0001$) (Fig. 3).

DISCUSSION

A novel approach to cancer prevention would involve LOX modulators which would shift the balance from pro-carcinogenic (5-,12-HETE) to anticarcinogenic (8-,15-HETE) metabolism of AA (3). The aim of the performed studies was to find out whether it would be possible to stimulate the production of 15-HETE by use of the appropriate dietary components. In our planned research the diet of the examined rats was supplemented with zinc and copper ions as well as with a polyphenolic compound, i.e. resveratrol. Irrespective of the applied supplementation with zinc only or zinc plus resveratrol, a 2-4-fold decrease of the 15-HETE level was observed in urine of rats with mammary cancer as compared with the rats from the control group (- DMBA) fed the same diet (weeks 10 and 14 - early stage of carcinogenesis) (Fig. 2). What is interesting is that the animals treated with DMBA and supplemented with zinc with resveratrol were



a - zinc versus zinc + resveratrol ($p = 0.02$)
 b - copper versus copper + resveratrol ($p = 0.0001$)

Fig. 3. Urinary 15-hydroxyeicosatetraenoic acid (15-HETE) (pg/mg creatinine) (mean±standard deviation) in DMBA-exposed rats fed diets with addition of zinc or copper separately or in combination with resveratrol.

characterized by a statistically significant lower level of 15-HETE in urine as compared with the animals that received a standard diet or only zinc. The first palpable tumor was found in one out of 10 rats in the group of animals that obtained Zn with resveratrol in the 13th week of their life (Table I). In spite of applying double the doses of DMBA during the 13th and 14th week in groups of animals that received a standard diet or a Zn supplemented diet, no tumors were observed until the 15th week.

Cancer and inflammation are critically linked, and among the enzymes that synthesize proinflammatory mediators from arachidonic acid are the cyclo- and lipoxygenases. The synthesis of proinflammatory molecules by COX (cyclooxygenase) and LOX is an important step for initiation of tumorigenesis. Thus, the inhibitory effects of resveratrol on COX-1/LOX catalytic activities and COX-2 transcription, with consequent decrease of COX-2 protein levels, are highly relevant to cancer prevention (13). Polyphenolic compounds have already been shown to be preventive against various stages of chemically induced carcinogenesis (14, 15). The use of resveratrol was additionally justified by the fact that this compound, depending on the dose applied, inhibits the 12-HETE production (16). Zinc is part of many transcriptions factors. This element is responsible for induction or moderation of apoptosis, activation or inhibition of enzymes as well as regulation of cellular signaling and maintaining a balance between oxidation and reduction processes (17). However, the supplementation of rat diet with those two dietary antioxidants rather unexpectedly quickened the rate of tumor growth in DMBA-treated rats and also resulted in a possibly related decrease of the 15-HETE level in urine of cancerous rats as compared with the control group, especially in the early stage of carcinogenesis (Table I). The question now arises as to whether this decrease is due to the reduced production of this biomarker, in favor of other LOX metabolites. The intracellular zinc release causes the activation of 12-LOX (18). Breast cancer is associated with zinc hyper-accumulation in breast tissue which is postulated to be potentiated by the over-expression of Zn importing proteins. Zinc transporter Zip6 [LIV-1 (estrogen regulated protein

LIV-1)] over-expression has been documented in estrogen receptor-positive (ER+) breast tumors (19). In the case of its imbalance in the body, zinc may act as a double-edged weapon and can lead to a number of metabolic disorders (19).

On the other hand, the procancerogenic activity of copper was found to be related to the formation of reactive oxygen radicals that impair DNA threads and to initiation of angiogenesis of the tumor. Copper metabolism is profoundly altered in neoplastic disease (20). Many investigations have been performed that confirmed the effectiveness of using copper chelators (e.g. penicillamines, tetrathiomolybdate, zinc anions) as anticancer agents (21, 22). In this research, resveratrol was used as the copper chelator because this compound occurs in nature and exhibits strong anti-oxidative properties (16). The hydroxyl groups of polyphenols, when reacting with free radicals, form more stable complexes. Furthermore, polyphenols complexate the Cu (II) and Fe (II) ions, and can thus inhibit the activity of enzymes, e.g. of xantine oxidase, and of the processes intensifying the formation of free radicals (16, 23). As a result of the present investigation it was found that in the group of rats supplemented with copper as well as with copper plus resveratrol, the first tumors appeared a week later than in the group supplemented with zinc plus resveratrol (in 14th week) (Table I). The rats that received copper together with resveratrol did not result in lower incidence of carcinoma but, on the contrary, the presence of resveratrol stimulated the process, which was evidenced by increase in the maximum tumor weight (in one rat) (11 g) and a number of tumors in one rat (2-6) (Table II). What is interesting is the concentrations of 15-HETE in urine of rats supplemented with copper and resveratrol (+ DMBA) increased with time of the experiment (Fig. 2).

Resveratrol exhibits a strong antioxidative activity as it inhibits the activity of 5-lipoxygenase enzyme. As mentioned previously, dioxygenase is one of the key enzymes in the arachidonic acid transformations. It catalyzes the reactions, a result of which eicosanoids and leukotrienes form, as well as the lipid oxidation reactions which are a notable constituent of oxidative stress in cells (24). As a result of those oxidative transformations, the reactive toxic aldehydes form, among which is

4-hydroxynonenal (4-HNE). The latter plays an important role in the process of signal transduction and cell proliferation and exhibits the unfavorable clastogenic activity which causes changes in chromosome structure. Chatterjee et al. (24) found that after a single DMBA injection the concentration of 4-HNE increased about 2.5-fold in comparison with the group of rats which were not treated with carcinogen. The concentration of lipid peroxidation products was reduced by 35% when the rats' diet was supplemented with resveratrol at a dose of 100 µg/rat. Peroxidation of arachidonic acid causes DNA double strand breaks, because in the course of peroxidation the hydroxy- and hydroperoxy-6,8,11,14-eicosatetraene acids (HETE and HPETE) form which cause damage to the genetic material of the cells. The metabolites of arachidonic acid are also responsible for the appearance of inflammatory disorders. Overexpression of 5-LOX was described mostly in pre-neoplastically transformed cells of breast cancer, but it was also found in prostate cancer, pancreatic cancer and esophageal cancer. Thus it seems that the inhibitors of that enzyme, to which resveratrol belongs, inhibiting cell proliferation and favoring the apoptosis of neoplastic cells, can be potential and promising drugs for the therapy and prevention of breast cancer (24). It is worth noting that during the perinatal period the organism is very sensitive and so the high level of exposure to estrogens during pregnancy increases the risk of mammary cancer in female offspring (25). It also seems that the character of the resveratrol effect on the organism varies and is dependent on the age of the animals when the resveratrol supplementation was introduced. Our rats were treated with DMBA at week 7 of life (on day 50 of life – it is considered that the animals become sexually mature at this time). With regard to resveratrol, there are few investigations concerning this period. In adult rats, resveratrol, when administered by a stomach tube or with the diet, significantly inhibited mammary oncogenesis induced by MNU (N-methyl-N-nitrosourea) or DMBA (26, 27). In our previous study it was shown that resveratrol when applied at a dose of 0.2 mg/kg bw inhibits the formation of tumors in rats induced by 7,12-dimethylbenz[a]anthracene (incidence 40%)

as compared with the rats that were fed only the standard diet (incidence 86%). The maximum tumor weight was much lower than in the control group (0.62 - 7.22 vs 0.49 - 23.57 g). However, at the same time, it was observed that one rat supplemented with resveratrol developed as many as 8 tumors, which shows that the response to the applied compound can vary from individual to individual (28). On the other hand, in the paper of Sato et al. (25) resveratrol at a dose of 100 mg/kg body weight accelerated the appearance of MNU-induced mammary carcinomas, but no such activity was observed when resveratrol was given at a dose of 10 mg/kg body weight. Cancer incidence and multiplicity were significantly higher in the high-dose group, whereas latency was unchanged. As the majority (80%) of MNU-induced mammary carcinomas are hormone-dependent, resveratrol appears to increase the ER - and/or PgR-positive cells presumed to be the progenitors of hormone-dependent carcinomas, resulting in a higher mammary carcinoma yield (29, 30).

Summing up the obtained results, it can be concluded that the level of 15-HETE in urine of the examined animals depends on the applied supplementation.

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EXPRESSION OF BONE-RELATED PROTEINS IN VASCULAR CALCIFICATION AND ITS SERUM CORRELATIONS WITH CORONARY ARTERY CALCIFICATION SCORE

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Since vascular calcification is considered a process regulated similar to that of bone tissue mineralization, we investigated the participation of bone formation proteins. We analyzed the correlation of serum circulating bone markers, osteoprotegerin (OPG) and receptor activator of nuclear factor κ B ligand (RANKL) in chronic kidney disease (CKD) patients, to coronary artery calcification score. We also considered the effect of inorganic phosphate on pro- and anti-calcifying tissue factors. We confirmed that circulating OPG is an independent calcium score predictor with its high serum concentration favoring high coronary artery calcification. In tissue samples of non-diseased human renal arteries, the expression of OPG and receptor activator of nuclear factor κ B (RANK) was positive, while expression of RANKL was absent. In atherosclerotic specimens and arteries with medial calcification, the most upregulated was expression of bone morphogenetic proteins, BMP-2 and BMP-7, as well as expression of RANK and RANKL. In the diseased arteries, OPG expression was present only in areas where bone structures were formed. In atherosclerotic and medial calcification arteries, loss of α -smooth muscle actin (α -SMA) expression was observed. These data suggest a possible regulatory role of the examined proteins, especially OPG and RANKL, in vascular calcification, as well as their possible clinical significance as circulating predictors of vascular calcification.

Vascular calcification is a process characterized by mineralization of soft tissues, triggered by disorders in calcium-phosphate homeostasis or activation of inflammatory cytokines. Furthermore, they suggest an osteoporotic bone loss, and the finding that OPG-null mice had osteoporosis and arterial calcification revealed a possibility that intimal and medial calcification are an active cell-mediated process, imitating osteogenesis (1).

Chronic kidney disease (CKD) is closely related

to vascular calcification, due to increased circulating phosphate levels throughout the disease (2). What is significant to observe in vascular calcifications, is that they present a great risk of mortality and morbidity (2).

Vascular smooth muscle cells (VSMC) are widely perceived as terminally differentiated cells in the vascular media. However, it has been shown that cultured VSMCs undergo dedifferentiation (3). Hyperphosphatemia has the potential to change the phenotype of human VSMCs from contractile to

Key words: calcification score; chronic kidney disease; OPG; RANKL; vascular calcification

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secretory (4). Vascular calcification is an imbalance, resulting from an imbalance between calcification promoters and inhibitors, whether free in the circulation or attached to the vascular wall.

The identified proteins with the role of a calcification inhibitor are osteopontin (OPN), fetuin-A, bone morphogenetic protein-7 (BMP-7) and osteoprotegerin (OPG); while recognized promoters of calcification are hyperphosphatemia, bone morphogenetic protein-2 (BMP-2) and receptor activator of a nuclear factor κ B ligand (RANKL) (5).

New insights into vascular biology are provided by assessing members of the tumor necrosis factor alpha (TNF α) family that present a functioning link between skeletal and vascular system - RANK/RANKL/OPG triade. In bone tissue, OPG is produced by osteoblasts and acts as a decoy receptor that blocks the interaction between RANK and its ligand, RANKL. In this same manner, OPG reduces the number of active osteoclasts which increases bone volume and, consequently, bone mineral density. Conversely, RANKL is expressed on osteoblasts as transmembrane protein. But then again, in soluble form it can also be found in the circulation (6). Besides their presence in bones, OPG and RANKL are significantly involved in the control of the vascular and immune systems.

We aimed to investigate whether the human renal arterial walls of non-diseased and diseased arteries differ significantly, considering the expression of the above-mentioned proteins. Also, in mild-to-moderate chronic kidney disease (CKD) the precise time-based relationship between circulating bone markers, OPG, RANKL, and coronary calcification prior to and after kidney transplantation remains mostly unknown.

Therefore, we observed patients with several stages of CKD and those with functioning renal graft to study vascular calcification signs and possible serum predictors assessing bone-related proteins.

MATERIALS AND METHODS

Patients and baseline data

We enrolled a total of 185 CKD patients who were recruited from the Clinical Hospital Rijeka, Croatia and grouped according to renal function. Three groups were formed: group I consisted of 42 patients with stages III

and IV of CKD; 105 patients with stage V of CKD were in a group II; and 38 patients in group III (Tx12 group - 12 months post-transplantation) were post-transplant patients followed-up 6 to 12-months post-transplantation.

A control group of 40 healthy individuals was matched for sex and age and was studied at the same time. Inclusion criteria for this group were no bone or renal diseases, no therapy with medication that could affect normal bone formation and parathyroid hormone (PTH) levels. All the otherwise hemodialyzed (HD) patients received regular hemodialysis three times a week with total duration of 12 h. The mean duration of dialysis treatment was 69 months. They received either high flux polysulfone HD or on-line hemodiafiltration treatments with bicarbonate-buffered dialysate. All the dialyzed patients were receiving concomitant drugs such as calcium carbonate (500mg to 3g/day) and calcitriol (0.25 μ g). They did not receive non-calcium-based phosphate binders. Transplanted patients had stable graft function (serum creatinine 1.5 $\pm$ 0.3 mg/dl). The immunosuppressive therapy included prednisone, mycophenolate mofetil, and tacrolimus.

All the experimental groups were included in the cross-sectional observational study on serum bone markers and its correlation to coronary artery score calcification score (CACS). Male/female ratio for group I was 20/20; for group II 45/45, group III or Tx12 group 14/16, while the controls were 20/20. Median age (years, range) across experimental groups was: for group I 72 (40 -82); for group II 64 (38-89); group III or Tx12 group 49 (28-67); and the control group 55 (29-74).

Laboratory measurements

Venous blood was assessed in the morning after overnight fasting. The samples were centrifuged, and sera aliquoted and stored at -80°C. No repeated thawing and freezing cycles occurred. Measurements of creatinine, total calcium, ionized calcium, phosphate, and albumin were provided at the Clinical Hospital, Rijeka by using a standard autoanalyzer (Olympus Apparatus, Rungis, France). Serum OPG and sRANKL were measured using a commercially available kit from Biomedica (Biomedica Gruppe, Wien, Austria).

Multidetector computed tomography

CACS was evaluated using multidetector computed tomography (MDCT) (16-slice technique on the model

Light Speed Ultra 16; GE Yokogawa Medical Systems, Tokyo, Japan). The timing of image acquisition was coordinated with the diastolic phase of the cardiac cycle at 70% or thereabout (total three phases) of the R wave to R wave interval (RR interval) as determined through electrocardiographic monitoring with a 2.5 mm gap between slices. Scanning time was about 30 s for the entire zone of interest that encompassed the whole heart. This equipment was capable of detecting lesions of a density of at least 130 Hounsfield units (HU) and a minimum area of 0.4882 mm². Total CACS was calculated using Smart score software (GE Yokogawa Medical Systems) in modified Agatston units (8).

Imaging procedure

From each studied group, 20 randomly selected subjects underwent a multi-row spiral CT (MSCT) to assess calcifications in coronary arteries. The Agatston scale (8) was used to interpret the results by CACS expressed in HU.

Tissue samples

Renal arteries (n=25) were sampled from 20 patients (14 male and 6 female) with renal cell carcinoma who underwent nephrectomy, and five patients who underwent nephrectomy because of traumatic lesions. Specimens were fixed in paraformaldehyde, embedded in paraffin, cut, stained with HE and Von Kossa silver nitrate, and examined by light microscopy. Specimens were distributed after histological examination in the following groups: control arteries without signs of calcifications (n=5), atherosclerotic arteries with intimal calcifications (n=10) and arteries with calcifications localized in the media (n=10). Control arteries were sampled from the patients aged 28-36 years without arterial hypertension, hypercholesterolemia, diabetes mellitus or chronic renal failure. Arteries with intimal and medial calcifications were sampled from the patients aged 50-95 years, most of whom had history of arterial hypertension, hypercholesterolemia, diabetes mellitus or chronic renal failure. The study was approved by the institutional ethics committee and written informed consent was obtained from all participants

Immunohistochemistry

Immunohistochemistry was performed by immunoperoxidase protocol on serial paraffin sections

(5 µm). The primary antibodies used were: OPG (1:25), RANK (1:50) and RANKL (1:50) (R&D Systems, Minneapolis, USA), BMP-2 (1:50) and BMP-7 (1:100) (Santa Cruz Biotechnology, CA, USA), OPN (1:100) and α-SMA (1: 100) were purchased from Dako, Glostrup, Denmark. Deparaffinized and rehydrated sections underwent antigen retrieval with Tris-EDTA for 15 min in a microwave oven (except OPG which had no pretreatment) and incubated with primary antibody for 1 h at room temperature. All slides were incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-goat secondary antibody (Dako, Glostrup, Denmark) and ABC reagent for 30 min. Reaction sites were visualized with diaminobenzidine tetrachloride and counterstained with hematoxylin. In negative controls incubation with primary antibody was omitted.

Human vascular smooth muscle cells (HVS MC)

HVSMC were maintained in Dulbecco's modified Eagle medium (DMEM) with 0.9 mM Pi, 1% Glut, P/S and supplemented with 15% FCS. Cells were maintained in culture at 37°C (5% CO₂, 90% humidity) and used from passage 3 to passage 8. Cells were exposed to 3 mM inorganic Phosphate (Pi) in DMEM for 14 days.

Western blot analysis of smooth muscle cells

After 14 days of incubation in 3 mM Pi, HVSMCs were lysed 10 min on ice. After cell lysis, protein concentration was determined by Bradford assay. Proteins were separated in 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The blots were then incubated overnight at 4°C with the following primary antibodies: anti-fetuin-A (1:1000), anti-OPG (1:500), anti-OPN (1:1000), anti-α-SMA (1:1000), anti-RANKL (1:500), anti-runX2 (1:1000) and anti-BMP2 (1:500). The blots were incubated with secondary antibody for 1 h at room temperature to detect immunoreactive bands. The membranes were developed with the enhanced chemiluminescence detection system and a specific, peroxidase-conjugated anti-IgG antibody. Band intensity was analyzed using the Kodak Bio Imaging System and protein levels were normalized against beta-actin protein expressions.

Statistical analysis

To evaluate the relation of serum bone markers to

vascular calcification, the subjects were classified into two groups according to CACS results; mild to moderate (CACS < 400HU) and severe to very severe calcification (CACS >400HU) group. This classification is a modification of the one proposed by Rumberger et al. (9) who recommended dividing coronary artery calcification scores into four categories. For this study the distribution was based on the results of Raggi et al. and Stompor et al. (10, 11).

All data are presented as mean, SD, median and range. Statistical methods included Mann-Whitney *U*-test for unpaired data, ANOVA for nonparametric data and Spearman's correlation coefficient. For polymerase chain reaction (PCR) and for Western blotting, data were analyzed using Kruskal-Wallis nonparametric test to determine significant differences ($p < 0.05$) in mean values between groups followed by Mann-Whitney *U*-test to evaluate the significance of differences between groups. Differences were considered to be significant when $p < 0.05$. Data was analyzed using Statistica 6.0. computer software.

Table I. Simple regression analysis between CACS and independent variables.

Independent variables	r	p value
Age (years)	0.22	<0.05
Gender	-	n.s
BMI	-0.22	<0.05
Dialysis duration	0.28	<0.01
Ca (mg/dl)	-	n.s
P (mg/dl)	0.24	<0.05
HDL (mmol/l)	-	n.s
LDL (mmol/l)	-	n.s
CaxPO ₄ (mmol ² /l ²)	-	n.s
CRP (mg/dL)	-	n.s
OPG (pmol/l)	0.46	<0.001
sRANKL (pmol/l)	-	n.s

CACS: coronary artery calcification score; BAP: bone alkaline phosphatase; iPTH: intact parathyroid hormone; CRP: C reactive protein; TRAP5b: tartrate-resistant acid phosphatase; iPTH: intact parathyroid hormone; BAP: bone alkaline phosphatase; OPG: osteoprotegerine; sRANKL: soluble RANK ligand; LDL: low-density lipoprotein; HDL: high-density lipoprotein.

RESULTS

Coronary artery calcification and risk factors

Coronary artery calcification was observed in 86% of the patients, with the median value of CACS equaled to 424 Agatston units (range 0 – 7338 units). Increase in CACS with CKD stage progression was observed ($p < 0.01$). ANOVA revealed significantly increased CACS values in the CKD5 group compared to the CKD3+4 and group III (Tx12 group) ($p < 0.001$) (Fig. 1).

In univariate analysis, CACS was significantly positively correlated with age, dialysis duration, P and OPG in CKD3+4 and CKD5 patients, and significantly negatively correlated with body mass index (BMI). No significant correlation was observed between CACS and gender, Ca, CaxPo₄, CRP, HDL, LDL or sRANKL (Table I).

Increased OPG bore greater CACS, whereas sRANKL did not have such implication – as per simple regression analysis revealed that in group III (Tx12 group) no significant correlation was found between CACS and any measured parameters (Table II).

Coronary artery calcification and risk factors: multivariate analyses

Potential independent variables in multivariate analysis were singled out based on the contributor's significance in simple regression analyses. After constructing two models in multivariable regression analysis with CACS as a dependent variable (using the stepwise forward method and another one adjusted for traditional risk factors such as age and gender).

Using that former model, OPG proved to be positively linked to CACS ($r=0.311$, $P=0.028$). Even after adjusting for eGFR and traditional risk factors, such as age and gender, OPG was still positively correlated with CACS (Table III). In multivariate analysis between CACS and the before-mentioned parameters only OPG remained an independent predictor of CACS.

Expression of bone-related proteins in the calcified arterial wall: parallel observations in atherosclerotic lesions and arteries with medial calcification

Histologically, vascular calcification is referred to atherosclerosis, Mönckeberg's sclerosis, calcification

Table II. Simple regression analysis for CACS at cut off value of 400HU by bone metabolism markers.

Independent variables	ca score		p value
	< 400 HU	> 400 HU	
OPG	5.81±0.33	11.27±0.92	<0.001
sRANKL	2.58±0.36	2.21±0.32	0.524

Simple regression analysis revealed that CACS was positively correlated with OPG but not sRANKL after adjusting CACS at cut off value of 400HU.

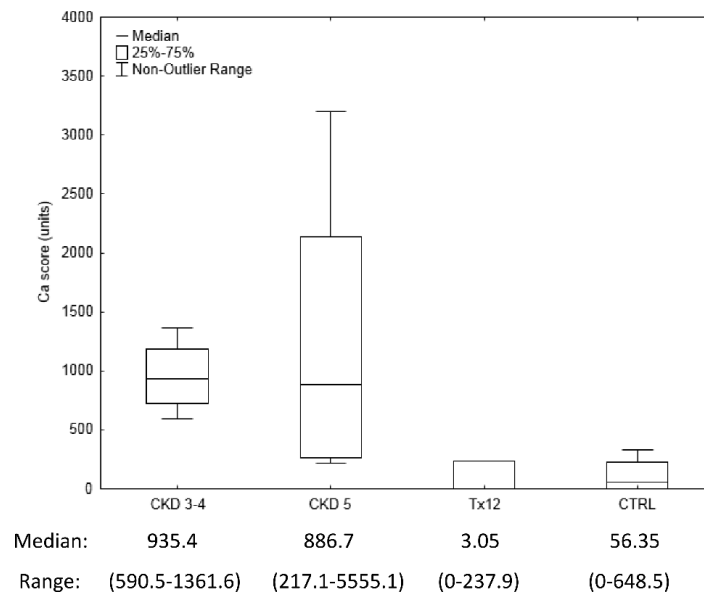


Fig. 1. Increase in coronary artery score calcification score (CACS) with chronic kidney disease (CKD) progression. As CKD stage progressed, the CACS increased significantly ($P<0.001$). CACS was significantly higher than in patients with CKD stage 5 than in patients with stage 3+4 and in patients one year after transplantation (group III or Tx12 group) ($P<0.001$). CACS in patients in group III or Tx12 group was similar to those in control group.

of cardiac valves and vascular calciphylaxis. Doherty et al. (12) identified two different types of arterial calcification, localized in the media or the intima. Medial and intimal calcifications are different entities. Intimal calcification was observed almost exclusively in the atherosclerotic plaques. To date, several molecular mechanisms of plaque calcification have been identified, with many similarities to processes of bone formation and resorption. Also, calcium mineral deposition in atherosclerotic plaques is the result of intra-arterial processes of osteogenesis. Medial calcification occurs independently of atherosclerosis and is observed with high frequency in Mönckeberg's sclerosis, end-stage renal failure

disease, and diabetes mellitus. The mechanism of medial calcification is still not clear (7).

In the group with medial calcification, we sampled renal arteries with calcification deposits only in the media. In the atherosclerosis group, the calcification was found in the intima of the arterial wall and coincided with an advanced stage of atherosclerotic changes.

Alpha-smooth muscle actin (α -SMA) is a marker of VSMC phenotype. Changing of VSMC phenotype is underlying of atherosclerosis and medial calcification pathogenesis. Since transformed VSMC lose contractile capabilities, the number of α -SMA positive cells decreased especially in

surrounding calcified areas in tunica media. We also found α -SMA marker in the intimal layer of renal artery which implies the migration activities of changed smooth muscle cells from the tunica media during the process of atherosclerosis.

Control arteries

Bone matrix proteins involved in bone formation BMP-2, BMP-7 and OPN were not detected in the control arteries, nor in medial and intimal vessel layer. We found positive staining for OPG and RANK in smooth muscle cells of the media. Also OPG positive staining was found in endothelial cells. RANK ligand was not detected in any observed control specimen.

Atherosclerotic specimens

All investigated proteins, except OPG and RANKL, showed immunoreactivity throughout initial and advanced stages of atherosclerosis, however, the localization of the proteins was diverse and depended on the cellular structures. Presence of calcification was observed in the intima.

Intimal smooth muscle cells which originated from tunica media showed the expression of BMP-2, BMP-7, RANK, and OPN. Foam cells forming the lipid core highly expressed RANK and OPN, while endothelial cells lining the arterial lumen showed immunoreactivity of BMP-2, BMP-7, and RANK. Also, localization of OPN, BMP-2, BMP-7, and RANK is associated with the areas of calcification in the extracellular matrix.

RANKL was only observed surrounding the mineral deposits in advanced atherosclerotic plaque lesions characterized by lamellar bone structure and chondrocyte-like cells (Fig. 2). Immunoreactivity of OPG was found only in early stages of atherosclerosis at the same localization as RANKL.

Medial calcification

In this group, we observed dispersed vascular calcifications in the medial layer of the vessel wall. Immunoreactivity of RANKL was localized similarly as in atherosclerotic plaque, mostly in the extracellular matrix but only in the specimens with decreased α -SMA expression in tunica media. The

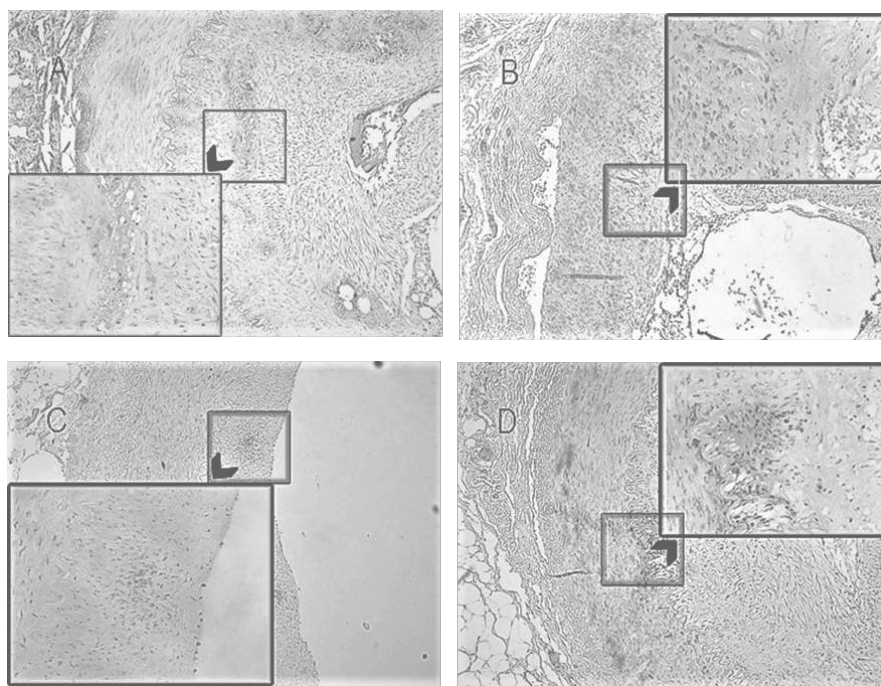


Fig. 2. Immunohistochemical staining for BMP-2 (A), BMP-7 (B), RANK (C) and RANKL (D) in renal artery atherosclerosis. Magnification $\times 100$ and $\times 400$ (insets), region of higher magnification is marked in black box.

expression of RANK was found in the cytoplasm of VSMC in tunica media, but RANK expression was evidenced in the early stage of calcification (Fig. 3). OPG expression was low and observed as faint staining in VSMC (data not shown).

Vascular calcifications in the media were linked with a decrease of α -SMA expression which signifies the changes of VSMC phenotype. OPN as specific osteoblast marker was expressed in all analyzed samples simultaneously with initial signs of calcification. Higher expression of OPN was associated with more severe cases of calcification (Table II). We noticed a negative correlation between OPN and α -SMA expression.

The expression of BMP-2 and BMP-7 was found in the cytoplasm of VSMC in calcified tunica media.

Expression of inhibitors and promoters of calcification in HVSMCs after exposure to phosphate

WB analysis of HVSMCs after 14 days of exposure to 0.9 and 3 mM Pi showed that in the condition of high phosphate protein, the expression of fetuin-A, OPG and α -SMA was decreased while the expression

of OPN, Runx2 and BMP-2 was increased compared to normal phosphate concentration (Fig. 4).

DISCUSSION

Vascular calcification is a biologically active process combined with the expression of many proteins that promote osteogenic differentiation in vascular tissue. Smooth muscle cells have an essential role in the secretion of new proteins in the media. The injury, which is induced by different etiological factors, causes changing of VSMC phenotype toward osteoblast-like cells. According to our results, this is manifested by a decrease of α -SMA, the key biomarker

Table III. Multiple linear regression analysis for CACS, adjusting for age and gender.

Independent variables	β -	<i>t</i> value	<i>p</i> value
Age (year)	0.0264413	0.95	0.3454
Gender (female)	0.104371	-0.35	0.7242
Osteoprotegerin (pmol/L)	1.4094878	2.16	0.035

$r^2=0.329$, $P<0.001$. CACS: coronary artery calcification score

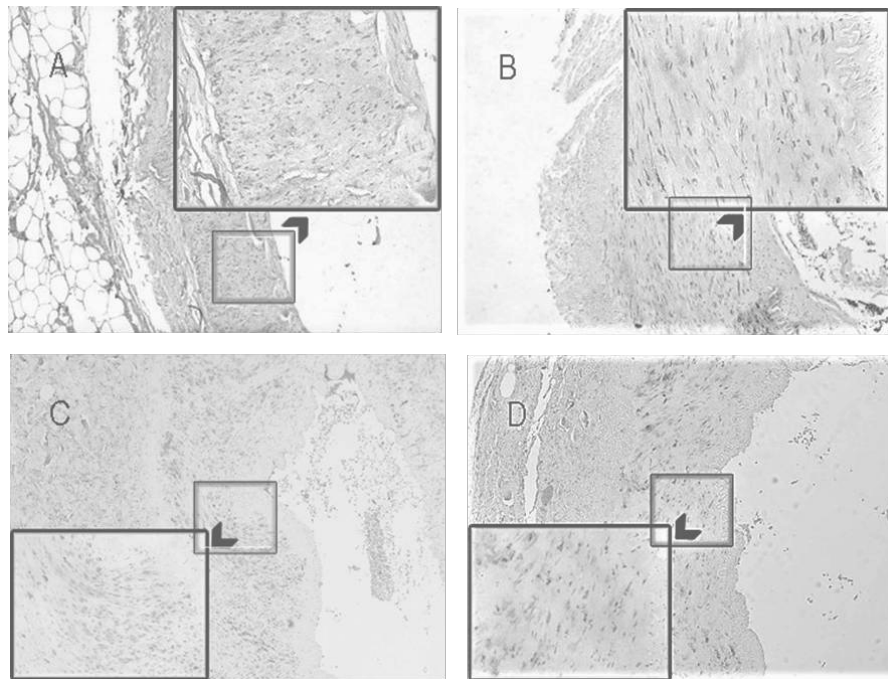


Fig. 3. Immunohistochemical staining for BMP-2 (A), BMP-7 (B), RANK (C) and RANKL (D) in medial calcification of renal arteries. Magnification x 100 and x 400 (insets), region of higher magnification is marked in black box.

of VSMC phenotype and by the expression of bone morphogenetic proteins 2 and 7 and osteopontin. Usually, VSMC does not express these proteins. Endothelial cells usually express osteoprotegerin that helps in endothelial survival. We found decreased osteoprotegerin expression in both atherosclerosis and medial calcification renal artery samples as well as in VSMC exposed to hyperphosphatemia.

In control samples of renal artery, RANK is expressed as well as in diseased artery with developed calcifications. The expression of RANKL occurs in the surrounding of the calcification process and its expression increases with the severity of calcification. However, in our VSMC, which was cultured with high phosphate for 14 days, there was no change in RANKL protein expression compared to control, which may indicate that the microenvironment of the vessel *in vivo* promotes RANKL expression, not only under the influence of phosphate but maybe under the influence of some other factors.

BMPs are studied, among other things, as essential factors in embryonal organogenesis. BMP-2 is capable of inducing bone formation through activation of transcription factors Runx2 and Osterix and is capable of differentiating mesenchymal stem cells

into osteoblasts (13, 14). BMP-2 is also studied in connection to vascular calcifications because of its role in either transformation of VSMCs into osteoblast-like cells, or inducing apoptosis, which is the initiator of calcification. It is already proved that BMP-2 is found only in calcified lesions and is absent in healthy vessels (15-17).

Our research also confirms the finding that in samples of renal arteries decreased expression of α -SMA positive cells was found, especially in calcified areas, which is probably caused by BMP-2 action. Whether the BMP-2 expression alone is substantial for induction of calcification, or whether it is accompanied with the loss of calcification inhibitors, remain to be elucidated. The same result of the protein expression was confirmed by Western blot in cells exposed to high phosphate.

The second most studied transforming growth factor (TGF)-beta member in vascular calcifications is BMP-7. During embryogenesis BMP-7 is responsible for kidney development, and is predominantly expressed by kidney tubular cells. Kidney injury decreases the BMP-7 expression (18- 20). This protein regulates skeletal remodeling and stimulates the expression of markers of VSMC phenotype, and also reverses vascular calcification in CKD (21). In our study, no BMP-7 expression was found in control arteries, but it is up regulated in calcified lesions along with BMP-2. Coexpression of two presumably opposite bone morphogenetic factors involved in vascular calcification could be explained as competing activity because they share common membrane receptors and signaling pathway.

Except for apoptosis, VSMC have the most important role in the process of vascular calcification. These cells are highly specialized in maintaining vascular tonus by contraction and relaxation. Moreover, they are capable of phenotypic change during development and, unlike other cells, do not terminate their differentiation. This is the reason why vascular tissue is competent in adaptation to various stimuli. In response to injury, these cells undergo phenotype transformation, lose their contractile capabilities and enters synthetic state producing large amounts of extracellular matrix (22). Smooth muscle actin is the most abundant protein in VSMC. We found decreased number of α -SMA

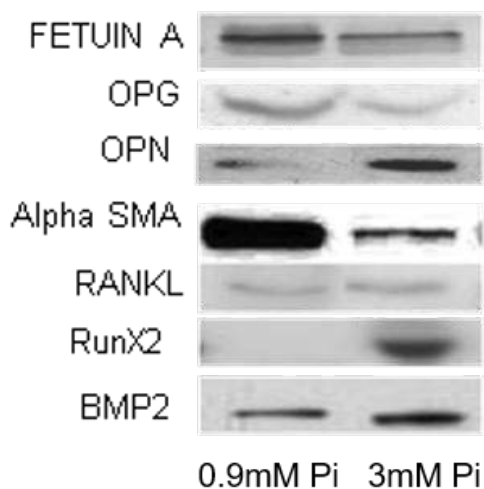


Fig. 4. Western blot representative bands obtained from HVSMS cells after 14 days exposure to 0.9 and 3 mM Pi conditions. In conditions of high phosphate protein expression of fetuin A, OPG and α -SMA was decreased while expression of OPN, Runx2 and BMP-2 was increased compared to normal phosphate concentration.

positive cells in calcified lesions compared to control renal arteries as well as decreased α -SMA protein expression in VSMCs in hyperphosphatemic condition.

At the same time, expression of OPN was up regulated and colocalized with sites of calcification. In control arteries OPN was absent. OPN was firstly identified in osteoblasts and later in a variety of acute and chronic inflammatory conditions. In atherosclerosis OPN is generally regarded as a proinflammatory and proatherogenic molecule. In medial calcification, OPN is a sign of osteoblast-like cell formation but its function is not clear, because the gathered results are opposing and describe OPN both as a positive and negative regulator of vascular calcification (23).

RANKL is a transmembrane protein expressed by T lymphocytes and osteoblasts. The main purpose of RANKL is to induce differentiation, activation and survival of osteoclasts through binding to RANK which is a transmembrane receptor expressed by macrophages, dendritic cells and osteoclasts. OPG is a soluble glycoprotein expressed by osteoblasts and acts as an inhibitor of osteoclast formation. These cytokines balance the sensitive homeostasis between bone formation and bone resorption (6, 24).

In normal arteries, OPG was expressed by endothelial cells and VSMC, while the expression of RANKL was either low or not even detectable in control vessels. No immunoreactivity of OPG was observed in calcified vessels, which confirms previous results that osteoprotegerin null mice developed severe vascular calcifications. Interestingly, RANKL was expressed in extracellular matrix surrounding calcium deposits. Contrary to atherosclerosis, medial calcification revealed decreased OPG staining presence and rare cases of RANKL positivity in calcified areas.

Our research reveals that RANK is the only protein whose expression was maintained from non-diseased arteries to disturbed vascular morphology. The role of RANKL as procalcifying agent is potentiated since levels of RANK expression persist in vascular calcification. Also, our results pointed out decreased OPG expression in calcified milieu, which enables RANKL acting and enhancing of osteogenic and inflammatory activations. In the literature, OPG is regarded as inhibitor of vascular calcification.

Many conflicting reports about circulating OPG

and RANKL levels in CKD have been published in the literature. Some authors state that elevated OPG levels inhibit stimulation of osteoclastogenesis induced by PTH, causing peripheral resistance to PTH (25). Others explain high OPG levels as compensatory mechanism that can prevent bone loss (26). Both are supported by our results, showing the negative correlation between OPG and iPTH values, also both markers correlated with age. The positive correlation between age and OPG was already noticed in a healthy population and in patients with end-stage renal disease (27). As shown previously, increased age and high OPG values are associated with occurrence of cardiovascular diseases, which is one of the leading causes of mortality in the HD population. Morena et al. report OPG levels as independent prognostic factor for vascular damage (28). Their results are in concordance with our findings that OPG is an independent predictor of high CACS.

This paper gives an overview of expression of calcifying promoters and inhibitors in atherosclerosis and medial calcification. Vascular calcification contributes to this cardiovascular disease, and currently there are very limited treatment or prevention options for vascular calcification. The process of vascular calcification is regulated by both the inhibitors and the promoters of calcification, but the promoting factors seems to overpower the inhibitors. The precise role of inhibitors remains to be determined.

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TRANSCRIPTOMIC PROFILE OF CELL CYCLE PROGRESSION GENES IN HUMAN OVARIAN GRANULOSA CELLS

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The ovarian granulosa cells (GCs) that form the structure of follicle undergo substantial modification during the various stages of human folliculogenesis. These modifications include morphological changes, accompanied by differential expression of genes, encoding proteins which are mainly involved in cell growth, proliferation and differentiation. Recent data bring a new insight into the aspects of GCs' stem-like specificity and plasticity, enabling their prolonged proliferation and differentiation into other cell types. This manuscript focuses attention on emerging alterations during GC cell cycle - a series of biochemical and biophysical changes within the cell. Human GCs were collected from follicles of women set to undergo intracytoplasmic sperm injection procedure, as a part of remnant follicular fluid. The cells were primarily cultured for 30 days. Throughout this time, we observed the prominent change in cell morphology from epithelial-like to fibroblast-like, suggesting differentiation to other cell types. Additionally, at days 1, 7, 15 and 30, the RNA was isolated for molecular assays. Using Affymetrix® Human Genome U219 Array, we found 2579 human transcripts that were differentially expressed in GCs. From these genes, we extracted 582 Gene Ontology Biological Process (GO BP) Terms and 45 KEGG pathways, among which we investigated transcripts belonging to four GO BPs associated with cell proliferation: “cell cycle phase transition”, “G1/S phase transition”, “G2/M phase transition” and “cell cycle checkpoint”. Microarray results were validated by RT-qPCR. Increased expression of all the genes studied indicated that increase in GC proliferation during long-term *in vitro* culture is orchestrated by the up-regulation of genes related to cell cycle control. Furthermore, observed changes in cell morphology may be regulated by a presented set of genes, leading to the induction of pathways specific for stemness plasticity and transdifferentiation *in vitro*.

Key words: human ovarian granulosa cells; cell cycle; progression; in vitro

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It is a fact that mammalian oocytes grow and develop during the stages of oogenesis and folliculogenesis within the follicular environment (1). Our previous experiment on porcine model indicated that proper oocyte maturation is dependent on unique cellular communication between the ovum and surrounding somatic cumulus cells (CCs), occurring through gap junction connections (GJCs). We have shown the expression of GJC markers, such as connexins (Cxs), between the oocyte and CCs. The aforementioned proteins are also expressed in follicular granulosa cells (GCs) (2). These observations suggest that both CCs and GCs may be involved in a unique cross-talk between the oocyte and somatic follicular cells during oocyte maturation, as well as proliferation and differentiation of surrounding cells. Therefore, it may be postulated that ovarian follicular GCs form not only the structure of follicular walls, but also display a role during gamete maturation, substantially supporting its growth and development (3–5).

Recent experimental data by Kossowska-Tomaszczuk et al. bring a new insight into the aspects of GC proliferation and differentiation *in vitro* (6). The result of the mentioned studies showed that GCs' long-term, *in vitro* culture, enriched with suitable stimulating factors, promoted GCs differentiation into osteoblasts, chondroblasts and neuroblasts. Based on these experiments, it is suggested that human GCs exhibit stem-like specificity and plasticity to differentiate into various, distinct cell types (7). This observation gives hope for GC-originating, newly differentiated cells that could find application in a wide spectrum of treatments related to reconstructive and regenerative medicine.

Moreover, the *in vitro* differentiability capability of human GCs was shown to be accompanied by changes in proliferation patterns. Noteworthy, GCs under physiological conditions are unable to proliferate longer than 10 days, since corpus luteum, which they form, degenerates after this time, stopping progesterone production (8). This brings attention to emerging alterations during the cell cycle, being a series of biochemical and biophysical changes within the cell. The cell cycle, leading to DNA synthesis and mitosis, consists of 4 basic phases: G1 (gap1), S (synthesis), G2 (gap2) and M (mitosis). Pre-division

cell growth and syntheses of macromolecules, like RNA and proteins, occur during the G1 phase, the synthesis of DNA during the S phase and cell growth, as well as chromosomal replication, control and repairs during the G2 phase (9). Although all the above steps are crucial for successful cell division, the gene expression profile describing cell cycle progression, including genes related to the G1/S phase transition, G2/M phase transition and/or cell cycle checkpoints, has never been investigated previously in the context of proliferation during long-term *in vitro* GC cultivation.

Therefore, this study was aimed to determine the expression profile of “cell cycle-progression”-related genes in human follicular GCs, as they may serve as a valuable model for investigation of their cellular proliferation capability during long-term *in vitro* culture.

MATERIALS AND METHODS

A large part of the materials and methods section is based on other, published work of the author, presenting results from the same cycle of studies describing human granulosa cells (4).

Patients and collection of granulosa cells

This study included 8 infertile patients aged 18–40 years, who were enrolled in the assisted reproduction program at the Division of Infertility and Reproductive Endocrinology, Department of Gynecology, Obstetrics and Gynecological Oncology, Poznan University of Medical Sciences, Poznan, Poland. All the patients were previously informed of the scope of research conducted and gave their written consent for the material collection and use in this particular experiment. All the experiments were approved by a local ethics committee (558/17 by Bioethical Committee at the Poznan University of Medical Sciences).

Patients with endometriosis, polycystic ovary syndrome (PCOS), and low ovarian reserve (indicated by a serum antimüllerian concentration (AMH) of less than 0.7 ng/mL or day 2–3 FSH serum level above 15 mU/mL) were excluded. Patients underwent controlled ovarian hyperstimulation protocol with human recombinant FSH (Gonal-F, Merck Serono) and highly purified hMG - HP (Menopur, Ferring) at a variable dose, adjusted according

to clinical and laboratory data. Pituitary suppression was based on the administration of cetrorelix acetate (Cetrotide, Merck Serono). Ovulation was triggered by subcutaneous administration of 6.500 U of hCG (Ovitrelle, Merck-Serono). The oocyte retrieval was performed trans-vaginally, 36 hours after hCG administration, under ultrasound guidance, in general anaesthesia. The aspirated follicular fluid, from follicles with an average diameter more than 16 mm, containing oocyte with corona radiata and granulosa cells was passed, without delay, to an embryologist. Follicular fluid, without the oocyte, was given to an employee of the Department of Anatomy, Poznan University of Medical Sciences, who performed the extraction of granulosa cells (GCs). Fresh follicular fluid with was centrifuged for 10 min at 200 g, to separate and collect GCs.

Primary cell culture

Collected GCs were washed and centrifuged at 200 x g for 10 min at RT, with culture medium. Medium consisted of Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, USA), 2% fetal bovine serum FBS (FBS; Sigma-Aldrich Co., St. Louis, MO, USA), 200 mM L-glutamine (Invitrogen, USA), 10 mg/ml gentamicin (Invitrogen, USA), 10,000 units/ml penicillin, and 10,000 µg/ml streptomycin (Invitrogen, USA). Cells were cultivated at 37°C under aerobic conditions (5% CO₂). Once adherent cells were more than 90% confluent, they were detached with 0.05% trypsin-EDTA (Invitrogen, USA) for 3-5 min and counted using a Z2 counter or Vi-Cell XR 2.03 cell viability analyzer (both Beckman Coulter, USA). Cells were cultivated for 30 days and the medium was changed twice a week. Total RNA, for further experiments, was isolated from GCs after 1, 7, 15 and 30 days.

Microarray expression analysis and statistics

Total RNA (100 ng) from each pooled sample was subjected to two rounds of sense cDNA amplification (Ambion® WT Expression Kit). The obtained cDNA was used for biotin labelling and fragmentation by Affymetrix GeneChip® WT Terminal Labeling and Hybridization (Affymetrix). Biotin-labelled fragments of cDNA (5.5 µg) were hybridized to the Affymetrix® Human Genome U219 Array (48°C/20 h). Microarrays were then washed and stained according to the technical protocol using the Affymetrix GeneAtlas Fluidics Station. The array

strips were scanned employing Imaging Station of the GeneAtlas System. Preliminary analysis of the scanned chips was performed using Affymetrix GeneAtlas™ Operating Software. The quality of gene expression data was confirmed according to the quality control criteria provided by the software. The obtained CEL files were imported into downstream data analysis software, as previously described (10).

All the presented analyses and graphs were generated using Bioconductor and R programming languages. Each CEL file was merged with a description file. To correct background, normalize, and summarize results, the Robust Multiarray Averaging (RMA) algorithm was used. To determine the statistical significance of the analyzed genes, moderated *t*-statistics from the empirical Bayes method were performed. The obtained p-value was corrected for multiple comparisons using Benjamini and Hochberg's false discovery rate. The selection of significantly altered genes was based on a p-value lower than 0.05 and expression higher than two-fold. The differentially expressed gene list (separated for up- and down-regulated genes) was uploaded to DAVID (Database for Annotation, Visualization and Integrated Discovery) software (11). The DAVID software analysis showed that differentially expressed genes belong to 582 Gene ontology groups and 45 KEGG pathways. In this paper, we focused on cell cycle-related genes belonging to the "cell cycle" pathway from Kyoto Encyclopedia of Genes and Genomes (KEGG) as well as from "cell cycle phase transition" (GO:0044772), "cell cycle G2/M phase transition" (GO:0044839), "cell cycle G1/S phase transition" (GO: 0044843) and "cell cycle checkpoint" (GO:0000075) gene ontology groups.

Interactions between cell cycle-related differentially expressed genes were investigated by STRING10 (Search Tool for the Retrieval of Interacting Genes) software (12). The list of gene names was used as a query for an interaction prediction. The search criteria were based on co-occurrences of genes/proteins in scientific texts (text mining), co-expression, and experimentally observed interactions. The results of such analyses generated a gene/protein interaction network where the intensity of the edges reflected the strength of the interaction score.

Real-Time q-PCR analysis

The RT-qPCR method was used to confirm the results

obtained in the analysis of expression microarrays. Based on the results obtained during the analysis of expression microarrays, three genes were selected from each heatmap: the ones showing highest, lowest, and intermediate-level of expression. Four biological samples of each gene were used for the analysis. Each biological test was performed in 3 replicates. Reverse transcription was based on the protocols and reagents of SABiosciences (RT2 First Stand Kit - 330401), using a Veritimer 96 well Thermal Cycler. 1 microgram of each gene's RNA transcript was used for reverse transcription. Real-time PCR was performed using the Light Cycler® 96 (Roche Diagnostic GmbH, Germany). RT2 SYBR® Green ROXTM qPCR Master Mix (Qiagen Sciences, Maryland, USA) and sequence-specific primers (Table I). *Glyceraldehyde-3-phosphate dehydrogenase (GADPH)*, *β-actin (ACTB)*, *hypoxanthine phosphoribosyltransferase 1 (HPRT1)* were used as housekeeping genes. Expression of all the genes was analyzed using the relative quantification (RQ) method. The q-PCR starters were designed using Primer3Plus software (<http://primer3plus.com/cgi-bin/dev/primer3plus.cgi>). The sequences of the respective genes were taken from the Ensemble database (<http://www.ensembl.org/index.html>), from which only the sequence of exons was exported, as the target sequence

of the designed starter was spread across the border of two adjacent exons. This approach was used as a precaution against the possibility of a non-specific DNA template-based product (DNAse contained in the reverse transcription kit was used as the other precaution). The primer attachment temperature was the geometric mean of all used primers.

RESULTS

To investigate the primary culture of human ovarian granulosa cell transcriptome changes following 1, 7, 15 and 30 days after the start of culture, we performed whole gene expression analysis by Affymetrix® Human Genome U219 Array. This array allowed us to examine the expression levels of more than 22481 human transcripts. As significantly changed we chose the genes with a fold change higher than the cut-off value (fold>|2|) and corrected p-value <0.05. From 2579 differently expressed genes, 626 genes were up-regulated and 831 were down-regulated after 7 days of culture, 829 were up-regulated and 936 were down-regulated after 15 days of culture and 926 were up-regulated and 1126 were down-regulated after 30 days of culture. These results are shown as volcano plots (Fig. 1). Interestingly, no significant expression changes were observed between 7, 15 and 30 days of culture.

The submission of all 2579 differently expressed genes to DAVID database resulted in extraction of 582 Gene Ontology Biological Process (GO BP) Terms and 45 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Among the enriched GO BP Terms and KEGG pathways we chose 39 genes that belong to the “cell cycle” KEGG pathway as well as 75 genes from “cell cycle phase transition” (GO:0044772), 33 genes from “cell cycle G1/S phase transition” (GO: 0044843), 33 genes from “cell cycle G2/M phase transition” (GO:0044839) and 34 genes from “cell cycle checkpoint” (GO:0000075) GO BP terms. Subsequently, the expression levels of genes belonging to “cell cycle” KEGG pathway were marked on the pathway and are presented in Fig. 2.

The expression data of genes belonging to “cell cycle phase transition”, “cell cycle G1/S phase

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

Gene	Primer sequence (5'-3')	Product size (bp)
CASP2	ACTGGTGTGAGCAATGTGC TGCATTTCTGTGCAGTCTG	150
ZWILCH	GGTTGAGACCTTGAAGCATGAC TTTGACACACAGTTGCTCAG	103
CDC6	TCAATTCTGTGCCCGCAAAG TAGCTCTCTGCAAACATCCAG	74
CDC25	ACATCAAGGGTGCAGTGAAC ACAACAATGACACGCTTGCC	100
PRIM2	GCCAGTCTGAAGAACTTAGACG AGCCTCAAATGCAATTGGC	128
BID	TAGCCAGAAATGGGATGGACTG TAGGAACGCTGTTGACATGC	125
PPM1D	TGGAAGAACTGGCGGAATG ACATCTTCATGCCCGAATG	97
FOXMI	ATTGCCCCAAGGTTTTTGG ACCAAAATCTCGCAGATCGC	81
CEP41	TTTTGCCAGCTGATCATCC AATCTCTCAGCTGTCACTTCC	70
GAPDH	TCAGCCGCATCTCTTTTGC ACGACCAAATCCGTTGACTC	90
ACTB	AAAGACCTGTACGCCAACAC CTCAGGAGGAGCAATGATCTTG	132
HPRT	TGGCGTCGTGATTAGTGATG ACATCTCGAGCAAGACGTTT	141

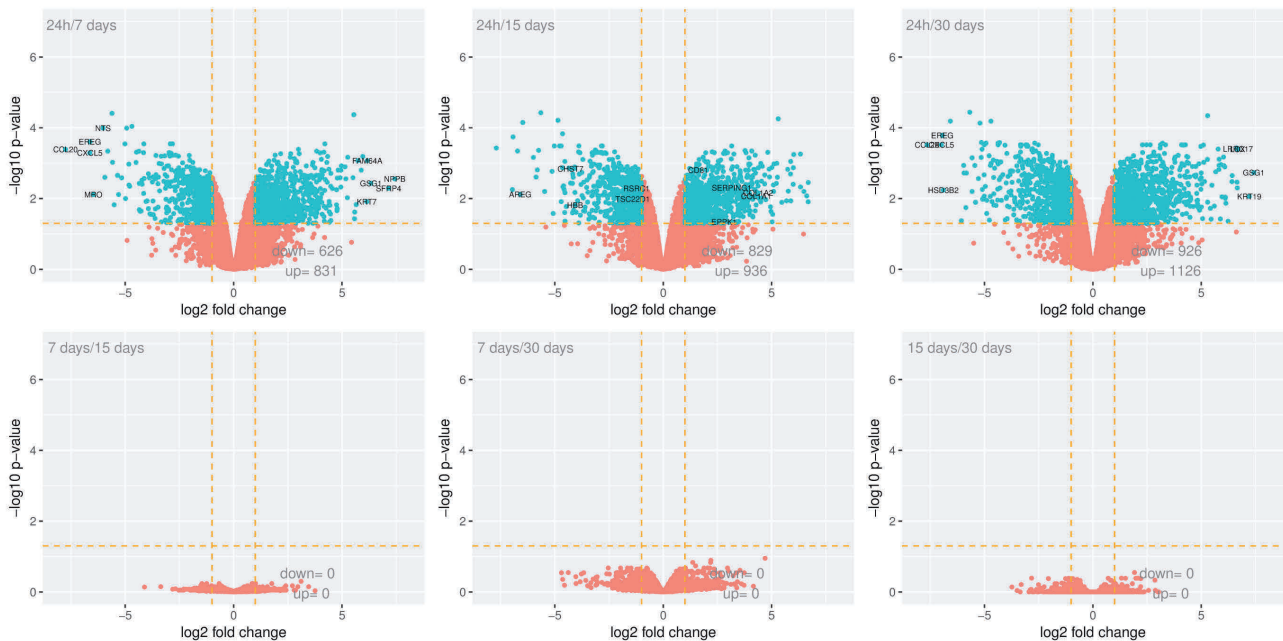


Fig. 1. Volcano plots - the amounts of differently expressed up- and down-regulated genes. Volcano plots show fold change and p value of all genes from Affymetrix® Human Genome U219 Array. The names of 10 genes with highest fold change and lowest p value are placed on the plots near the adequate dots.

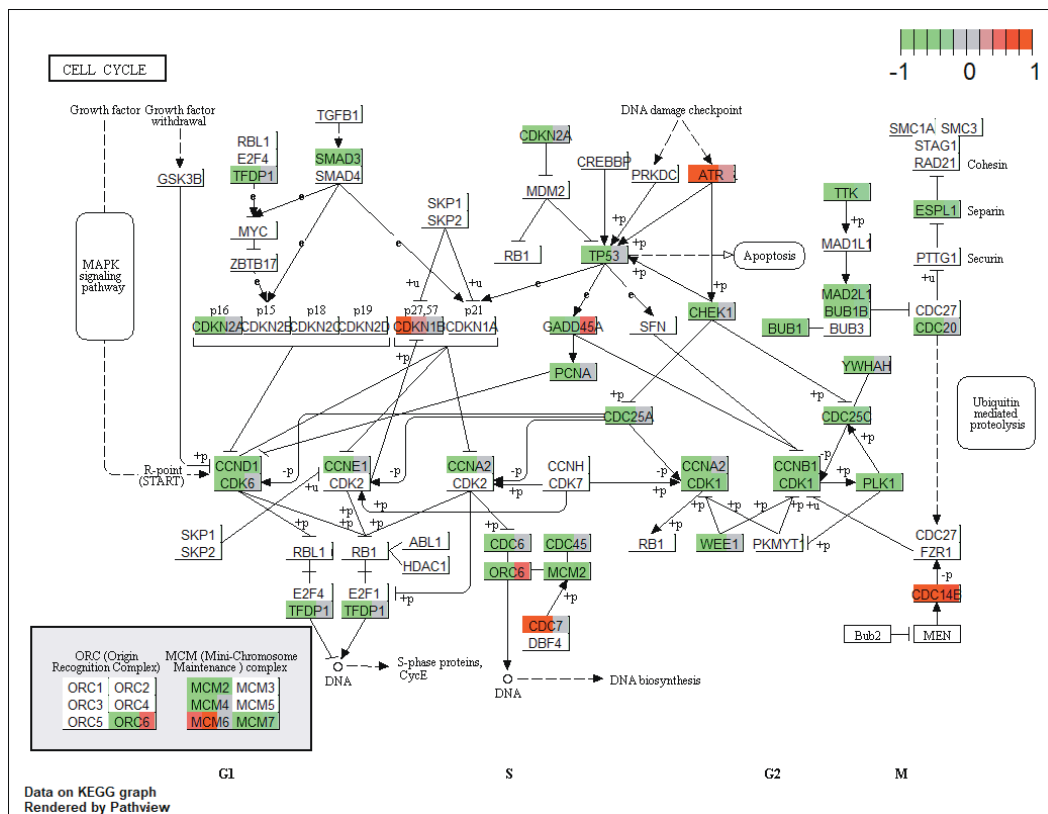


Fig. 2. The “cell cycle” KEGG pathway with marked expression levels of differently expressed genes. Arbitrary signal intensity acquired from microarray analysis is represented by colors (green, higher; red, lower expression). The boxes with names of genes were separated for three parts with representation of gene expression from the 7, 15 and 30 days of culture.

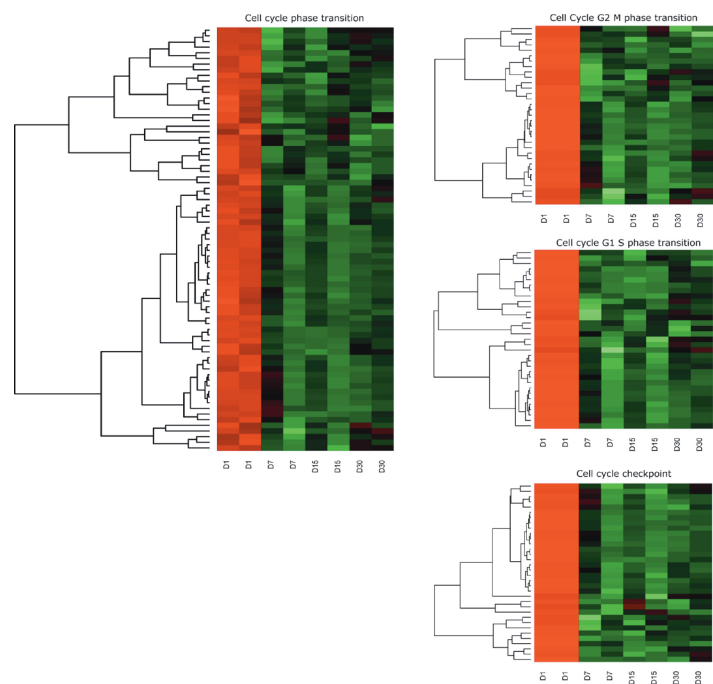


Fig. 3. Heatmaps – Gene ontology groups. Heatmap representation of differentially expressed genes belonging to the to “cell cycle phase transition”, “cell cycle G1/S phase transition”, “cell cycle G2/M phase transition” and “cell cycle checkpoint” Gene Ontology groups. Arbitrary signal intensity acquired from microarray analysis is represented by colors (green, higher; red, lower expression). Log2 signal intensity values were resized to Row Z-Score scale for any single genes (from -2, the lowest expression to +2, the highest expression).

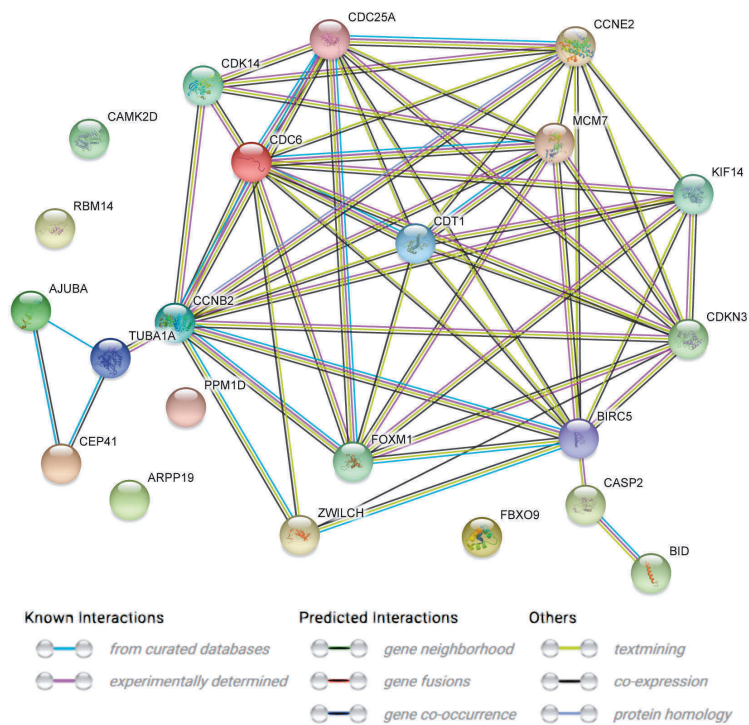


Fig. 4. STRING-generated interaction network between differentially expressed genes (genes described in the discussion and used to validate microarray results). Applied prediction methods: text mining, co-expression, experimentally observed interactions.

transition”, “cell cycle G2/M phase transition” and “cell cycle checkpoint” GO BP terms were subjected to a hierarchical clusterization procedure, with their expression values presented as a heatmaps (Fig. 3).

Heatmap results indicate, that all the genes observed were up-regulated in all the analyzed time periods of primary cultures, as compared to Day 1, which was used as a reference. There are several points on the heatmaps that present abnormal results such as Day 15 of *PEA15* and *ZWILCH*, however, they do not prevail in the second biological repeat.

To further explore known interactions between genes of interest, STRING-generated interaction network was generated among differentially expressed genes involved in cell cycle regulation. Applied prediction methods included textmining, co-expression and experimentally observed interactions (Fig. 4).

Most of the genes, subjected to STRING analysis, showed mutual interactions with at least one of the other analyzed genes. There were a few exceptions, such as *CAMK2D*, *RBM14*, *ARPP19*, *PPM1D* and *FBXO9*, that did not show any interactions with other genes subjected to analysis but may interact with them through other genes detected in our research, that were not analyzed by STRING, or through other genes that are not part of the described gene ontologies.

Additionally, RT-qPCR analysis was performed

to quantitatively validate the results obtained through the microarray approach. The list of chosen genes and RT-qPCR primers can be found in Table I. From each heatmap, few genes, which exhibited similar degree of variation in fold change of mRNA expression in microarray analysis, in each of the examined culture time periods, were chosen.

The results are presented as a bar graph (Fig. 5). Overall, the direction of fold changes was confirmed by the RT-qPCR analysis. However, there are significant quantitative differences between the results from both analyses. The RT-qPCR results, while usually maintaining the day-to-day patterns observed through the microarrays, presented values that were generally higher. Fig. 6 shows the specificity of the products obtained as a result of PCR amplification.

In addition, the results of our research indicate that granulosa cells, in *in vitro* primary culture, change their morphology over time (Fig. 7). As can be observed, the cells first exhibit epithelial-like morphology that undergoes a transition to fibroblast-like structures in the later periods of culture. This occurrence has been observed before and was deemed proprietary to the granulosa long-term *in vitro* cultures (6).

All of the analyses allowed for selecting of a group of genes of interest that were further described and related to the literature in the Discussion section.

Table II. Fold changes, adjusted p-values, ENTREZ gene IDs and gene ontologies of the genes analysed in the manuscript.

Gene	Fold change D7/24h	Fold change D15/24h	Fold change D30/24h	adj.p.value D7/24h	adj.p.value D15/24h	adj.p.value D30/24h	ENTR EZ GENE ID	GENE ONTOLOGY GROUPS
<i>BID</i>	3.131750	2.877270	4.106849	0.018015498	0.0202330774	0.007169286	637	CCPT; G1/S
<i>CASP2</i>	2.130807	1.871144	1.640512	0.021111880	0.0333535622	0.060610607	835	CCPT; G1/S
<i>CDC25</i>	2.273391	2.059917	1.504914	0.035017029	0.0458197145	0.185158836	993	CCPT; G1/S; G2/M
<i>CDC6</i>	2.895325	2.859848	1.898467	0.023258338	0.0218962096	0.083754124	990	CCPT; G1/S; CCC
<i>CEP41</i>	2.061151	1.534255	1.616602	0.0200761698	0.0828629683	0.0551186456	95681	CCPT; G2/M
<i>FOXMI</i>	2.494756	3.466317	3.498170	0.0323466245	0.0112051319	0.0098587792	2305	CCPT; G2/M
<i>PPM1D</i>	2.776618	2.556595	4.122938	0.015080302	0.017226499	0.0041369054	8493	CCPT; G2/M
<i>PRIM2</i>	2.510909	2.326185	1.948706	0.019566283	0.022777393	0.0419643025	5558	CCPT; G1/S
<i>ZWILCH</i>	2.115303	1.750544	1.978641	0.0498285386	0.0992286180	0.0528847391	55055	CCC

CCPT: cell cycle phase transition; G1/S: cell cycle G1/S phase transition; G2/M: cell cycle G2/M phase transition; CCC: cell cycle checkpoint.

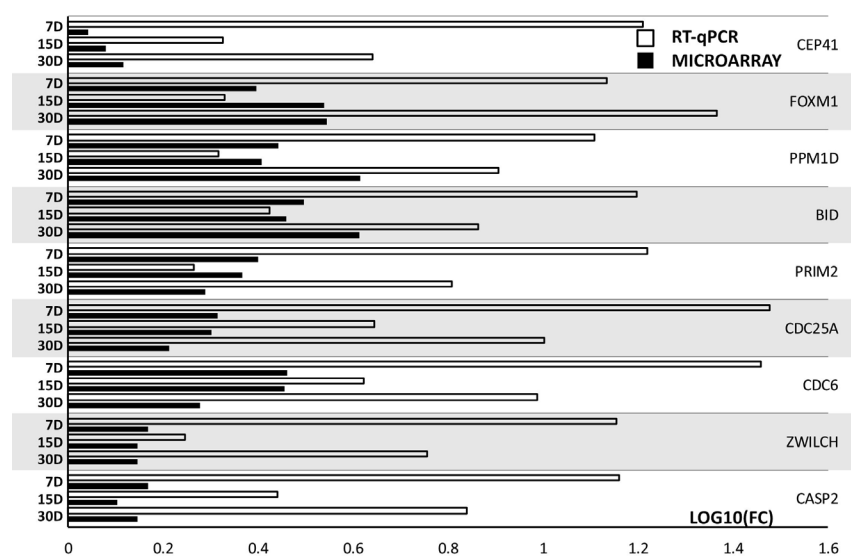


Fig. 5. Results of the RT-qPCR validation presented in the form of a bar chart with comparisons to the results obtained with microarray. All the values presented are the relative changes of gene expression, as compared to day 1 of primary culture. D: Day of Culture.

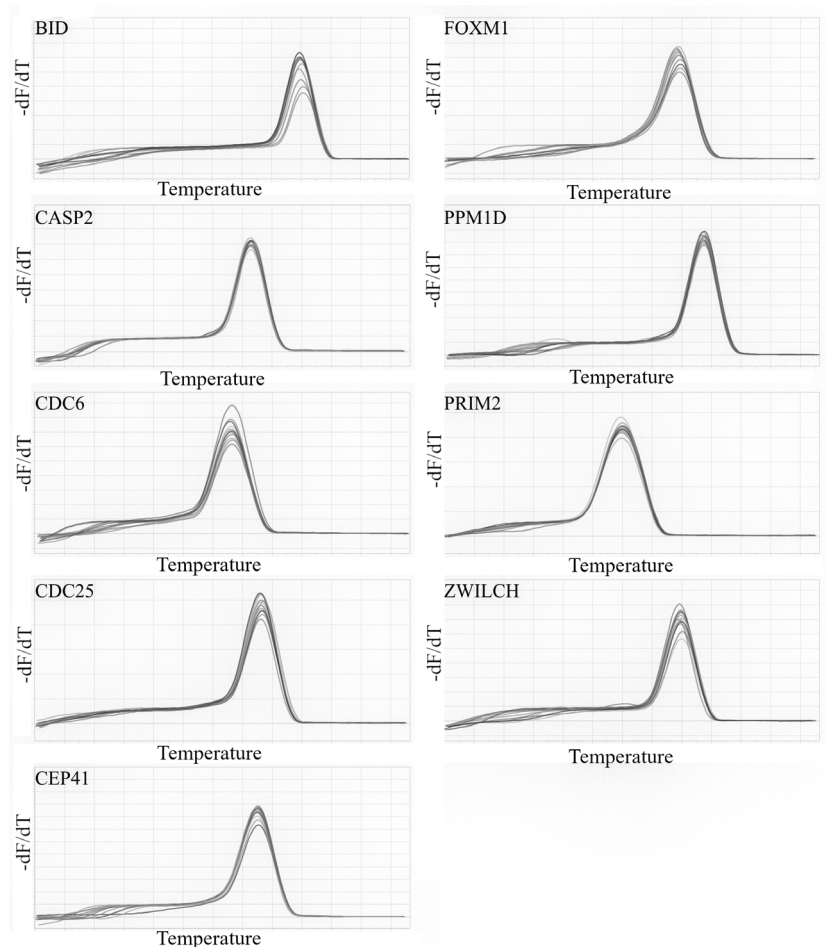


Fig. 6. Figure showing dissociation curves that confirm the specificity of the amplification products obtained in the RT-qPCR analysis.

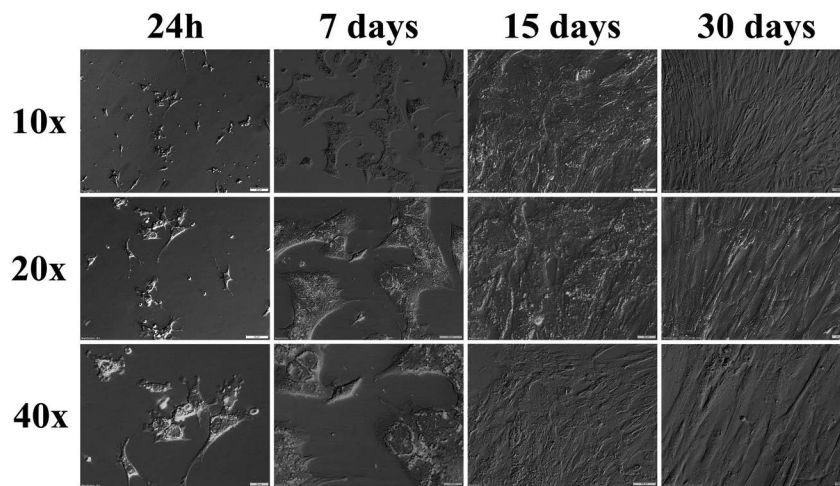


Fig. 7. Morphology changes of granulosa cells in primary long-term *in vitro* culture. Photographs were taken after 1, 7, 15 and 30 days of culture in 3 magnifications (10x, 20x, 40x).

All of the genes from that group were upregulated throughout the culture time. Most of them belonged to more than one gene ontology group, with *ZWILCH* being the only exception. None of the genes belonged to all of the analyzed gene ontologies. The majority of genes of interest presented comparable upregulation, with *BID*, *FOXMI* and *PPMID* showing noticeably higher change in transcript levels. Data concerning the genes of interest that were chosen for analysis were compiled and are presented in Table II.

DISCUSSION

Observations of our team, concerning long-term, *in vitro* GC culture, revealed not only significant alterations in cell morphology, but also up-regulated expression of genes related to cell cycle, its transition phases and checkpoints. Importantly, we defined this set of genes as representative for the GC cell cycle under *in vitro*, long-term primary conditions, as opposed to GC proliferation *in vivo*, which ends about 10 days after ovulation.

Kossowska-Tomaszczuk et al., among two GC populations collected from follicular fluid, indicated one less differentiated with great potential for long-term proliferation and differentiation towards other cell types, such as chondroblasts, osteoblasts and neuroblasts (6). The GCs in our culture were collected

from growing to mature follicles, thus presenting a wide spectrum of luteinisation levels. Interestingly, while luteinizing GCs are considered terminally differentiated, stem cells exhibiting FSHR activity have also been identified in GC populations collected during the *in vitro* fertilization procedure (6,13). Although we have not used differentiating factors, we clearly observed a change in GC morphology, from epithelial-like to fibroblast-like spindle-shaped, which suggests cell transformation. Based on previous reports, describing stemness properties of GCs (6) and our results of loss of FSHR and LHR expression after 7 days of culture (unpublished data), we defined the cells after this time point as granulosa-originating cells (GOCs). Keeping this in mind, we sustain the theory describing prolonged proliferative abilities of GCs kept *in vitro* and, therefore, their great potential to differentiate into other cell types.

Varras et al. conducted studies in which luteinized GCs directly surrounding the oocyte - cumulus cells (CCs) were used. They showed that almost half of these GCs express the *Oct-4* gene, being a characteristic marker of mesenchymal stem cells. This observation let them assume that luteinized GCs consist of stem cells that do not originate from primordial germ cells, which was supported by lack of expression of their marker-*DAZL* gene (14). In this study, we used exclusively the GCs that were

suspended in follicular fluid. Due to the fact that some of the follicles were growing, the granulosa cells still had the ability to proliferate – similar results can be found in researches carried out by Hirshfield and Rodgers (15).

Apart from morphological changes, the present study indicated the most adequate genes related to cell cycle, its transition phases and checkpoints. We discuss those which, in our opinion, are the most relevant to cell proliferation and differentiation or have been already described in the aspect of GCs biology. Chronologically, G1 to S phase transition caught our attention, revealing genes such as: *KANK2*, *MCM7*, *CCNE1*, *CCNE2*, *CDT1* and *CDKN3*. Specific gene of GC cell cycle G1/S transition during proliferation is *KN Motif and Ankyrin Repeat Domains 2 (KANK2)*. Its overexpression was shown to decrease the proliferation of breast adenocarcinoma cells in humans. Moreover, Zhang et al. found that *KANK2* is a negative regulator of estrogen-induced cell proliferation, and its function is regulated by casein kinase 2-dependent phosphorylation (16). Increased expression of this gene in our research may also indicate changes occurring in GCs and their potential for differentiation. Another important gene that plays a key role in the G1/S transition is the *Minichromosome Maintenance Complex Component 7 (MCM7)*. This gene guarantees proper placement of the replication fork on chromosomal DNA, ensuring single genome replication during one cell cycle (17). Our experiment on GCs showed the strongest expression of this gene between 7th and 15th day of *in vitro* culture. Similarly, *Cyclin E1 (CCNE1)* and *Cyclin E2 (CCNE2)* were also expressed at comparable levels. These genes, apart from G1/S transition regulation, are also highly expressed in the testis, thymus and brain. Geng et al. presented *CCNE1* and/or *CCNE2* null mice model. Males with deficiency of the mentioned cyclins produced fewer spermatozoa and subsequently presented decreased fertility (18). The results of our study, in which the expression of the *CCNE1* and/or *CCNE2* gene increases from 7th to 15th day of IVC, indicate that both genes might be important for the proliferation of GCs, as well as GOCs, and may also affect their count and therefore indirectly

influence the differentiation process and/or even the correctness of the folliculogenesis *in vivo*. Some of the presented genes showed an increased expression only on the 30th day of culture, such as *Chromatin Licensing and DNA Replication Factor 1 (CDT1)* gene, which is responsible for replication of the DNA in the mature ovary (19). The results of our study indirectly confirm a theory that the gene is also expressed in GCs, likely influencing their proliferation and differentiation during GOC formation. Thus, it cannot be excluded that *CDT1* gene may be also involved in ovarian follicle growth and development.

Among the analyzed genes, some enhance progression of G1/S phase transition, while overexpression of others disrupts this process. An example of the last phenomenon, resulting in inhibition of GC proliferation in long-term *in vitro* culture, is *Cyclin-dependent Kinase Inhibitor 3* gene (*CDKN3*) (20).

As already mentioned, the cell must pass through all individual phases of the cell cycle to properly divide. Along our long-term *in vitro* cell culture, we were able to indicate genes of altered expression, which are associated with another phase transition during cell cycle - G2/M. Our findings suggest that the above switch may be regulated by: *CAMK2D*, *CDK14*, *AJUBA*, *TUBA1A*, *ARPP19*, *KIF14*, *BIRC5* and *CCNB2*.

The first of these genes, *Calcium/Calmodulin-dependent Protein Kinase II-Delta (CAMK2D)*, encodes a family of protein kinases, which are responsible for programmed body reconstruction during growth, a key element in the body's adaptation to environmental conditions (21). Notably, this gene is also responsible for the cell transition from G2 phase to mitosis. This indicates that *CAMK2D* may be responsible for adapting GCs to a new environmental condition, resulting in intense cell division upon *in vitro* culture condition. Previously, Yang et al. discovered that *Cyclin Dependent Protein Kinase 14 (CDK14)* gene is highly expressed in *in vitro* cultured cells, including those of ovarian origin (22). In addition, this gene, taking part in the Wnt pathway, may act as an inhibitor of the proliferation and migration of cancer cells. The *Ajuba LIM*

Protein (AJUBA) gene has been found in totipotent embryonic stem cells. It was first identified in the skin, brain, and urogenital organ of fetal mouse. Moreover, *AJUBA* is responsible for activation of numerous protein complexes and is involved in several cellular processes, like proliferation, migration and, above all, differentiation. In addition, it is liable for chromatin condensation during cell division. Thus, expression of *AJUBA* gene in GOCs may indicate their potential for differentiation into different cell types (23).

Another important gene related to G2/M transition in GOCs during IVC, and potentially in GCs during folliculogenesis, is *Tubulin Alpha 1 A (TUBA1A)*. Tubulins are main components of microtubules, which are critical throughout the cell mitosis. Anchored at the centrosome, they constitute to mitotic spindle formation and proper cell division. TUBA1A was associated with neuronal migration in brain and its mutations were shown to cause cortical malformations in humans (24). Similarly to *TUBA1A*, *cAMP-Regulated Phosphoprotein 19 (ARPP19)*, is strongly expressed in the nervous system (25), however its predominant role is regulation of mitosis. Its activity leads to inactivation of Protein Phosphatase 2A (PP2A), which is a crucial condition to keep high cyclin-B1-CDK1 activity during M phase (26). There is no information about either of the mentioned genes regarding GC proliferation capability, however, their strong relation to cell cycle events allows us to assume that they may also serve as specific markers of GC proliferational potential and differentiation towards GOCs in long-term IVC.

The *Kinesin Family Member 14 (KIF14)* belongs to the kinesin superfamily of microtubule associated motors, which play an important role in intracellular transport and cell division (27). Thus, expression of this gene might be important for communication among GCs, as well as between GCs and the oocyte, enabling transport of the substances to and from the oocyte (28).

Under physiological conditions, during the folliculogenesis process, some of the follicles undergo atresia at an early stage of development. This is related to the acquisition of a dominant role by one follicle (29). The *Baculoviral IAP Repeat*

Containing 5 (BIRC5) gene - also known as *survivin* is a member of the inhibitors of apoptosis protein (IAP) family. Studies show that *survivin* is involved in maintaining cell function during long-term *in vitro* cultivation. However, this gene is also an inhibitor of cell apoptosis, playing a key role in regulation of cell cycle in GCs. It is suggested that under physiological conditions, this gene and encoded protein are also recognized as markers of functional, non-luteal phase in human GCs (30).

Interestingly, the *Cyclin B2 (CCNB2)* gene exhibits increased expression not only during cell division, but also in embryonic stem cells (ESCs). This may be related to the multiplicative properties attributed to GCs by Kossowska-Tomaszczuk et al. (6). The above data confirms our assumptions that GCs in long-term *in vitro* cultivation change their properties.

However, it needs to be noted that this research is, in many ways, an entry level molecular analysis of granulosa cells. While it may serve as a valuable reference for future proteomic, *in vivo* and clinical studies, the results need to be confirmed by the outcomes of further experiments. Despite that, the microarrays, while being a very comprehensive source of data, especially when it comes to qualitative analysis, can also serve as a source of quantitatively accurate measurements when accompanied by RT-qPCR validation.

In conclusion, we suggest that the control of the human ovarian GC cell cycle in long-term *in vitro* culture is regulated primarily by genes belonging to the pathway and ontological groups of cell cycle, its phase transitions and checkpoints. Substantially increased expression of all selected genes may indicate their share in granulosa, as well as granulosa-originating cells prolonged proliferation. Most of the genes, in addition to their classical role in the transition of cells from one phase of cell cycle to another, display other significant functions, related to differentiation of cells and tissues. Thus, the substantial changes in cell morphology observed may be regulated by the presented set of genes, leading to induction of pathways specific for stemness plasticity and transdifferentiation *in vitro*. Summarizing, while the results give us insight into

the molecular processes, underlying the functioning of GCs *in vitro*, they need much further validation and additional research to be applied in the clinical environment.

ACKNOWLEDGEMENTS

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SCAFFOLDING ADAPTER PROTEIN *Gab1* IMPAIRS bFGF-INDUCED BIO-ACTIVITY VIA AFFECTING PI3K-AKT PATHWAY

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The role of Grb2-associated binder 1 (*Gab1*) in bFGF-activated PI3K-AKT pathway of endothelial cells remains largely unknown. To elucidate this role, a set of studies with siRNA knockdown of *Gab1* was performed. Knockdown of *Gab1* using siRNA was performed in fused endothelial cell line EA.hy926 and the low level of *Gab1* was confirmed with quantitative R-T PCR and Western blotting. Effects of *Gab1* down-regulation were examined on several aspects: bFGF-induced AKT phosphorylation, proliferation, migration and vessel tubing formation of EA.hy926 cells. The bFGF-induced AKT phosphorylation of wild-type EA.hy926 cells was both dose-dependent and time dependent with a peak at 10 ng/ml and about 30 min after bFGF treatment. The AKT activation was significantly reduced in *Gab1* siRNA-treated EA.hy926 cells. The blocking of *Gab1*-AKT path resulted in a set of biological alterations of EA.hy926 cells: (i) reduced proliferation; (ii) impaired migration; (iii) decreased vessel tubing formation in both 2D and 3D culture. All data support that *Gab1* is associated with angiogenesis function of EA.hy926 endothelium cells via PI3K-Akt signaling pathway.

Critical limb ischemia (CLI) is the one of the major complications of diabetes, atherosclerosis or peripheral arterial occlusive diseases (PAOD), which causes pain on walking (claudication) or/and at rest, and non-healing ulcers (1). Although the standard treatment is surgical endovascular revascularization, the lack of sufficient runoff vessel supply results in no surgical options other than pain control or amputation in a considerable number of patients (2).

The discovery of vessel regeneration therapy with angiogenic cytokines has been proposed as a new strategy for treating CLI (3). Some clinical

studies have shown that the administration of basic fibroblast growth factor (bFGF) alone or combined with bypass surgery or percutaneous catheter-based revascularization is a safe and potentially effective therapy to improve ischemia (2). We have found that over-expression of bFGF could improve the perfusion in lower limb ischemic rat model (4). Other groups using direct injection of bFGF into ischemic tissue also showed a promising result of enhancing angiogenesis and improving the perfusion of ischemic tissue (2, 5). However, the detailed molecular mechanism that affects the signal pathways remains largely unknown.

Key words: Gab1; angiogenesis; bFGF; PI3K-AKT pathway; vascular diseases

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Grb2-associated binder 1 (Gab1), a scaffolding adaptor and a member of the insulin receptor substrates 1 (IRS1)-like multi-substrate docking protein family, is highly expressed in vascular endothelial cells (6). Upon stimulation of growth factors, Gab1 provides the binding sites for phosphatidylinositol 3-kinases (PI3K) regulatory subunit P85 to recruit and activate PI3K-AKT pathway, which are crucial to cell proliferation, differentiation and angiogenesis (7). Recent *in vitro* and *in vivo* studies showed that Gab1 is essential for vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) signaling in promoting cell migration, survival, and tube formation (8, 9). However, to date, no direct evidence has demonstrated the role of Gab1 in bFGF-induced angiogenesis.

We hypothesize that Gab1 participates in the bFGF-induced angiogenesis. To prove this, we utilized siRNA to knockdown Gab1 in endothelial cells and then examined their response to bFGF-induced activation of PI3K-AKT pathway, proliferation, migration and vessel tube formation. Our data demonstrated that Gab1 regulated bFGF-induced angiogenesis via affecting PI3K-AKT signaling.

EA.hy926 is a fusion cell line of human umbilical vein endothelial cells (HUVEC) and human lung cancer A549 cells (10) and possesses many characteristics of primary cultured endothelial cells (11, 12). Due to its easiness to maintain, EA.hy926 was chosen to study the role of Gab1 in bFGF-induced angiogenesis via a serial test for alterations of biological functions following the knockdown of Gab1, which was otherwise difficult to carry out over a relatively long time (a few months) in primary endothelial culture system.

MATERIALS AND METHODS

Reagents

Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (USA); the antibodies against Gab1, PI3K subunit P85, phospho-AKT (Ser473), AKT and GAPDH from Cell Signaling Technology (USA); human recombinant bFGF from PeproTech (USA).

Cell culture

The fused human endothelial EA.hy926 cells (CRL-2922, ATCC, USA) were cultured in DMEM supplemented with 10% FBS (Hyclone, USA), 100 U/ml of penicillin and 100 µg/ml of streptomycin in a humidified 5% CO₂ incubator (Thermo Forma, USA) at 37°C.

Lentiviral Gab1 siRNA infection

EA.hy926 cells (2.5×10^5) were seeded in T25cm² flasks one day before infection. Specific lentiviral expression systems for both Gab1 siRNA (knockdown group, KO) or scramble siRNAs (scramble control group) with GFP gene were purchased from Shanghai Genechem Co., Ltd. (Shanghai, China). After overnight culture, the cells were fed with the same media but antibiotic-free and then infected with either lentiviral Gab1siRNA for knock-down of Gab1 or scramble siRNA as controls at a multiplicity of infection (MOI) of 100 with 5 µg/ml of polybrene (Genechem, China) according to the manufacturer's instructions. After incubation for 12 hours, the cells were rinsed twice in PBS and then cultured in complete DMEM medium (supplemented with 10% FBS and 1% antibiotic) for additional 72 hours. The transfected cells were observed for GFP expression under an inverted fluorescence microscopy (Nikon, Japan) to confirm the successful infection. The cells were cultured with puromycin to select and maintain the Gab1 knock-down cells.

Quantitative RT-PCR

To confirm the knockdown of Gab1, the total RNA was extracted from EA.hy926 cells with Trizol (Invitrogen, USA) following the manufacturer's protocol. The cDNA was synthesized from 2 µg of total RNA with Moloney murine leukemia virus reverse transcriptase (Promega, USA) according to the manufacturer's instruction. Quantitative RT-PCR was performed using Power SYBR Green PCR Master Mixture (Takara, Japan). The primer sequences used in the experiment were as follows: Gab1-F: 5'-TGGCAGCTCTTTACAAGCACC-3', Gab1-R: 5'-TGATGAGCAACAGGTAGTCTTGA-3'; GAPDH-F: 5'-TGACTTCAACAGCGACACCCA-3', GAPDH-R: 5'-CACCTGTGCTGTAGCCAAA-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as control and target gene expression was calculated using the 2^{-ΔΔCt} comparative method (ΔCt is equal to the value of Gab1 ΔCt minus GAPDH; -ΔΔCt is equal to the average value of ΔCt of

negative control group subtracting the corresponding sample ΔC_t ; $2^{-\Delta\Delta C_t}$ reflects the level of Gab1 mRNAs compared with negative control group).

Western blotting for AKT phosphorylation

After serum-starvation in DMEM containing 1% BSA overnight, EA.hy926 cells were stimulated with 0 or 10 ng/ml bFGF (PeproTech, USA) for the indicated time periods. Whole-cell protein extracts were prepared using RIPA lysis buffer (25mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100) with PMSF protease inhibitor. Protein concentration was quantified using the BCA method. A total of 25 μ g proteins/sample were separated using 10% SDS-PAGE (Beyotime, China) as described elsewhere (7). After transferring to PVDF membrane, the specific antibody (Cell Signaling Technology, USA) against phosphorylated AKT (S473) or PI3K (p85) at 0.2 μ g/ml was used to detect corresponding proteins on the membrane followed by incubating with horseradish peroxidase (HRP)-conjugated secondary antibodies (cell signaling technology) and developing with ECL kit (Genview, USA). The emitted light was detected using Chemidoc XRS⁺ gel imaging system (Bio-Rad, USA). The obtained digital images were analyzed and quantified using Quantity-one 4.6 software (Bio-Rad, USA).

Cell proliferation

The wild type controls, scramble or Gab1 siRNA cells were seeded onto five 96-well plates at a density of 4×10^3 cells/well and 6 wells per group. Cells were cultured at 37°C in an atmosphere containing 5% CO₂. Cell proliferation assays were performed on days 1, 2, 3, 4 and 5 after bFGF treatment. Twenty μ l of 5 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Cat# 11465007001, Sigma, USA) were added to each well. The cells were incubated for additional 4 h at 37°C and then the media was carefully removed. Without disturbing the cells, 150 μ l DMSO (Sigma, USA) were added into each well and the plate was shaken at room temperature for 15 min. Absorbance was read at 570 nm. The experiments were repeated for 3 times.

Cell migration assay

The migration of EA.hy926 cells was evaluated using the Transwell migration technique (Corning, USA). EA.hy926 cells (5×10^4 in 500 μ l complete medium) were

placed in the upper chamber. A total of 600 μ l complete medium containing 10 ng/ml bFGF was added into each lower chamber. After 24 h, the cells in the upper chamber were removed, and the cells in the lower chamber were washed with PBS. Then, the cells migrated into the lower chamber were fixed with ice cold acetone and stained with hematoxylin. Migration activity was evaluated as the mean number of migrated cells in five random high-power fields ($\times 200$) per chamber and compared among three groups. The experiments were repeated for 3 times.

Endothelial cell tube formation assay

Tube formation of endothelial cells on gelled basement membrane extract was performed according to Arnaoutova's report (8). Briefly, 150 μ l Matrigel (BD, USA) was pipetted into each well of 48-well cell culture plates at 4°C. The plates were then incubated at 37°C for 30 min to allow the Matrigel to form gel. The wild type cells or scramble or Gab1 EA.hy926 siRNA transfected cells at a concentration of 1.5×10^5 cells per ml with 10 ng/ml bFGF were gently applied to the top of Matrigel in the above 48-well plates (15,000 cells in 100 μ l per well). The tube formation was captured with an inverted microscope. Total capillary tube length was measured in five view fields, which covered most of the well. The vessel length was measured with *image pro-plus* program. Data are representative of 6 tests performed in three separated experiments.

Statistical analysis

All experimental data are presented as mean $\pm$ SD. SPSS 19.0 software (IBM Corp., Armonk, N.Y., USA) was used for all statistical analyses. Comparisons between two groups were analyzed by two-tailed Student's *t*-test and among more than two groups by ANOVA. $P < 0.05$ were considered statistically significant.

RESULTS

Generation of endothelium-specific Gab1 knockout cells

To elucidate the functional role of Gab1 in the endothelium angiogenesis, we generated specific Gab1 knockdown endothelial cells using the lentiviral siRNA system. After infection with lentiviral siRNA at a MOI of 100, cell RNA and proteins were prepared and were subject to quantitative RT-PCR and Western blotting, respectively. As shown in

Fig. 1, Gab1 mRNA and protein were significantly decreased to less than one third of those in the wild-type control or scramble siRNA infectants ($P < 0.05$). These cells provided the ideal models to study the biological function of Gab1.

Gab1 is involved in the activation of PI3K and AKT by bFGF in EA.hy926 endothelium

To define whether bFGF was involved in the AKT activation in EA.hy926 cells and its optimal concentration and peaked time, the wild type cells were treated with different concentrations (0, 1, 5, 10, 15, 20 ng/ml) of bFGF, harvested 30 min later and then subjected to Western blotting for phosphorylation of AKT (S473). Results (Fig. 2, A and B) showed that Gab1 was involved in activation of PI3K and AKT by bFGF in EA.hy926 cells. The optimal concentration of bFGF for activating pAKT was 10 ng/ml. Then, cells treated with bFGF at 10 ng/ml were harvested at different time points (15, 30, 60, or 120 min) and tested for the optimal time of pAKT activation. The data (Fig. 2 C and D) showed that the peaked time for bFGF-induced pAKT was at 30 min.

To examine the role of Gab1 in bFGF-dependent activation of AKT pathway, we treated the wild type controls, scramble or Gab1 siRNA cells side-by-side with bFGF at 10 ng/ml for 30 min and examined the levels of pAKT (S473) and pPI3K (P85) with Western blotting. The result (Fig. 2, E-G) showed that while the activation of pAKT (S473) and pPI3K (P85) was greatly triggered upon the treatment of bFGF on the wild type controls and scramble infected cells, the extent of the activation was much lower in the Gab1 siRNA cells as compared to the counterparts ($P < 0.05$), suggesting that the down-regulation of Gab1 leads to an impaired PI3K/ AKT signaling.

Gab1 protein is required for the proliferation of EA.hy926 endothelial cells

To explore the effect of impaired PI3K/AKT signaling associated with Gab1 down-regulation on biological function of EA.hy926 endothelial cells, several assays were carried out.

The endothelial proliferation is a good marker for potential angiogenesis. The same number of the wild type control cells, scramble or Gab1 siRNA

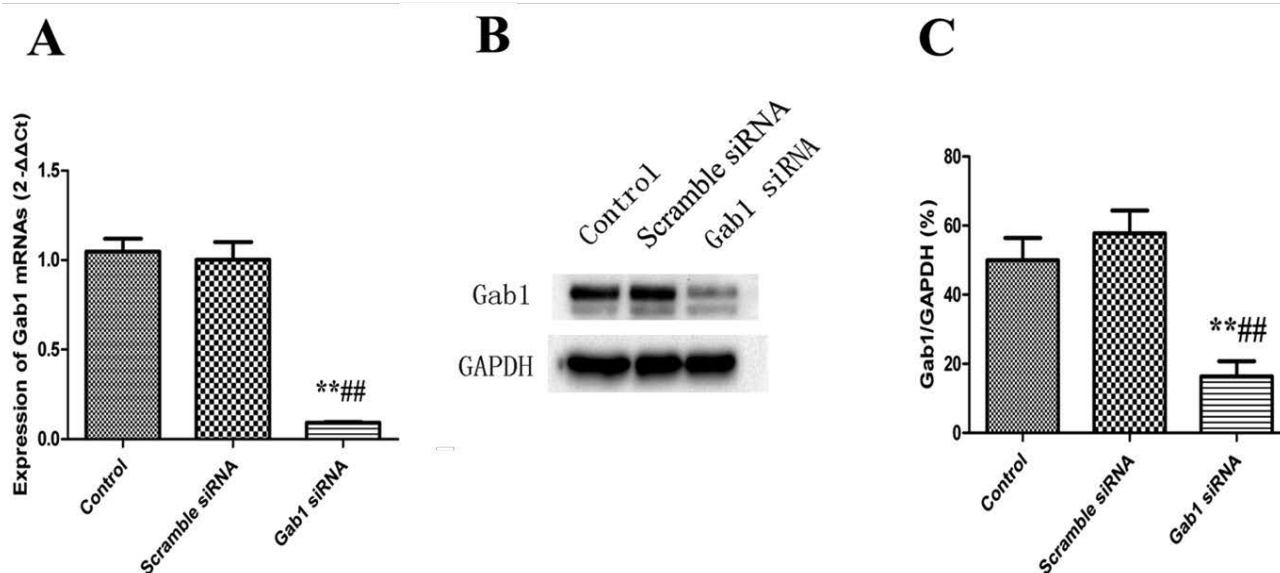


Fig. 1. Knockdown of Gab1 using siRNA. EA.hy926 endothelial cells were infected with lentiviruses with expression for Gab1 siRNA (knockdown group) or scramble siRNA (scramble group). **A**) The mRNA expression levels of Gab1 mRNAs in wild type control, scramble siRNA and Gab1 siRNA groups were measured by qRT-PCR assay (3 samples per group); **B**) The protein levels of Gab1 in wild type control, scramble siRNA and Gab1 siRNA groups were measured by Western blotting; **C**) Quantitation of Gab1 normalized with GAPDH. ** $P < 0.001$ vs wild-type control, ### $P < 0.001$ vs scramble siRNA. Data were representatives of three independent experiments.

infectants were seeded in corresponding wells on 96 well plates and stimulated with bFGF for 5 days. The proliferating viable cells in triplicates were measured every day with MTT assay.

The data revealed that on day 2, the Gab1 siRNA cells started growing more slowly than wild type controls and scramble siRNA cells (Fig. 3). This growth tendency was maintained for 5 days and the maximal difference was shown on day 5. The slope of cell growth was small for Gab1 siRNA cells compared

with that of their counterparts ($P < 0.05$), suggesting that the decreased expression of Gab1 affects the proliferation of EA.hy926 endothelial cells.

Gab1 protein is associated with bFGF-induced migration of EA.hy926 endothelial cells

Migration is a characteristic of endothelial cells. To explore the effect of Gab1 protein on EA.hy926 cell migration, the cells were seeded in serum free medium in the upper chamber of Transwells, and

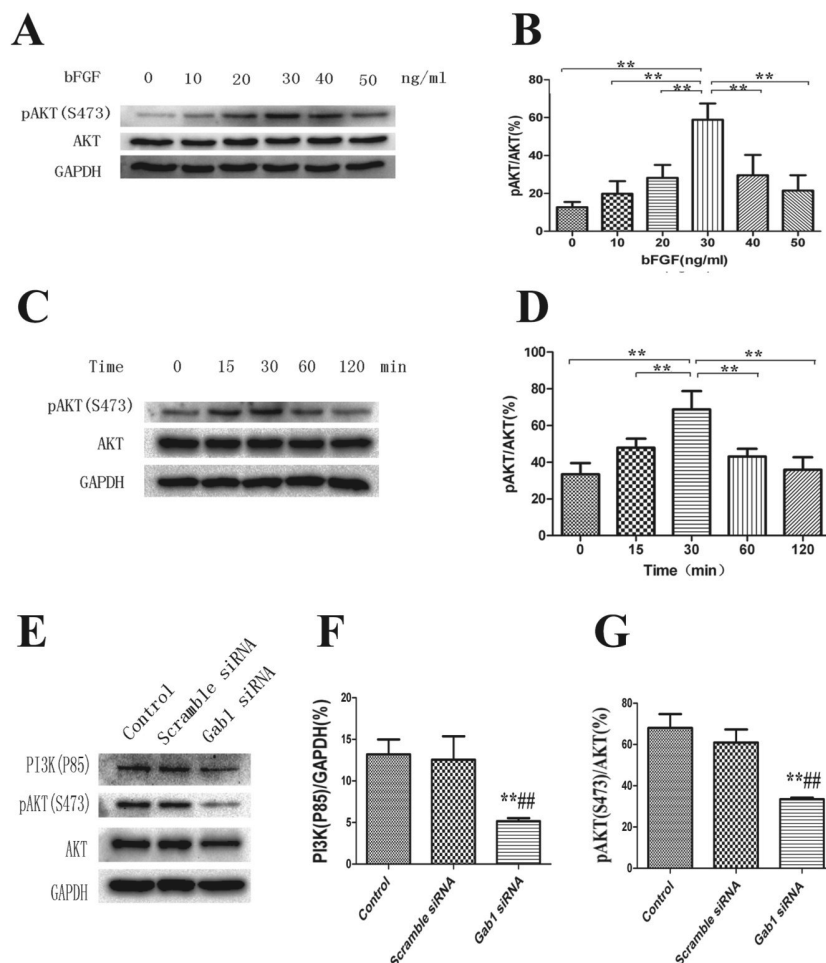


Fig. 2. Gab1 was required for bFGF-induced activation of PI3K and AKT. The EA.hy926 cells in three groups were treated with b-FGF and p-AKT and p-PI3K were determined with Western blotting. **A-B**) Optimal dose of b-FGF in activation of p-AKT was defined using a serial doses of b-FGF as indicated. Cells were harvested and their lysates were prepared at 30 min for phosphorylation of AKT (S473) by Western blotting and quantified against total AKT and GAPDH ($n=3$). **C-D**) Optimal timing for b-FGF activation of p-AKT was defined by harvesting cells at different time points after treated with 10 ng/ml b-FGF. p-AKT was quantified by Western blotting ($n=3$); **E-G**) Effect of down-regulation of Gab1 on p-AKT and p-PI3K: 30 min after indicated cells were treated with 10 ng/ml b-FGF, the cell were harvested and analyzed for the levels of Gab1, p-AKT and p-PI3K. Their expression level were quantified by Western blotting ($n=3$). ** $P < 0.01$, vs wild-type control; ### $P < 0.01$ vs scramble siRNA.

10 ng/ml bFGF was used as a chemo-attractant in the lower chambers. The results showed that Gab1 siRNA cells had a lower capability to migrate towards bFGF containing low chambers compared to the wild type control cells and scramble siRNA cells (Fig 4).

Effect of Gab1 protein on bFGF-induced tubing formation of EA.hy926 endothelial cells

The unique morphological characteristic of endothelial cells is to form vessel tubing in Matrigel. To elucidate the effect of Gab1 on the tubing formation, EA.hy926 cells were cultured in Matrigel with addition of 10 ng/ml bFGF, and monitored using microscopes with both phase contract and dark field (fluorescence). The images of tubing formation were taken and processed for the total length in the same size of area. The data were compared among these three groups. The results showed that upon the induction of bFGF the Gab1 siRNA cells formed the shortest and least tubing among the three types of cells (Fig. 5A and B). Similarly, the observation of tube-formation under a fluorescence microscope presented the same

result, less tubing formation in Gab1-knockdown EA.hy926 endothelial cells (Fig. 5C and D).

DISCUSSION

Understanding of intracellular signaling mechanisms underlying angiogenic cytokines-induced angiogenesis would be instrumental in designing new treatment for diabetes feet, atherosclerosis or peripheral arterial occlusive diseases. It is well accepted that bFGF is a key member of the growth factor family in promoting angiogenesis (2), which has widely been used in experimental studies and in clinical treatment with relatively high effectively and safety (2). However, the downstream molecules and pathways that promote angiogenesis have not been fully defined. Angiogenesis is mainly an adaptive response to tissue hypoxia, and a process of generation and expansion of blood vessels from a preexisting vascular network (9). Upon endogenous or exogenous stimulation, the endothelial cells loosen their intracellular connections. They sprout, migrate, proliferate and

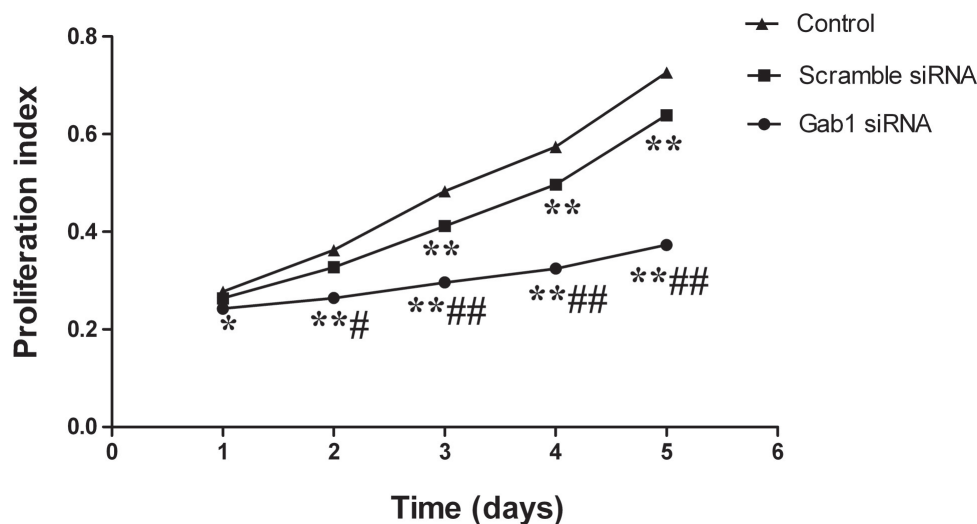


Fig. 3. Knockdown of Gab1 inhibited the proliferation of EA.hy926 endothelial cells. The cells were seeded in 96-well plates (4×10^3 /well, 6 wells/treatment group, repeated for 3 times). The MTT assay (570 nm absorbency) was used to measure the cell proliferation after treated with 10 ng/ml of bFGF at the different time points as indicated. * $P < 0.05$, ** $P < 0.01$ vs wild-type control, # $P < 0.05$, ### $P < 0.01$ vs scramble siRNA. Data were representatives of three experiments.

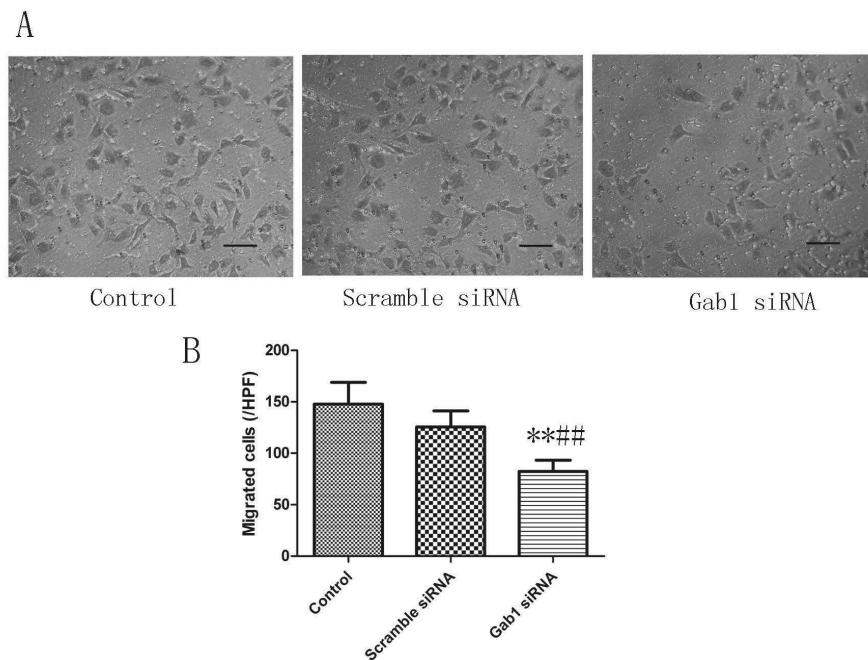


Fig. 4. Knockdown of *Gab1* reduced the migration of EA.hy926 endothelial cells. Cells were seeded on upper chamber of transwells (5×10^4 /well, 6 replicates per group, repeated for 3 times). After 24 hours culture, the cells migrated to the lower chamber containing 600 μ l complete medium with 10 ng/ml bFGF were fixed with ice-cold acetone and stained with hematoxylin, then captured and counted. **A)** the cells migrated to the lower chamber were captured (scale bar, 100 μ m); **B)** the number of cells migrated to the lower chamber were counted in five random high-power fields ($\times 200$) per chamber. The results were compared among three groups. $^{***}P < 0.01$ vs wild-type control, $^{\#}P < 0.05$, $^{##}P < 0.01$ vs scramble siRNA. Data are representatives of three experiments.

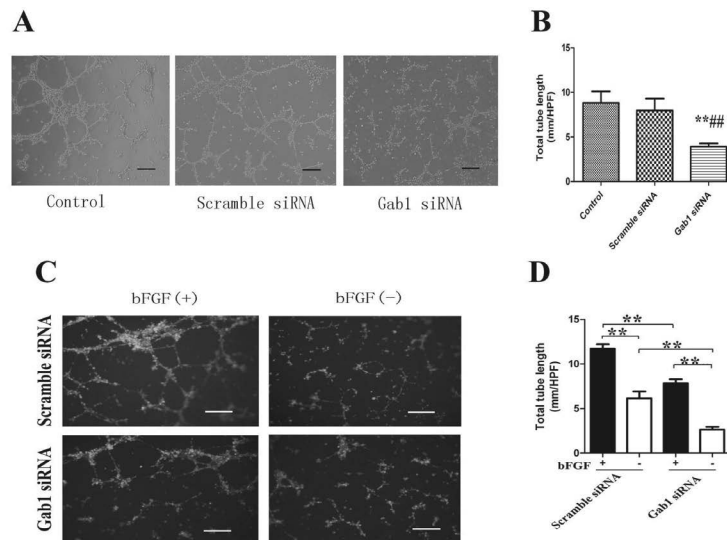


Fig. 5. Knockdown of *Gab1* impaired the vessel tubing formation of EA.hy926 endothelial cells. Cells were seeded in Matrigel with the presence of 10 ng/ml of bFGF and the tubing formation was pictured. The total capillary tubing length was measured in five view fields. The vessel length was measured with image pro-plus program. **A)** The representative image of Matrigel tubing formation induced by bFGF (scale bar, 100 μ m); **B)** The average length of total tubing network per high power field (6 replicates per treatment group, repeated for 3 times). **C)** Representative image of Matrigel tubing formation assay (scale bar, 100 μ m). **D)** The average length of total tubing network per high power field ($n=6$ replicates per treatment group). $^{***}P < 0.01$ vs wild type control, $^{##}P < 0.01$ vs scramble siRNA.

then form new thin-wall vessels. The scaffold adapter protein is involved in amplification and integration of signal transduction evoked by growth factors. The current study indicates that Gab1 is necessary in angiogenesis (2).

Although the HUVEC maintain all characteristics of primary endothelial cells, they tend to senescence after passing several generations *in vitro* and are difficult to be used for a series of tests in a relatively long period of time. Therefore, like other previous reports (18, 26, 27), EA.hy926 cell line was selected for Gab1 gene knockdown study. Since EA.hy926 cell line has a uniform gene background and similar characteristics of differentiated endothelial cell functions, such as angiogenesis, homeostasis/thrombosis, and could respond to inflammation (11, 12, 19-22), we were able to compare the wide type and scramble siRNA treated cells with the Gab1 low-expressing cells to draw a clear-cut conclusion of effect of Gab1 on bFGF-induced angiogenesis via PI3K-AKT Pathway in EA.hy926 cells.

Gab1 has been intensively studied as compared to Gab2, 3 and 4, since the Gab1^{-/-} mice are not viable and only reach day 14 to 18 of gestation (23), while Gab2^{-/-} and Gab3^{-/-} mice live to a normal age (24, 25), indicating that Gab1 is critical for angiogenesis during development. Thus, we focused on the effect of Gab1 on the survival, migration and tube formation of endothelial cells. Our results demonstrated that Gab1 was closely associated with the bFGF-induced angiogenesis in several aspects.

First, at the molecule level, we found that the bFGF-induced activation of AKT at an optimal induction condition of 10 ng/ml bFGF (28, 29) for 30 min was greatly reduced with the down-regulation of Gab1 as evidenced by a reduced level of pPI3K (P85) and pAKT (S473) (Fig. 2). Secondly, at the cell proliferation level, we demonstrated that a greatly impaired proliferation was associated with Gab1-deficient EA.hy926 cells (Fig. 3). In addition, the migration capability of Gab1-deficient EA.hy926 cells was significantly reduced (Fig. 4). Furthermore, the capacity of forming vessel tubes was significantly impaired (Fig. 5).

Taken together, these studies demonstrated that the scaffolding adapter protein Gab1 regulates

bFGF-induced angiogenesis through the PI3K-AKT pathway.

Several studies have been carried out regarding the involvement of VEGF/ HGF and Gab1 in angiogenesis. Shioyama group generated endothelial-specific Gab1 knockout (Gab1 ECKO) mice displaying impaired limb perfusion and enhanced necrosis in a hind limb ischemia (HLI) model because of reduced HGF/c-Met signaling (13). Lu et al. reported that Gab1 was involved in ischemic and VEGF-induced angiogenesis using the strategy for endothelial deletion of Gab1 (14). Zhao et al. used the Gab1ECKO mouse model to further confirm the requirement of Gab1 for postnatal angiogenesis via stimulation of HGF (15). We studied the effect of siRNA knockdown of Gab1 on the bFGF-induced angiogenesis via PI3K-Akt pathway, which was not explored by previous studies (16). Therefore, our new finding aids in the understanding of the network process of bFGF--Gab1-- PI3K/Akt-- angiogenesis, which might be utilized for new strategy of treatments of CLI and PAOD,

To date, the use of angiogenic cytokine, including bFGF, in the treatment of lower limb ischemic diseases primarily relies on the bolus administration of the growth factor or direct injection into the blood stream (16). The results of existing studies are not consistent. Preclinical trials of direct administration of angiogenic cytokines have shown some limitations, whereby bFGF stimulated vessel growth for a transient period, but resulted in fragile and leaky vessels that were prone to regression. To enhance the treatment effect of bFGF, a new strategy is needed to enhance the down-stream signaling path (17), such as Gab1 PI3K-Akt signaling pathways. However, how to deliver the beneficial factor is a "bottle neck" for clinical practice, which relies on more modern molecular biological means.

As this set of studies demonstrated that the Gab1 is needed for angiogenic effect of bFGF, a great effort should be focused on how to enhance the function of Gab1 and PI3K-Akt signaling pathways, which might bring a better result to prolong and increase the effect of bFGF on treatment of lower limb ischemic diseases, such as diabetes feet, atherosclerosis or peripheral arterial occlusive diseases.

Since a relatively long-term cell culture was required for the multiple procedures and assays in this study, immobilized EA.hy926 endothelial cells were used, which might not completely reflect the bio-functions of primary endothelial cells. Further study on primary endothelial cells is needed to confirm the results.

Taken together, Gab1 is associated with angiogenesis function of EA.hy926 endothelial cells via PI3K-Akt signaling pathway. Down-regulation of Gab1 impairs the bFGF-induced AKT phosphorylation, proliferation, migration and vessel tubing formation of EA.hy926 cells.

ACKNOWLEDGEMENTS

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JIA-WEI-SI-MIAO-WAN ALLEVIATES ACUTE GOUTY ARTHRITIS BY TARGETING NLRP3 INFLAMMASOME

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Gout is a common metabolic disease and acute gouty arthritis (AGA) is one of the important complications. Jia-Wei-Si-Miao-Wan is a newly developed drug for treating acute gouty arthritis, but the molecular mechanism has not been completely clarified. Thus, this study was aimed to explore the regulation of Jia-Wei-Si-Miao-Wan on NLRP3 inflammasome and TLR/NF- κ B signaling, which are two important signaling pathways in inflammation. AGA rat model was established by injecting monosodium urate into the right knee. Colchicine and Jia-Wei-Si-Miao-Wan were administrated by gavage. The circumference of the knee was measured. IL-1 β and IL-18 level in the flushing fluid was detected by enzyme linked immunosorbent assay (ELISA). Western blot, immunohistochemistry and quantitative real-time RT-PCR were used to detect the protein and mRNA expression of TLR4, NLRP3, ASC, caspase-1, NF- κ B and p-NF- κ B. The results showed that IL-1 β and IL-18 level in the flushing fluid was increased and TLR4, NLRP3, ASC, caspase-1, NF- κ B and p-NF- κ B expressions were up-regulated after the establishment of AGA rat model. Colchicine and Jia-Wei-Si-Miao-Wan administration could alleviate the inflammation in the knee by inhibiting NLRP3 inflammasome and TLR/NF- κ B signaling. *In vivo* data showed that the therapeutic effect of Jia-Wei-Si-Miao-Wan could be comparable with colchicine but had lower hepatic and renal toxicity. In conclusion, Targeting NLRP3 inflammasome and TLR/NF- κ B signaling by Jia-Wei-Si-Miao-Wan could be effective in treating AGA.

Gout is one of the most common metabolic disorders, which is characterized by increased serum uric acid (UA) level (1). Along with the westernized lifestyle, the incidence of gout in China, which is around 0.15-0.67%, has been on the increase in recent years. Furthermore, epidemiological studies have observed an early onset age of gout (2). This disease has a recurrent course and there is no effective curative treatment, which could greatly impair the patients' quality of life. Thus, it is

obvious that gout as a metabolic disorder has become a serious threat to the health of the general population. Furthermore, a large proportion of these patients will develop acute gouty arthritis (AGA), the typical clinical manifestations of which include pain, swelling and deformity of the joint and even immobility (3, 4).

At present, the treatments for AGA are mainly to alleviate the symptoms at the acute onset and prevent recurrence by decreasing serum uric acid level. The

Key words: acute gouty arthritis; inflammation; NLRP3 inflammasome; traditional Chinese medicine; therapy.

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treatment used for this, such as colchicine, steroids and non-steroidal anti-inflammatory drugs, may lead to severe gastrointestinal side effects, as well as hepatic, renal or cardiac toxicity, which limit their clinical application (5). Chinese herbs have been widely introduced in the treatment of multiple human inflammatory and malignant diseases and their clinical efficacy and safety are widely acknowledged (6-8). Based on clinical experience, Jia-Wei-Si-Miao-Wan was developed and used to treat AGA patients, and has been shown to have anti-inflammatory effects. However, the specific mechanism of this concoction in alleviating the inflammatory response of AGA has not been completely understood, therefore this study aimed to further explore the regulatory effect of Jia-Wei-Si-Miao-Wan on NLRP3 inflammasome and TLR/NF- κ B in AGA rat model, which is a key signaling in inflammation. These findings could help enrich the current understanding on the pathogenesis of AGA and provide evidence for the usage of Jia-Wei-Si-Miao-Wan as a recommended drug for treating such disease.

MATERIALS AND METHODS

Jia-Wei-Si-Miao-Wan preparation

Jia-Wei-Si-Miao-Wan was prepared by the traditional Chinese Medicine Pharmacy (The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, China) at the concentration of 1.2g/ml. The main recipe of Jia-Wei-Si-Miao-Wan per dosage was 15 g rhizoma atracylodes, 10 g golden cypress, 20 g semen coicis and 15 g radix achyranthis bidentatae. The study was approved by the Ethics Committee of The First Affiliated Hospital of Zhejiang Chinese Medical University and was in accordance with the Office for Protection from Research Risks (OPRR) and Animal Welfare Act Guidelines.

Animal rat model

Monosodium urate suspension was prepared at a concentration of 25mg/ml and stored at 4°C. A total of 108 6-week-old Sprague Dawley rats with a body weight of 200±15g were purchased from the Shanghai Laboratory Animal Center, China. The animals were acclimatized for one week to the new habitat with standard conditions of 23±1°C temperature, humidity 40±5 and 12-h light/dark cycle. After acclimatization, the animals were randomly divided into 6 groups: blank control group (Group A, n=18); AGA group (Group B, n=18); colchicine treatment group (Group C, n=18); low-dosage Jia-Wei-Si-Miao-Wan treatment group (Group D, n=18), middle-dosage Jia-Wei-Si-Miao-Wan treatment group (Group E, n=18) and high-dosage Jia-Wei-Si-Miao-Wan treatment group (Group F, n=18). The animals received food and water ad libitum. After acclimatization, the AGA rat model was established using the Coderre method. A volume of 0.2 ml 2.5% monosodium urate suspension was injected into the right joint cavity of the rats to establish the AGA rat model in Groups B, C, D, E and F. In Group C, colchicine was given at 0.325mg/(Kg•d) by gavage in a volume of 4 ml twice a day for 4 days and this group served as positive control. In Groups D, E and F, a dosage of 8, 16 and 24g/kg/d Jia-Wei-Si-Miao-Wan was diluted into 4 ml and was given by gavage twice a day for 4 days. Rats in Groups A and B received 4 ml normal saline solution by gavage twice a day for 4 days. The circumference of the right knee was measured at 1 h before (baseline circumference, A) and 4 h, 48 h after the model establishment and 4 h after the final gavage (B), and swelling index was calculated as (B-A)/A.

The rats were anesthetized 4 h after final gavage and the joint capsule in the right knee was cut. 1 ml normal saline solution was used to rinse the joint cavity and the flushing fluid was collected to be observed under microscopy. The

Table I. *Quantitative real-time RT-PCR primers.*

	Forward primers (5'→3')	Reverse primers (5'→3')
TLR4	CTCACAACCTTCAGTGGCTGGATTTA	GTCTCCACAGCCACCAGATTCTC
NLRP3	GAGTTCTTCGCTGCTATGT	ACCTTCACGTCTCGGTTT
ASC	GGACAGTACCAGGCAGTTTCG	GTCACCAAGTAGGGCTGTGT
caspase-1	CACGAGACCTGTGCGATCAT	GCGCCACCTTCTTTGTTTCAG
GAPDH	GACAACTTTGGCATCGTGGA	ATGCAGGGATGATGTTCTGG

synovium tissue was fixed by 4% paraformaldehyde. The synovium tissue specimen was cut into 4- μ m slices and hematoxylin-eosin (HE) staining was performed.

RNA extraction and quantitative real-time RT-PCR

Total RNA from the collected synovium tissue from the rats was extracted using Trizol (Invitrogen, USA) and quantified; 1 μ g total RNA was reverse transcribed into cDNA, which was used as DNA templates. TLR4, NLRP3, ASC and caspase-1 mRNA were detected using quantitative real-time RT-PCR and GAPDH served as the internal control according to the protocol. The RT-PCR kit and SYBT Green I kit (Takara, USA) were used and the data was analyzed by $\Delta\Delta C_t$ method. The primers are listed in Table I.

Laboratory tests and cytokine detection

Eye blood was also collected for the detection of alanine aminotransferase (ALT, U/L), aspartate aminotransferase (AST, U/L), UA (μ mol/L) and creatinine (CREA, μ mol/L). The IL-1 β and IL-18 levels in the flushing fluid were detected by enzyme linked immunosorbent assay (ELISA) (Roche, USA) according to the manufacturer's instructions, and were compared among the different groups.

Western blot

Total protein from the synovium tissue was prepared using RIPA lysis buffer (Applygen, China) and quantified by bicinchoninic acid (BCA) assay (Applygen, China). 30 μ g total protein sample was loaded into each well and run by electrophoresis. The blot was incubated in the 1:1000 diluted primary anti-TLR-4, anti-NF- κ B, anti-p-NK- κ B, anti-NLRP3, anti-ASC and anti-caspase-1 antibodies (Affbiotech) overnight at 4°C, and then in secondary antibody for 1 h at room temperature. The intensity band was recorded and analyzed. GAPDH served as the internal control.

Immunohistochemistry

NLRP3, ASC and caspase-1 expression in synovium tissue was measured by immunohistochemistry. The primary antibodies included anti-NLRP3, anti-ASC and anti-caspase-1 antibodies (Affbiotech), which were diluted into 1:500. DAB method was used and the staining intensity was scored: score = A x B where A was defined as the percentage of the positive staining cells: 0 (0-1%), 1 (1-10%), 2 (10-50%), 3 (50-80%) and 4 (80-100%), and B referred to the staining intensity of the positive staining cells: 0 (negativity), 1 (weak positivity), 2 (positivity) and 3 (strong positivity).

Table II. Swelling index among different groups.

Groups	4 h after injection	48 h after injection	4 h after final gavage
A	0.0107 $\pm$ 0.0026	0.0124 $\pm$ 0.0027	0.0247 $\pm$ 0.0034
B	0.2984 $\pm$ 0.0357 [▲]	0.4889 $\pm$ 0.0591 [▲]	0.4291 $\pm$ 0.0496 [▲]
C	0.3016 $\pm$ 0.0292 [▲]	0.3987 $\pm$ 0.0420 ^{▲**}	0.1583 $\pm$ 0.0333 ^{▲**}
D	0.2887 $\pm$ 0.0322 [▲]	0.4609 $\pm$ 0.0514 [▲]	0.3936 $\pm$ 0.0453 [▲]
E	0.3021 $\pm$ 0.0330 [▲]	0.4426 $\pm$ 0.0465 ^{▲*}	0.2565 $\pm$ 0.0527 ^{▲**}
F	0.2979 $\pm$ 0.0287 [▲]	0.4242 $\pm$ 0.0553 ^{▲**}	0.1868 $\pm$ 0.0321 ^{▲**}

Notes: [▲] $P < 0.01$ when compared with Group A; ^{*} $P < 0.05$ when compared with Group B; ^{**} $P < 0.01$ when compared with Group B.

Statistical analysis

All the statistical analyses were performed using SPSS 17.0 software. The continuous and categorical data were shown as the mean $\pm$ standard deviation (SD) and percentage (%), and the differences were tested by one-way analysis of variance (ANOVA) with Turkey post-hoc comparison, and *chi*-squared test or Fisher exact probability test if appropriate, respectively. Two-tailed $P < 0.05$ was considered to be statistically significant.

RESULTS

Establishment of AGA rat model

The success rate of AGA rat model establishment was 100%. Synovium specimen was collected and examined by pathology (Fig. 1). In Group A, it was observed that the synovial cells were regularly arranged and the structure of the tissue was clear without inflammatory cell infiltration and proliferation. In Group B, obvious tissue swelling, irregular arrangement, inflammatory cell infiltration with capillary proliferation and fibroblasts were

detected, which were alleviated in Groups C, D, E and F to various degrees. The alleviating effect of Jia-Wei-Si-Miao-Wan on the inflammation in synovium tissue was in a dose-dependent manner. The swelling index of the knee was calculated (Table II) and it was shown that Jia-Wei-Si-Miao-Wan could decrease the swelling of the knee in a dose-dependent manner. Serum ALT, AST and CREA levels were comparable among the different groups (all $P > 0.05$), while serum UA level in Group B was significantly higher than that in the other groups ($P < 0.05$) (Fig. 2). These data indicated that Jia-Wei-Si-Miao-Wan could exert an anti-inflammatory activity without hepatic and renal toxicity.

Jia-Wei-Si-Miao-Wan decreased IL-1 β and IL-18 levels in joint cavity flushing fluid

In order to better clarify the molecular mechanism of Jia-Wei-Si-Miao-Wan in treating acute gouty arthritis, its effect was detected on IL-1 β and IL-18 level in the joint cavity flushing fluid (Fig. 3A-B), which are two important cytokines in

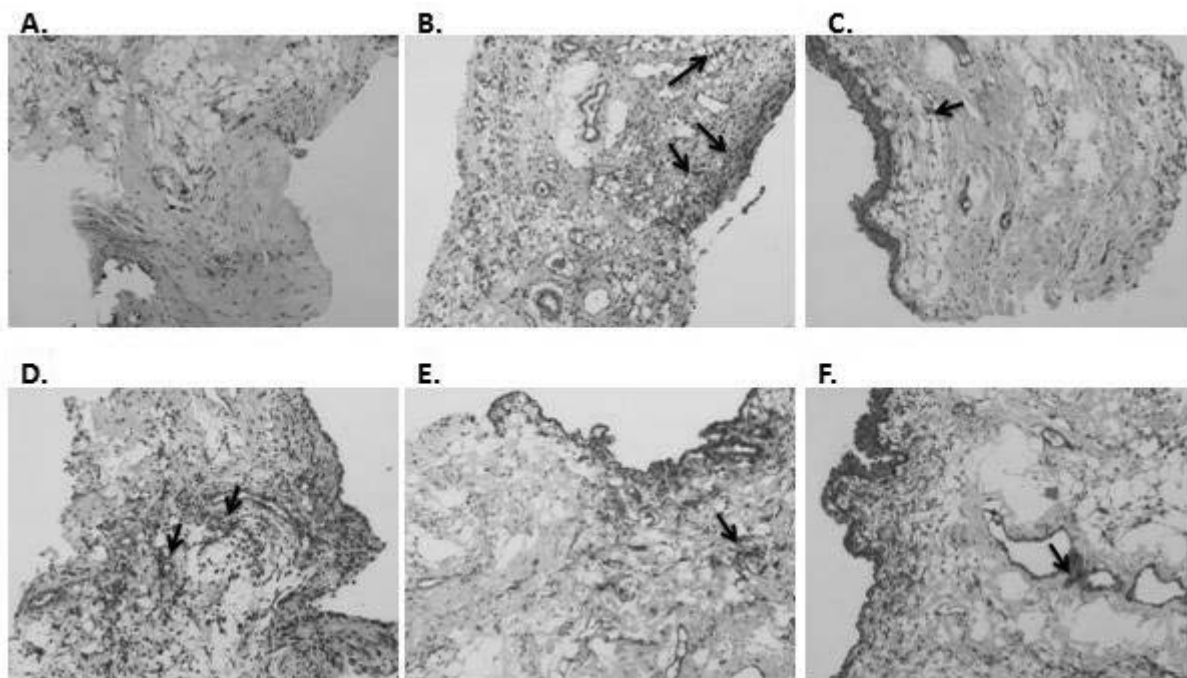


Fig. 1. HE staining of synovial tissue among different groups observed obvious tissue swelling, irregular arrangement, inflammatory cell infiltration with capillary proliferation and fibroblasts after the AGA rat model establishment, which were alleviated by colchicine and Jia-Wei-Si-Miao-Wan treatment.

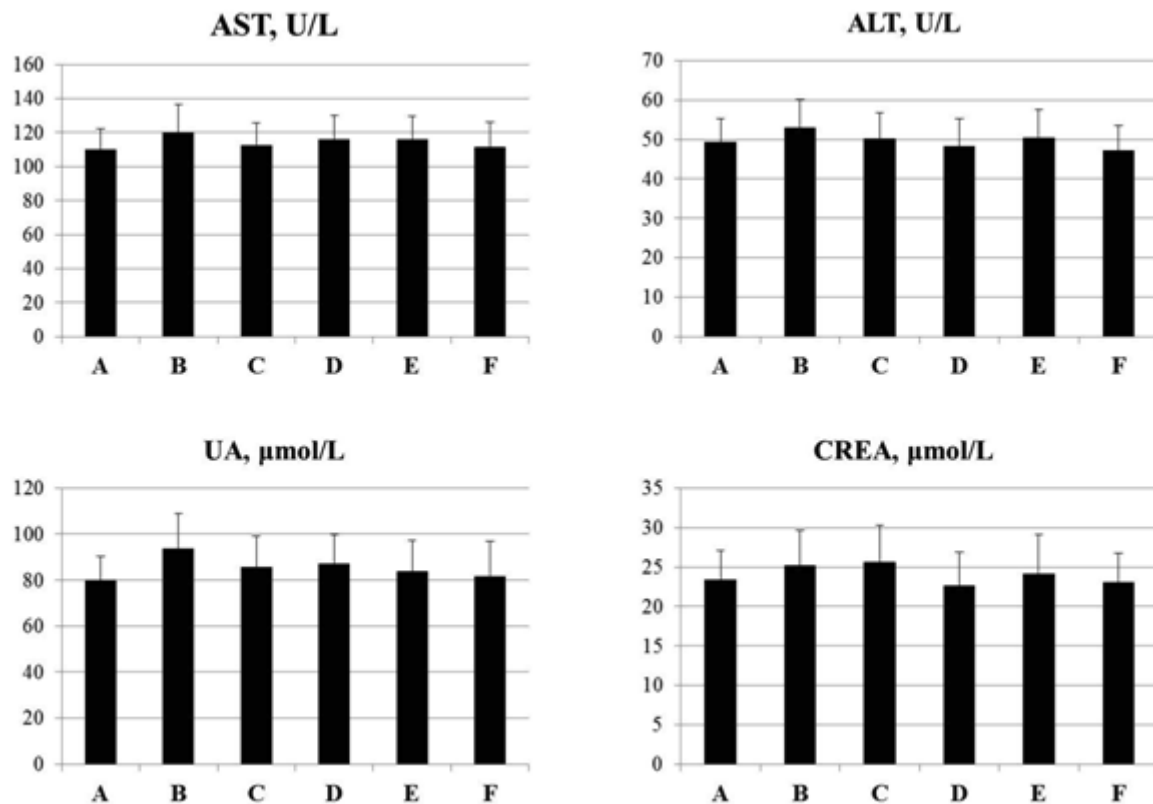


Fig. 2. Serum AST, ALT, UA and CREA levels were detected, which were not significantly increased by Jia-Wei-Si-Miao-Wan.

the inflammatory reaction induced by TLR/NF- κ B signaling (9,10). IL-1 β and IL-18 levels in Group B were higher than those in other groups ($P < 0.01$). IL-1 β and IL-18 levels in Group C and Group F were comparable, and were greatly decreased compared with those in Group B ($P < 0.01$), indicating that Jia-Wei-Si-Miao-Wan could decrease IL-1 β and IL-18 levels in joint cavity flushing fluid in a dose-dependent manner.

Jia-Wei-Si-Miao-Wan alleviated inflammatory response by targeting NLRP3 inflammasome and TLR/NF- κ B signaling

NLRP3 inflammasome has been proved to be associated with multiple human inflammatory diseases. Caspase-1 is essential for the maturation of IL-1 β and IL-18 precursors. Based on the effect of Jia-Wei-Si-Miao-Wan on IL-1 β and IL-18, it is hypothesized that Jia-Wei-Si-Miao-Wan could

inhibit the inflammation in AGA by suppressing the induction of NLRP3 inflammasome and TLR/NF- κ B signaling on IL-1 β and IL-18. Western blot and real-time RT-PCR showed that TLR4, NLRP3, ASC and caspase-1 protein and mRNA expressions were all significantly increased in Group B, when compared with those in Group A (Fig. 3C-D). NF- κ B and p-NF- κ B protein level were also up-regulated in Group B. Up-regulations of TLR4, NLRP3, ASC, caspase-1, NF- κ B and p-NF- κ B were decreased by colchicine and Jia-Wei-Si-Miao-Wan in a dosage-dependent manner after the establishment of AGA rat model. These findings were consistent with IHC analysis. Similar effects of Jia-Wei-Si-Miao-Wan on NLRP3, ASC and caspase-1 expression in synovium tissue were found (Fig. 3-6). Taken together, the therapeutic action of Jia-Wei-Si-Miao-Wan for AGA were associated with targeting NLRP3 inflammasome and TLR/NF- κ B signaling pathway.

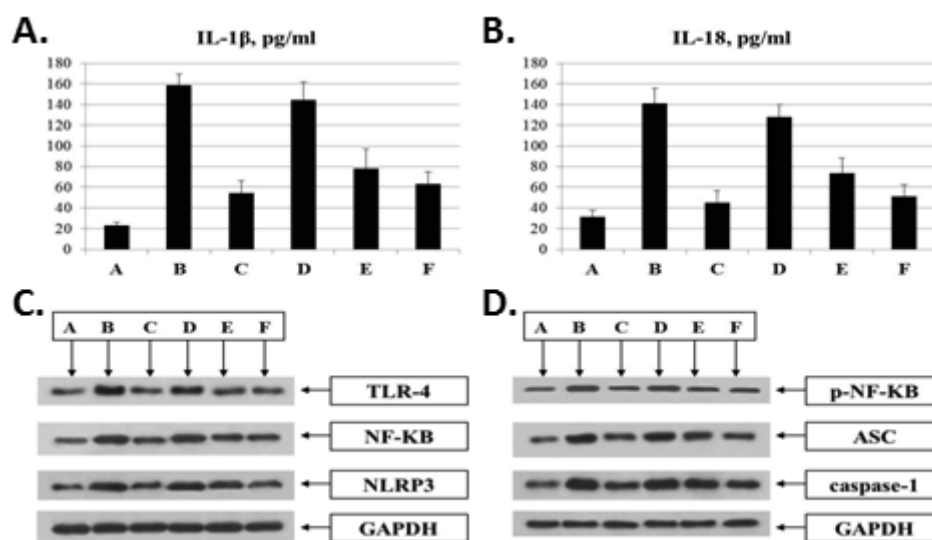


Fig. 3. IL-1 β (A) and IL-18 levels (B) in the joint cavity flushing fluid were detected by ELISA. TLR4, NLRP3, ASC, caspase-1, NF- κ B and p-NF- κ B protein expression was measured by Western blot (C-D).

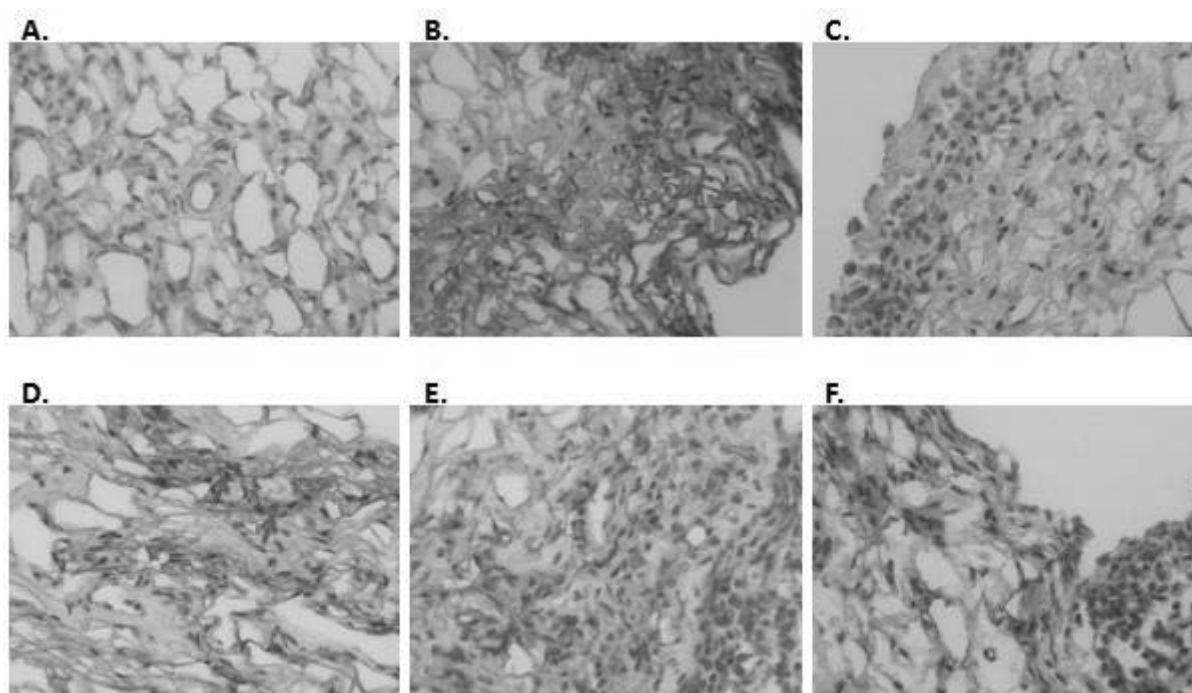


Fig. 4. NLRP3 level in synovial tissue was detected by IHC. Jia-Wei-Si-Miao-Wan decreased NLRP3 expression in a dose-dependent manner.

DISCUSSION

Gout is one of the most common metabolic disorders and there is still no cure. Patients with gout are prone to develop AGA (11, 12). Colchicine is effective in alleviating the clinical symptoms by

exhibiting anti-inflammatory effects, but the risk of bone marrow inhibition and hepatorenal toxicity is relatively high, and should be closely monitored during the treatment (13). A new Chinese recipe was newly developed called Jia-Wei-Si-Miao-Wan, which was effective in treating AGA in clinical

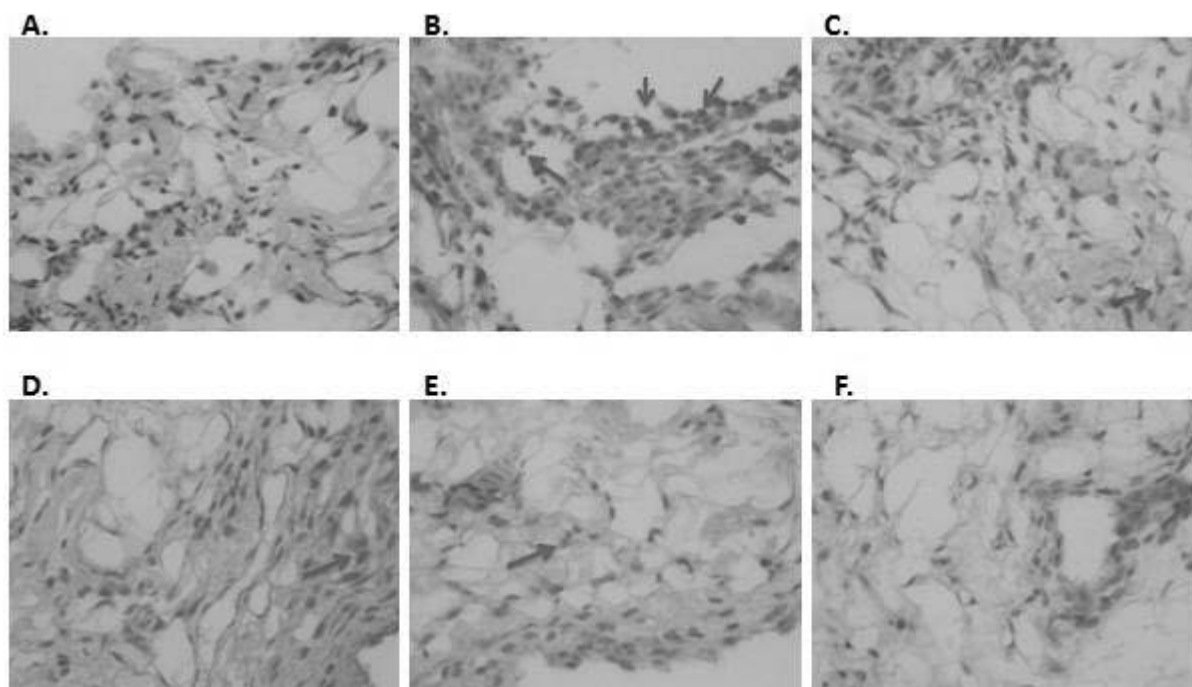


Fig. 5. ASC level in synovial tissue was detected by IHC. Jia-Wei-Si-Miao-Wan down-regulated NLRP3 expression in a dose-dependent manner.

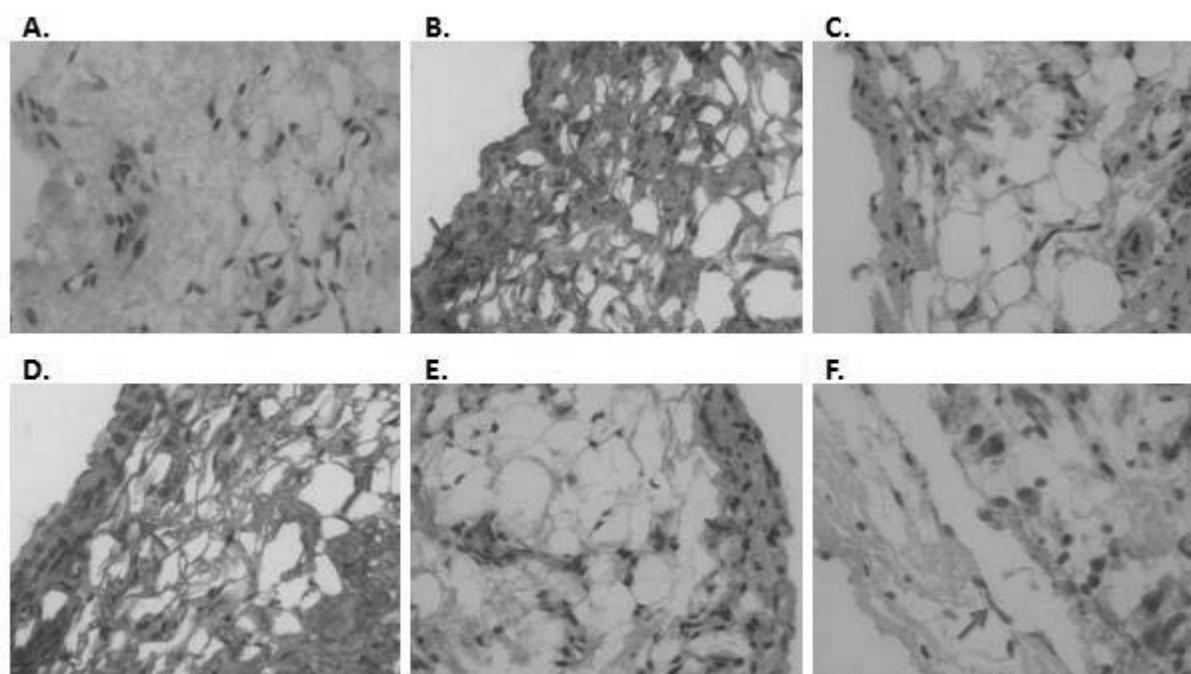


Fig. 6. The down-regulation of Jia-Wei-Si-Miao-Wan on the caspase-1 expression in synovial tissue was in a dose-dependent manner; which was detected by IHC.

practice. The present study proved that Jia-Wei-Si-Miao-Wan could suppress inflammation in AGA rat model by targeting NLRP3 inflammasome and TLR/NF- κ B signaling pathway.

NLRP3 inflammasome signaling and TLR/NF- κ B pathway play a important role in the inflammation response, which could lead to the release of a series of cytokines such as IL-1 β and IL-18 (14, 15). IL-1 β is closely associated with gouty arthritis, which is an inflammatory initiator. The binding of IL-1 β with specific receptors could activate the downstream signaling, causing neutrophil infiltration and osteoclast differentiation (16, 17). NLRP3 is one member of the NLR family and the structure is mainly composed of 3 structural domains including LRR domain, NACHT domain and effective domain (18). ASC has a PYD structural domain in N-terminal and a CARD recruiting domain in C-terminal. ASC is an important adaptor protein, and PYD domain of ASC could bind with PYD domain of NLRP3. The CARD domain could recruit the downstream caspase-1 to form the complex of NLRP3 inflammasome. Caspase-1 can convert IL-1 β and IL-18 precursor into mature IL-1 β and IL-18, and the formation of NLRP3 inflammasome is essential for the activation of caspase-1. The production of IL-1 β precursors is induced by TLR/NF- κ B pathway, and the IL-1 β and IL-18 maturation is controlled by caspase-1 in NLRP3 inflammasome (19, 20). NLRP3 inflammasome and TLR/NF- κ B inhibition can suppress the maturation of IL-1 β and IL-18, which has the activity of initiating the inflammatory response (21-23).

In this study, AGA rat model was first established and synovial tissue was collected. IL-1 β and IL-18 level in joint cavity flushing fluid was increased after the model establishment, supporting that IL-1 β and IL-18 contribute to the development of acute gouty arthritis. In the study, colchicine was used to treat AGA and the therapeutic effect of Jia-Wei-Si-Miao-Wan was compared with that of colchicine. The regulation of colchicine and Jia-Wei-Si-Miao-Wan on TLR4, NLRP3, ASC, caspase-1, NF- κ B and p-NF- κ B expression was evaluated in the synovial tissue from the rats; TLR4, NLRP3, ASC, caspase-1, NF- κ B and p-NF- κ B protein and mRNA expressions

were up-regulated in AGA rat model, and decreased after colchicine and Jia-Wei-Si-Miao-Wan treatment. Jia-Wei-Si-Miao-Wan had relatively low hepatic and renal toxicity, which is a promising therapeutic drug for acute gouty arthritis.

In summary, *in vivo* experiments demonstrated that Jia-Wei-Si-Miao-Wan could decrease inflammation by inhibiting the maturation of IL-1 β and IL-18 in AGA rat model. The therapeutic effectiveness of Jia-Wei-Si-Miao-Wan was comparable to but safer than colchicine. The data provided new insight into understanding the molecular mechanism of Jia-Wei-Si-Miao-Wan in the treatment of gouty arthritis, however, its therapeutic efficacy in treating AGA should be further validated in a large-scale clinical trial before being recommended in clinical practice.

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EFFECTS OF T CELL SUBSETS WITH DIFFERENT PROPORTIONS ON RENAL FUNCTION AND BLOOD LIPIDS IN PATIENTS WITH PREECLAMPSIA

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This study aimed to investigate the effect of T cell subsets with different proportions on renal function and blood lipids in patients with preeclampsia (PE). Ninety pregnant women were included in this experiment and classified into 3 groups: group A (30 with severe PE), group B (30 with mild PE) and group C (30 healthy pregnant women). Clinical characteristics were examined. The ratio of T cell subsets in the three groups was detected by flow cytometry. The relationship between PE and T cell subsets was determined. The results showed that no significant difference was found in age or gestational age ($P>0.05$). The number of CD4⁺ T cells (CD4 is mainly expressed in helper T cells, referred to as TH) in group A increased significantly compared with group B and group C ($P<0.05$), while the number of CD8⁺ T cells (CD8⁺ T cell, also known as cytotoxic T cell (CTL) in group A decreased noticeably in comparison to group C ($P<0.05$). The ratio of CD4⁺ cell number to CD8⁺ cell number (TH/CTL) in group A was elevated significantly compared with group B and group C ($P<0.05$). The uric acid (UA) concentration of group A was noticeably elevated compared to group C ($P<0.05$), which differed insignificantly between group B and C ($P>0.05$). Glomerular filtration rate (GFR) of group A was obviously impaired in comparison to group B and group C ($P<0.05$), which differed insignificantly between group B and C ($P>0.05$). Total cholesterol (TC) concentrations in group A, C and B were examined. TC concentrations in the former two groups were slightly higher compared to the latter ($P<0.05$), and those in the former two groups were also higher than the normal range ($P<0.05$). Mean triglyceride (TG) concentrations in all 3 groups were above the normal range, and those in group A and B were significantly higher compared to group C ($P<0.05$). TG concentrations differed insignificantly between group B and C ($P>0.05$). Serum TG, UA and TC in PE patients were positively related to TH/CTL ($P<0.05$). In conclusion, PE is related to T cell subsets, and T cell subsets are closely related to kidney injury and dyslipidemia.

Preeclampsia (PE) is a disease which specifically attacks pregnant women (1, 2). Approximately 2-8% of gravida worldwide develop PE (3, 4), which is classified into mild and severe, according to the severity of the disease (5). PE can cause

inflammation in many systems, which may lead to damage of the liver, kidneys, lung, central nervous system and other organs (6, 7). Understanding of PE etiology is currently insufficient. There are several main causes, including hypogenesis of uterine spiral

Key words: T cell subsets; preeclampsia; uric acid; kidney injury and dyslipidemia

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arterioles, inflammatory immune over-activation, vascular endothelial injury, genetic factors, insulin resistance and nutritional deficiency (8, 9). It has been suggested that pathogenesis of PE is related to the immune mechanism (10, 11). Thus various studies have investigated immune related factors. At the same time, since T cells are the main participants in cellular immunity, T cell subsets have recently gained attention (12). At present, it is generally accepted that the imbalance of T cell subsets is related to renal diseases and lipid changes in PE (13, 14). The number and proportion of T cell subsets were examined to verify their correlation with PE and to assist PE management.

MATERIALS AND METHODS

Patients

A total of 90 pregnant women admitted to the maternity ward of Zhejiang Provincial People's Hospital from March 2017 to July 2018 were included in the study, and were classified into 3 groups (30 patients in each group): severe PE group (group A), mild PE group (group B) and normal pregnancy group (group C). Mild PE was defined as systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg with proteinuria ≥ 0.3 g/24h [or random urinary protein (+)] subsequent to twenty weeks of gestation, accompanied by upper abdominal discomfort, headache and other symptoms. Severe PE was characterized by SBP ≥ 160 mmHg and/or DBP ≥ 110 mmHg with proteinuria ≥ 2.0 g/24h [or random urinary protein (+)], serum creatinine > 106 μ mol/L, platelet $< 100 \times 10^9$ /L, persistently increased LDH, increased serum ALT or AST, and persistent headache and epigastric discomfort. Informed consent was signed by all patients and this study was approved by the Ethics Committee of Zhejiang Provincial People's Hospital.

Specimen collection and data statistics

Clinical features of all participants were collected. Venous blood samples were taken, and UA, TC, creatinine and TG levels were detected and recorded immediately after admission.

According to the formula of glomerular filtration rate (GFR) = $186 \times \text{serum creatinine value}^{-1.154} \times \text{age}^{-0.203} \times 0.742$, the GFR value of each group was calculated.

Detection of T cell subsets

In this study, the number and proportion of T cell subsets in the three groups were detected by flow cytometry (Beckman Coulter, USA). 2 mL of peripheral blood of pregnant women were taken immediately before delivery, and were equally divided into five tubes for each pregnant woman, and each tube was added with 100 μ L EDTA (Thermo Scientific, USA) anticoagulant; 20 μ L of CD45-FITC (Thermo Scientific, USA), IgG-PE (Thermo Scientific, USA), 10 μ L IgG-ECD (American Diagnostica Inc) and 10 μ L IgG-PC5 (Coulter Immunotech, France) were added to No.1 tube, respectively; 20 μ L of CD45-PE (Thermo Scientific, USA), CD45-ECD (Coulter Immunotech, France), and CD45-PC5 (Beckman Coulter, USA) were added to No.2, No.3 and No.4 tubes, respectively; 10 μ L of CD45-FITC, CD4-PE (BDpimg, USA) CD8-ECD (Beckman Coulter, USA), and CD3-PC5 (Beckman Coulter, USA) were added to No. 5 tube. After mixing, they were incubated for 15 min at room temperature. 500 μ L of red blood cell lysis buffer was supplemented to the test tube, which was mixed and preserved in the dark for 10 min, followed by centrifugation for 5 min. The supernatant was abandoned, and 1 μ L of normal saline was added before another 5-min centrifugation. The supernatant was discarded, then after adding 170 μ L normal saline again, the cells were prepared to a suitable concentration. 100 μ L Flowcount counting microspheres (Beckman Coulter, USA) were added, and quality control microspheres were used for flow cytometry within 1 h without light. The ratios of TH, CTL and TH/CTL in each group were recorded.

Statistical analysis

SPSS 22.0 software was used for statistical analysis, and one-way analysis of variance and Pearson's correlation analysis were employed. $P < 0.05$ indicated significant difference.

RESULTS

General data

There were no significant differences in ages and the times of pregnancy among the 3 groups ($P > 0.05$), while gestational age differed significantly ($P < 0.05$), as shown in Table I. There was no significant difference in gestational age between groups B and C ($P > 0.05$).

Comparison of T cell subsets in each group

TH and CTL were detected by flow cytometry, and TH/CTL was calculated. The results of flow cytometry in each group are shown in Figs. 1-3.

As revealed in Fig. 1-3 and Table II, TH was remarkably enhanced in group A compared with group B and group C ($P<0.05$), which differed insignificantly between the latter 2 groups ($P>0.05$). CTL was noticeably suppressed in group A compared with group B and group C ($P<0.05$), which differed insignificantly between the latter 2 groups ($P>0.05$). TH/CTL was elevated significantly in group A compared with group B and group C ($P<0.05$), which differed insignificantly between group B and group C ($P>0.05$).

Comparison of UA, GFR, TC and TG levels in blood of each group

As revealed in Table III, UA was remarkably enhanced in group A compared with groups B and

C ($P<0.05$), and differed insignificantly between groups B and C ($P>0.05$). There was significant difference in GFR between group A and group B or group C ($P<0.05$), which differed insignificantly between group B and group C ($P>0.05$). TC levels were slightly elevated in groups A and C compared to group B ($P>0.05$), but no significant difference was found between group B and group C ($P>0.05$). TG levels of all 3 groups were above the normal range, which differed significantly among the 3 groups, and TG levels in group A and B were higher than those in group C ($P<0.05$). Alteration in renal function was more noticeable in group A, with TG concentrations higher than group C.

Correlation analysis

The correlation between UA, GFR and TG, TC and TH, CTL and TH/CTL in severe PE patients was analyzed, and is shown in Fig.4.

UA of patients with severe PE was positively

Table I. Comparison of general information in each group.

Groups	Group A	Group B	Group C	P
Age (years)	32.88±4.17	31.451±3.37	30.73±3.16	0.122
Gestational times	1.76±0.91	1.78±0.76	1.55±0.69	0.389
Gestational age (days)	248.26±23.33	269.31±11.15	272.51±11.42	0.001

Table II. Comparison of mean values of TH, CTL and TH/CTL in blood.

Groups	Group A	Group B	Group C	P
TH	1167.45±354.22	903.56±231.63	822.54±308.15	0.001
CTL	313.57±138.86	878.49±487.26	941.35±416.55	0.001
TH/CTL	4.28±1.75	1.71±1.15	1.09±0.58	0.001

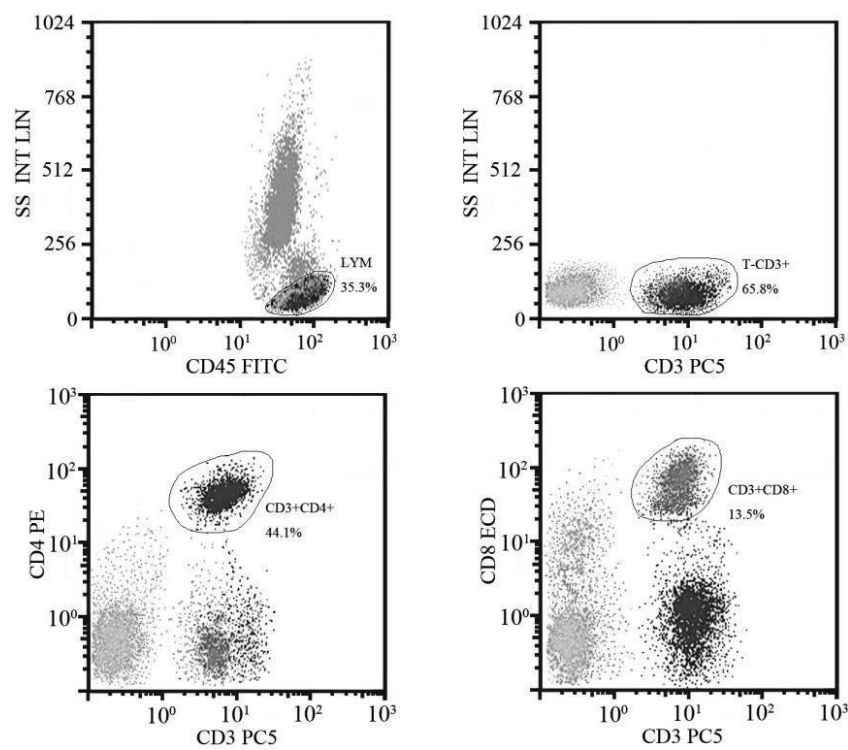


Fig. 1. Flow cytometry detection in group A.

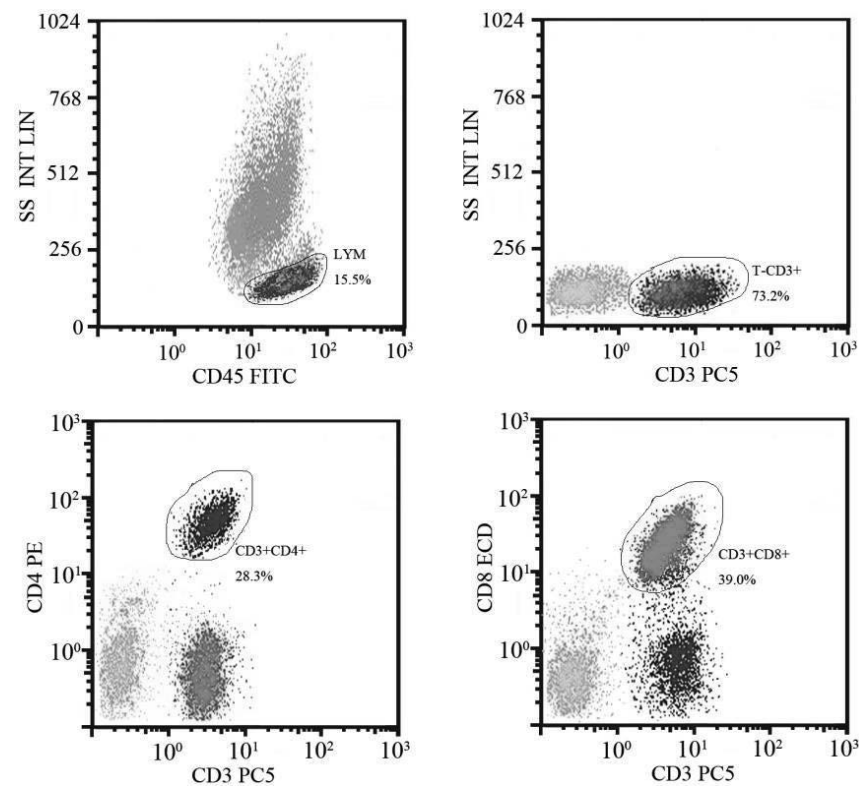


Fig. 2. Flow cytometry detection in group B.

correlated with TH ($P < 0.05$) (Fig.4). GFR was negatively correlated with TH/CTL ($P < 0.05$); TC was also positively correlated with TH, and TG was positively correlated with TH ($P < 0.05$). Since UA, GFR, TC and TG were linked with T cell subsets, it was suggested that T cell subsets were closely associated with kidney injury and lipid changes of patients with severe PE.

DISCUSSION

It has been shown that PE is associated with immunity, and T cells are an important component of cellular immunity (15). T cell subsets are divided into $CD4^+$ T cells (TH) and $CD8^+$ T cells (CTL) according to expressions of CD4 or CD8 molecules on the cell surface (16, 17). Our study proved that TH of group A was remarkably increased compared to group B and group C. The main function of $CD4^+$ T cells is to induce helper B cells, killer T lymphocytes

and other immune cells by secreting cytokines to enhance the immune response. Therefore, the number of $CD4^+$ T cells in PE patients was increased significantly. CTL was significantly suppressed in group A compared with group B and group C, which differed insignificantly between group B and group C. The decreased number of $CD8^+$ T cells may be attributed to two mechanisms. On one hand, for the fetus, the decreased number of suppressive T cells may weaken the maternal immunosuppressive status and affect the normal growth of the fetus. On the other hand, for the mother, the reduction of cytotoxic T cells weakens the clearance and killing effect of the mother to the external antigen, and brings about injury, which has a serious impact on maternal health. In terms of TH/CTL ratios, the ratio in group A was elevated significantly, which was far above the normal range. It can be considered that the pathogenesis of group A is obviously different from that of groups B and C. It has been indicated that the

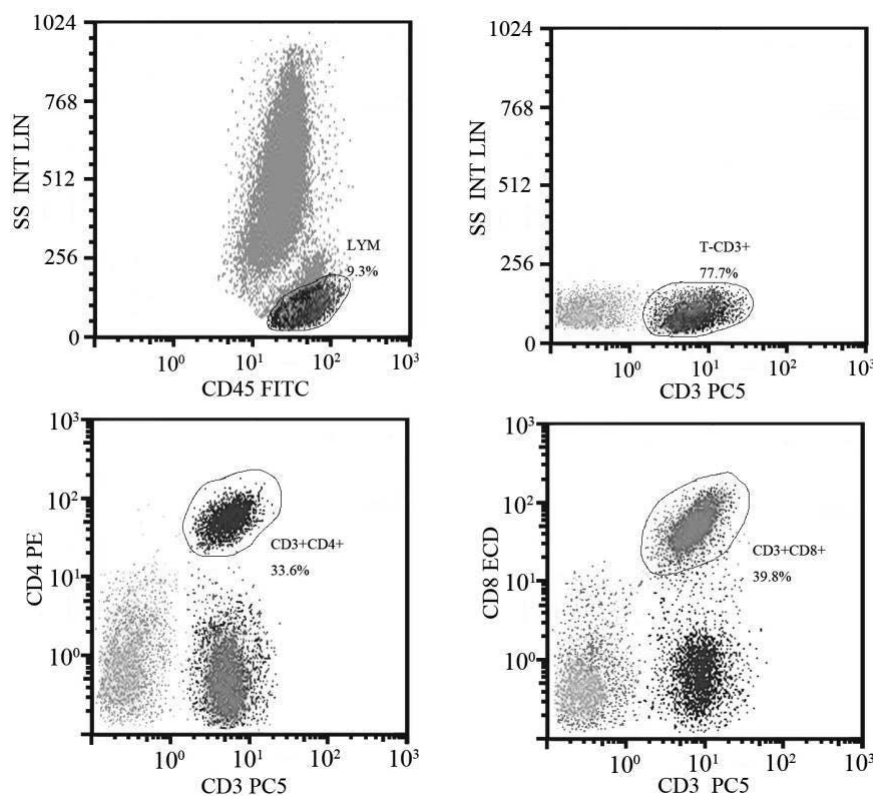


Fig. 3. Flow cytometry detection in group C.

Table III. Comparison of UA, GFR, TC and TG levels in blood.

Groups	Group A	Group B	Group C	P
UA (umol/L)	418.04±63.35	358.94±56.11	344.16±47.02	0.001
GFR (ml/min*1.73m ²)	115.62±15.87	136.27±20.08	144.46±24.13	0.001
TG (mmoL/L)	3.12±0.56	3.11±0.87	2.56±0.93	0.022
TC (mmoL/L)	5.48±1.23	5.17±0.74	5.49±1.04	0.593

Note: UA: normal range (155-357umol/l); GFR: normal range (133-153ml/min/1.73m²); TC: normal range (<5.20mmol/l); TG: normal range (<1.70mmol/l).

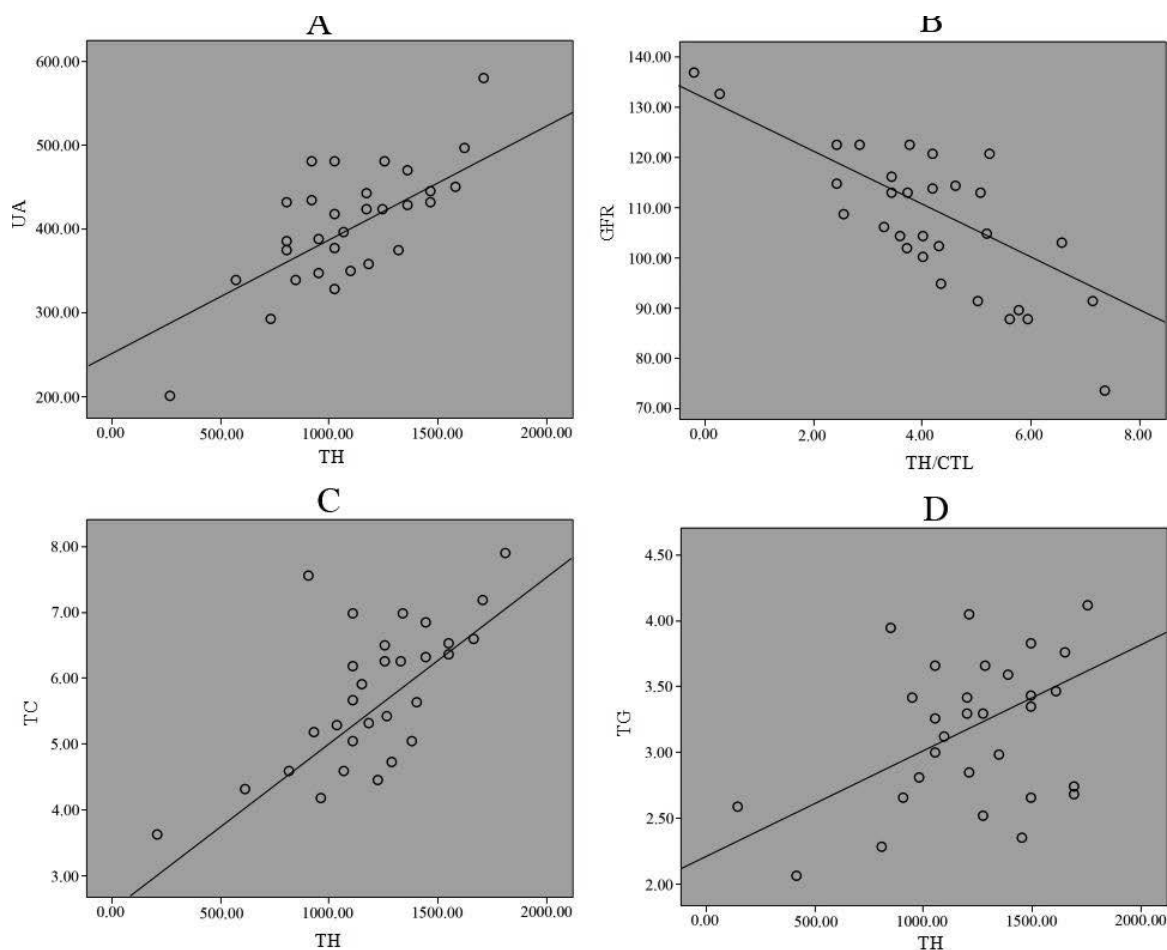


Fig. 4. Correlation analysis charts. **A)** UA and TH scatter plots. UA and TH were positively correlated with each other, with the correlation coefficient (r) of 0.749. **B)** GFR and TH/CTL ratio scatter plots; GFR and TH/CTL ratio was negatively correlated with each other, and $r = -0.598$ showed moderate correlation. **C)** TC and TH cell number scatter plots. TC and TH were positively correlated with each other, and $r = 0.703$ showed positive correlation. **D)** TG and TH scatter plots. TG was positively correlated with the number of TH cells, and $r = 0.588$ showed moderate correlation)

degree of maternal fetal immune balance is reflected by TH/CTL. The disease is aggravated with a higher TH/CTL ratio (18). It was found that the ratio of TH/CTL in group A was elevated significantly compared to group B, supporting the conclusion that the ratio of TH/CTL participates in pathological process of PE, and that the ratio of TH/CTL reflects PE severity. It is inferred that severe PE is closely related to T cell subsets, and it is a special disease of pregnancy caused by an abnormal number or proportion of T cell subsets.

In patients with PE, UA concentrations are used as a reliable predictor of early renal injury (19, 20). UA is a protective antioxidant in PE patients, but it can also induce inflammatory responses and lead to endothelial dysfunction (21). The increased UA often propels progression of the disease, including reducing the level of GFR. GFR levels and UA values are important indicators of the kidney injury. There was significant difference in UA and GFR between group A and group C, indicating that UA of patients with PE was significantly increased and GFR levels were decreased. Serious damage of renal function is induced by long-term immune responses due to severe immune hyperactivity in PE patients *in vivo*. At the same time, it was found that the difference in UA and GFR was insignificant between groups B and C, and that UA was increased in group A compared to group B. It is concluded that UA is of great importance for early prediction of severe PE. Meanwhile, the changes of GFR in groups B and A were significant for assessment on the severity of PE.

Enquobahrie et al. found that abnormal blood lipids during early pregnancy especially increased serum levels of TG and LDL (Low Density Lipoprotein), and were associated with an increased risk of PE (22). This study suggested that the levels of TC and TG were increased significantly. TC levels were elevated significantly in group A and group C compared to group B or the normal range, but no significant difference was identified between group A and group C. TG levels were above the normal range in all 3 groups, which differed significantly among the 3 groups, but no significant difference was found between group A and group B. Meanwhile, it was found that TC and TG were positively correlated

with the number of CD4⁺ T cells ($P < 0.05$). The level of blood lipids in patients with severe PE was generally higher than the normal value. The results also showed that the increase of TC and TG in patients with PE was significantly related to T cell subsets. Immune related factors have a great impact on the changes of blood lipids in patients with PE. Therefore, investigating the level of blood lipids may reflect the situation of T cell subsets. There was significant correlation between increased serum lipids and T cell subsets, which supports that immune factors lead to increased serum lipids.

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LETTER TO THE EDITOR

HETEROTOPIC PREGNANCY FOLLOWING *IN VITRO* FERTILIZATION AND EMBRYO TRANSFER PROCEDURE

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To investigate the pathogenesis, diagnosis and treatment of heterotopic pregnancy (HP), a retrospective analysis of the clinical data for *in vitro* fertilizations and embryo transfer procedures (IVF-ET) was performed at the Hospital of Jilin University. In this case study, the subject was admitted to the hospital for monitoring the IVF-ET results by color Doppler ultrasonography and found to have an intrauterine pregnancy combined with the income of the annex area. Postoperative pathology confirmed the HP. The patient was provided with appropriate expectant management, and finally cesarean section was performed for delivery of a full-term infant. IVF-ET patients should be alerted to the occurrence of HP. If HP is diagnosed, a comprehensive assessment based on the patient's clinical and extrauterine pregnancy characteristics should be undertaken to determine the treatment plan. Expectant management may also lead to a good outcome for mother and infant.

To the Editor,

Heterotopic pregnancy (HP) is a condition defined by co-existence of intrauterine and ectopic gestations (1). However, cervical, ovarian, cornual and abdominal heterotopic pregnancies have been reported (2). Extremely rare multifetal heterotopic pregnancies have also been reported (2). *In vitro* fertilizations (IVF) with embryo transfer (ET) have been linked to a higher rate of ectopic pregnancies compared to spontaneous pregnancies. Between 2.1% and 8.6% of all clinical pregnancies after IVF-ET have been reported as ectopic (3, 4), compared to 2% ectopic pregnancy rate after natural conception (5). Recent studies showed a remarkable increase in the incidence of ectopic pregnancies, which may be due to several factors including growing incidence of pelvic inflammatory disease, endometriosis,

tubal surgery, intrauterine device usage and, most importantly, the increasing use of ovulation induction and assisted reproductive technology (ART) (6). In recent years, the improvements in IVF-ET technology have made the treatment courses more or less similar to the natural processes. The Society for Assisted Reproductive Technology (SART) reported that the incidence of ectopic pregnancy following IVF-ET decreased from 2.1% in the year 2000 to 1.8% in 2001 (7) due to risk of ectopic pregnancy after IVF-ET rather than natural conception. Therefore, heterotopic pregnancy is a rare clinical type of ectopic pregnancy, the probability of which is just 1/30,000. In recent years, with the implementation of assisted reproductive technology and the growing use of ovulation drugs, the incidence of HP has been increasing significantly. It has been reported that

Key words: heterotopic pregnancy; in vitro fertilization and embryo transfer; expectant management

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the probability of HP after *in vitro* fertilization and embryo transfer procedure is approximately 1% (8). In the present article, we report a case of ectopic pregnancy at the ipsilateral tubal stump following IVF-ET with a prior uterine scar. The case was diagnosed in our hospital in November 2017.

Case report

A 29-year-old female was admitted to the Department of Obstetrics and Gynecology, The First Hospital of Jilin University, Changchun, Jilin, China. The patient was at approximately 38±2 weeks of pregnancy after IVF-ET procedure, and gave informed written consent for the publication of this case report.

Medical history

The patient had previously been pregnant four times but had only one live birth (male) delivered 5 years ago by caesarean section (gravidity and parity - G4P1). Two of her pregnancies (7 and 2 years ago) were terminated by abortion. The patient had partial resection of the right ovary and right fallopian tube due to right ovary mucinous cystadenoma 11 years ago. She also had ductus arteriosus repair surgery due to congenital heart disease 15 years ago. The subject had a regular menstrual pattern, 4-5d/28-30d. She had no dysmenorrhea. The last menstrual cycle was on March 03, 2017. Due to secondary infertility, the frozen embryo implantation was performed in the reproduction center of our hospital on March 16, 2017, and 2 embryos were implanted into the uterine cavity. The levels of human chorionic gonadotropin (HCG, 1106 mIU/ml) and progesterone (38.77 ng/ml) were measured 12 days after implantation of the embryo. Color ultrasonography examination (Shenzhen Greatrend Technology Co., Guangdong, China) showed an intrauterine early pregnancy 36 days after the embryo implantation. No ectopic pregnancy lesions were found by regular pregnancy tests. Ninety-eight days after implantation of the embryos, color ultrasonography showed a cyst measuring 3.3 × 2.9 cm in the right side of the mother's uterus (Fig. 1). Since the possibility of HP could not be ruled out, the patient was referred to the Department of Obstetrics and Gynecology, The

First Hospital of Jilin University, Changchun, Jilin, China. The patient and her family decided to take expectant management. Regular color ultrasound inspections were conducted to closely monitor any changes in the attachment area.

The size of the cyst was changing as follows: 18 weeks of pregnancy - 3.4 × 2.6 cm with mixed echoes and within 2.3 × 1.4 cm echo-free; 19 weeks of pregnancy - 3.1 × 2.4 cm with mixed echo and within 2.0 × 1.1 cm echo-free; 21 weeks of pregnancy - 1.7 × 1.5 cm with mixed echo and within 1.0 × 0.5 cm echo-free; 23 weeks of pregnancy - 2.4 × 1.5 cm with low echo and some blood flow around the signal; 30 weeks of pregnancy - 2.5 × 1.9 cm with mixed echo; 33 weeks of pregnancy - 2.1 × 2.0 cm with mixed echo. No obvious mass was identified after 38 weeks of pregnancy.

Clinical examination

The patient showed stable vital signs, no abnormal heart and lung auscultation, no abdominal distention, no abdominal third trimester, no abdominal tenderness or rebound tenderness and muscle tension. At the time of diagnosis and admission to the Department of Gynecology, the patient was at 38 ± 2 weeks of pregnancy, the baby's head position was down. The patient had a scar in the uterus due to previous surgery. Fundal height was 33 cm, and abdominal circumference was 106 cm. No contractions were observed. The cervical canal was regressed 50%. The uterus was not open, the fetal membrane was not broken, and the fetus was exposed at S=-3.

Post-operative treatment

When the patient was admitted to the hospital, the thickness of the scar on the uterus wall was 0.18 cm. No obvious abnormalities were found during the laboratory examination. On the 7th day of hospitalization (39±1 weeks of pregnancy), irregular contractions occurred. Taking into account the facts that the patient had congenital heart disease, the patient's ductus arteriosus was repaired, and the uterine scar was weak, the decision was taken to urgently perform cesarean section under combined spinal-epidural anesthesia. During surgery, the infant was born (weight 2980 g, length 49 cm, Apgar score

was performed 1 min and 10 min after birth). The child was hand-pulled using the LOP mechanism. The intraoperative exploration found that the right ovary was lacking, the right cervix stump had a 3×2 cm cyst and the left side had no abnormalities.

After consulting with the patient and her family, a right salpingo-oophorectomy was performed. The capsule of the cyst was found to be intact and smooth. It was sent for pathological examination. The operation was successful, and treatment for postoperative uterine contractions, prevention of infection, as well as symptomatic treatments was given. Postoperative pathology (right fallopian tube stump) was consistent with the heterotopic pregnancy. The tissue had undergone mechanical degeneration, and there was highly variable villous residual image and trophoblastic cells. Therefore, the final clinical diagnosis was pregnancy at the stump of the right side of the fallopian tube.

DISCUSSION

Heterotopic pregnancy refers to the presence of embryo outside the uterus, combined with the presence of intrauterine embryos. Extra-uterine pregnancies occur mostly in the fallopian tubes, but can also take place in the ovary, uterine horn, cervical and uterine scars. About 95% of extra-uterine pregnancies occur at the fallopian tubes, and 75% - at the fallopian tube ampulla (9). The risk of HP following the IVF-ET is influenced by several factors,

including pelvic inflammatory disease and the history of pelvic surgery and ectopic pregnancy. It has also been reported that after IVF-ET, the HP may also be induced by other factors, such as large number of embryos (2-3 implants), graft volume, fast embryo implantation, high embryo placement. The migration of the embryo itself and the uterus contractions during implantation may move the embryo into other parts (10, 11). If two or more embryos are transplanted at a lower position and all embryos survive at the area of placenta, the risks of placenta previa and cesarean scar pregnancy (CSP) increase. But if two or more embryos are transplanted at higher levels, the risk of extra-uterine pregnancy may become elevated. In our case, the pregnancy was induced by frozen embryo transplantation, and two embryos were implanted into the uterine cavity. The embryos moved and settled at the right salpingo-tubal stump, resulting in the development of HP. When the patient has a scarred uterus and a history of tubal surgery, it might be important to allow only one embryo to be transplanted into the uterine cavity during the embryo transfer. The position of the embryo may need to be well grasped, as this can reduce the risk of HP and placenta previa.

When a health care practitioner transplants two or more embryos into the uterine cavity in order to increase the success chances of the embryo transfer procedure, color Doppler ultrasound should be used, not only to verify the intrauterine single-child survival, but also to closely monitor for the existence of other embryos and thus prevent the occurrence of HP.

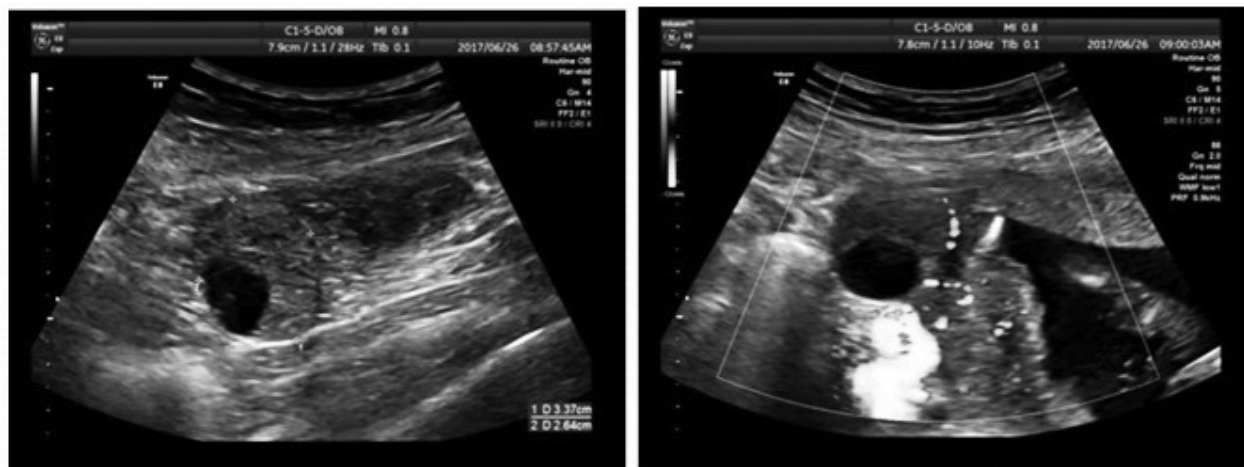


Fig. 1. Ultrasonography of embryos 98 days after implantation. A cyst measuring 3.3×2.9 cm in the uterus can be seen on the right.

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LETTER TO THE EDITOR

DIAGNOSTIC VALUE OF SERUM C-REACTIVE PROTEIN FOR SEPTIC ACUTE KIDNEY INJURY: A RETROSPECTIVE STUDY

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Acute kidney injury (AKI) is often associated with adverse clinical outcomes and is difficult to diagnose in the early stages in septic patients. This study aims to investigate the correlation between serum C-reactive protein (CRP) levels and AKI severity diagnosis in the patients with sepsis. This retrospective study included 60 patients, who were divided into an AKI group and a non-AKI group. CRP levels were compared between the two groups. CRP was evaluated by analysis of the area under the receiver operating characteristics curve (ROC, AUC=0.648, Z=1.965, P=0.0494, 95%CI 0.514-0.767). CRP levels were significantly different between the AKI group, 99.79(49.26-172.59) mg/L, and non-AKI group, 23.13(5.80-65.49), p=0.001, Z=-3.0409. CRP had a sensitivity of 55.26% and a specificity of 81.82% at the optimal cut-off value. Subsequent multivariate logistic regression analysis indicated that CRP could predict AKI severity in septic patients (OR=1.01; 95%CI=1.00-1.02; P=0.001). High level of CRP correlates with more severe AKI in patients with sepsis. In conclusion, CRP could be an attractive predictor in early diagnosis of AKI in septic patients.

To the Editor,

Acute kidney injury (AKI) is a very common complication in critically ill patients with sepsis, especially in severe sepsis and sepsis shock patients (1). AKI often shows poor outcome, high mortality and high medical costs. The mortality rate of septic AKI reaches up to 40%. Early diagnosis of septic AKI has become an unsolved problem in critically ill patients (2). Although Neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), cystatin-C (Cys-C) and other serum markers have been proved to be potential biomarkers in septic AKI (3), there is often limited or no access to them. Recent evidence has confirmed that inflammatory response plays an important role

in the development of septic AKI, in which kidney dysfunction might be caused by cytokines and other inflammatory mediators released by immune cells (4). The severity degree of inflammatory response was associated with the development of kidney injury. Some studies focusing on inflammatory biomarkers have reported that C-reactive protein (CRP), an acute phase reactant in response to inflammation (5, 6), is frequently measured to indicate severity of septic patients. Because of its wide availability and low cost, CRP has been investigated as an attractive biomarker to diagnose sepsis (7).

However, restricted studies on the relationship between CRP and septic AKI have been reported. Therefore, we proposed the hypothesis that serum

Key words: C-reactive protein; acute kidney injury; sepsis; biomarker

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CRP levels are associated with AKI severity in septic patients and CRP could be an attractive biomarker in the diagnostic process.

MATERIALS AND METHODS

Patients and methods

This retrospective study was conducted from June 2016 to April 2017 on septic patients admitted to the intensive care unit of Suzhou Integrated Chinese and Western Medicine Hospital. All subjects gave informed, signed consent to participate in the study, or informed written consent was given by the family. Demographic data were obtained from the electronic medical records including ages, genders, anamneses, Acute Physiology and Chronic

Health Evaluation (APACHE) II scores, Sequential Organ Failure Assessment (SOFA) Scores. Blood tests were assayed by routine laboratory methods including complete blood count, CRP, and serum creatinine. Diagnosis criteria of sepsis was based on the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). CRP value measurement while sepsis diagnosis based on sepsis 3.0. Because we were unable to collect information of patients' urinary output (UO), diagnosis and stages of AKI according to the KDIGO Clinical Practice Guidelines were based on serum creatinine levels. Exclusion criteria included chronic kidney disease, tumor, <48 hours in the intensive care unit, age <18 years. eGFR based on serum creatinine were estimated by Chronic Kidney Disease-Epidemiology Collaboration (CKD-EPI). According to

Table I. Baseline clinical and laboratory characteristics of the study subjects.

	AKI (n=29)	Non-AKI (n=31)	P value
Age (years)	69.55±16.21	70.48±16.49	0.851
Male gender (%)	23(79.3)	15(48.4)	0.001
Hypertension (%)	9(30.0)	13(41.9)	0.381
COPD (%)	15(51.7)	14(45.2)	0.611
MAP (mm Hg)	86.03±21.71	92.01±18.81	0.698
Heart rate	107.72±23.21	104.81±26.52	0.072
Temperature °C	37.18±1.31	36.60±0.69	0.001
Respiration rate	21.07±4.73	21.00±5.61	0.703
CRP (mg/L)	99.79(49.26-172.59)	23.13(5.80-65.49)	0.001
WBC	9.65(7.47-16.08)	11.31(8.53-14.26)	0.594
Platelet	141(95.5-194.5)	203 (125-267)	0.058
Hemoglobin (g/L)	114(94.5-140.5)	119 (102-140)	0.853
Albumin(g/L)	32.43±6.31	31.75±5.88	0.678
Bilirubin direct (μmol/L)	5.20(3.25-8.90)	4.10(2.40-8.60)	0.304
Total bilirubin (μmol/L)	13.70(10.55-24.50)	12.10(8.50-24.60)	0.473
Glucose (mmol/L)	7.63(6.04-8.94)	7.02(5.84-9.28)	0.923
Creatinine (μmol/L)	141.1(108.40-211.60)	63.80(50.40-77.90)	0.001
Urea nitrogen (mmol/L)	10.35(7.44-14.75)	6.25(4.10-11.19)	0.002
K ⁺ (mmol/L)	4.04±0.80	3.55±0.62	0.300
Na ⁺ (mmol/L)	138.93±26.03	137.23±5.85	0.023
Cl ⁻ (mmol/L)	104.59±10.99	97.90±6.19	0.045
APACHE II score	18(14-21.5)	14 (11-16)	0.004
SOFA score	6(4-8.5)	4(3-5)	0.001

COPD: chronic obstructive pulmonary disease; MAP: mean arterial pressure; CRP: C-reactive protein; WBC: white blood cell; APACHE II: Acute Physiology and Chronic Health Evaluation II; SOFA: Sequential Organ Failure Assessment.

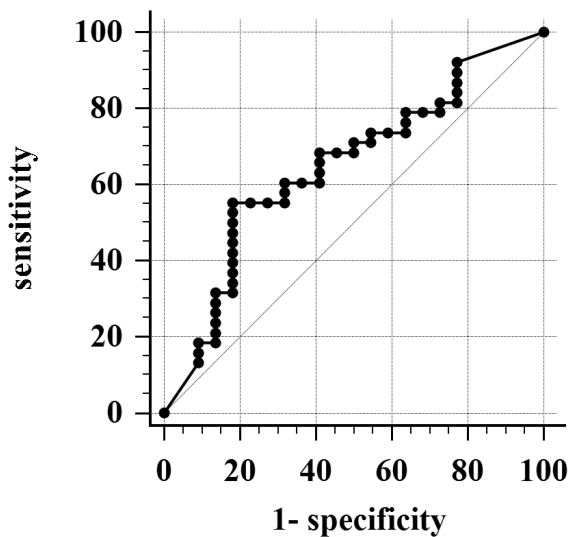


Fig. 1. Receiver operating characteristic (ROC) curves of CRP for predicting AKI in septic patients.

the KDIGO Clinical Practice Guidelines, septic patients were divided into an AKI group and a non-AKI group. Serum CRP levels were recorded on the day of AKI occurrence in the AKI group and on the day of diagnosis of sepsis in the non-AKI group.

Statistical analysis

The data were analyzed by SPSS, version 19.0 for Windows (SPSS Inc., Chicago, IL, USA). Continuous variables with normal distribution were presented as mean $\pm$ standard deviation (SD); non-normal variables were reported as median [interquartile range (IQR)]. Mean of two continuous normally distributed variables were compared by independent samples *t*-test. Mann-Whitney U test was used to compare means of non-normally distributed variables. Kruskal-Wallis test was used to compare CRP levels stratified by KDIGO classes in septic AKI patients. The frequencies of categorical variables were compared using χ^2 exact test. CRP, considered as an AKI predictor, was evaluated by analysis of the area under ROC. ROC was performed using MedCalc software (MedCalc Software, Ostend, Belgium). Collinearity between CRP and eGFR/serum creatinine was evaluated using the Spearman correlation coefficient. Risk factors with $P < 0.1$ from univariate analysis entered into

multivariate logistic regression. A value of $P < 0.05$ was considered to indicate significant differences.

RESULTS

Sixty patients (mean age: 70.03 ± 16.23 years, 38 males 63.33%) with sepsis were enrolled in the final analysis. Based on the KDIGO Clinical Practice Guidelines, twenty-nine patients (48.33%) were grouped into the AKI group and thirty-one patients (51.67%) into a non-AKI group. Among all subjects in the AKI group, KDIGO I, II, III occurred in 20 (68.97%), 7 (24.14%), 2 (6.90%) patients, respectively, in which most were classified as KDIGO I – II. The AKI group showed higher male rate, temperature, serum creatinine, urea nitrogen, K^+ , Na^+ , Cl^- , APACHE-II score and SOFA score than the non-AKI group. CRP levels were statistically significantly different between the AKI and non-AKI groups, $99.79 (49.26-172.59)$ mg/L vs $23.13 (5.80-65.49)$ mg/L, $p=0.001$, $Z= -3.409$. The baseline and clinical laboratory characteristics of the two groups are shown in Table I.

Receiver Operator Characteristic (ROC) Curve analysis demonstrates that CRP could be a predictor for septic AKI. The area under the curve was AUC 0.648, $Z=1.965$, $P=0.0494$, 95%CI (0.514-0.767) (Fig. 1). At the optimal cut-off value of 65.49 mg/L, CRP in the diagnosis of AKI has a sensitivity

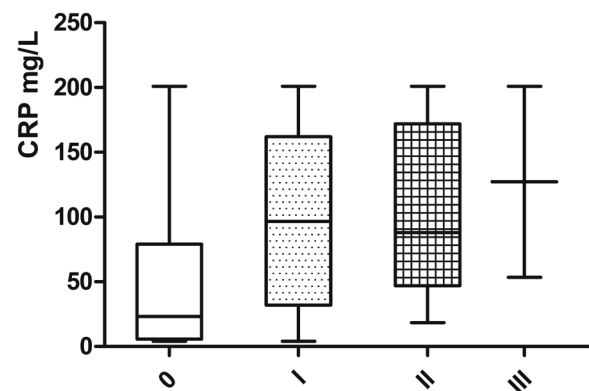


Fig. 2. CRP levels stratified by KDIGO classes in septic AKI patients.

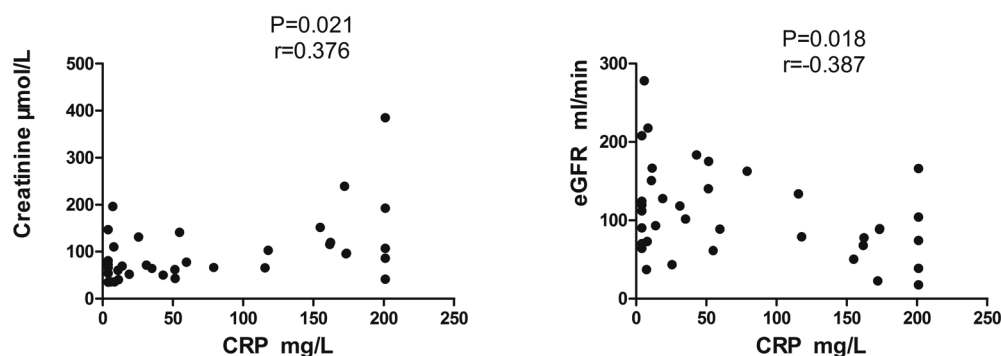


Fig. 3. Relationship between CRP and creatinine, eGFR.

of 55.26% and a specificity of 81.82%, positive likelihood ratio 3.04, negative likelihood ratio 0.55. A boxplot presenting CRP measurements among KDIGO classes shows a significant difference in different classes, $p=0.017$ (Fig. 2), which indicates CRP is able to discriminate AKI severity in sepsis patients. Univariate logistic analysis shows that gender, CRP, CI-, APACHE-II score, and SOFA score could enter into multivariate logistic regression analysis, which shows that CRP could predict AKI in sepsis patients ($OR=1.01$; $95\%CI=1.00-1.02$; $P=0.001$). The relationship between CRP and eGFR/serum creatinine is shown in Fig. 3, ($r= -0.387$, $P=0.018$; $r= 0.376$, $P=0.021$)

DISCUSSION

AKI is a common complication during occurrence and development of sepsis. A multicenter study indicated that more than 47% septic patients have AKI. Sepsis-related AKI was previously thought to be caused by changes in renal blood flow (8). It was found that inflammatory mediators can directly induce renal epithelial cell injury, which can cause renal vascular dysfunction and immune function injury (9). A number of studies proved that the increase of inflammatory mediators (IL-6, IL-10, IL-1 and TNF- α) are positively correlated with septic AKI events (10). We found that CRP levels were significantly higher in the AKI group than in the non-AKI group [99.79(49.26-172.59) mg/L vs

23.13(5.80-65.49) mg/L, $p=0.001$]. At the cut-off value of 65.49 mg/L, CRP was able to predict AKI with an AUC of 0.648. CRP levels were negatively related to eGFR. Multivariate logistic regression showed that CRP levels correlated with renal injury. In our study, CRP was not only an active indicator in response to sepsis, but also a beneficial biomarker for predicting AKI in septic patients. There were some constraints in our study. Firstly, as it was an observational study, we did not collect information of patients' OU, diagnosis and stages of AKI according to the KDIGO Clinical Practice Guidelines. Secondly, this was a single center retrospective study, there were a few cases that were missed, and we could not test biomarkers at multiple time points, so we did not explore the dynamic changes in CRP concentrations. Lastly, the relationship between CRP and creatinine, eGFR were weak. In conclusion, our results indicate that CRP could be an attractive biomarker for septic AKI diagnosis and severity assessment based on serum creatinine. CRP is also associated with eGFR in septic patients with AKI. Nevertheless, the CRP levels could not predict the mortality in septic patients with AKI. Larger multicenter prospective studies should be included in further work.

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LETTER TO THE EDITOR

EFFECTS OF OXYCODONE HYDROCHLORIDE ON IMMUNE FUNCTION AND BIOCHEMICAL INDEXES IN PUERPERAS UNDERGOING CESAREAN SECTION

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The aim of this study was to investigate the effect of oxycodone on the immune function and biochemical parameters in puerpera undergoing cesarean section. The puerperas undergoing cesarean section were randomly divided into two groups, namely, the oxycodone hydrochloride group (group A) and the morphine group (group B). After the operation, patient controlled intravenous analgesia (PCIA) was used for medication. The analgesic effects at different time points, after 1 h, 2 h, 6 h, 12h, 24 h and 48 h, were recorded. The score of the numerical rating scale (NRS) of uterine contraction pain was evaluated. At the same time, peripheral blood was taken at different time points after analgesia (immediately after surgery, then 24 h, 48 h and 72 h after surgery). Th1 and Th2 cell levels and biochemical parameters were determined. Compared with the morphine group, the Th1 cell level in the oxycodone hydrochloride group was significantly increased at 24 h and 48 h after surgery ($P<0.05$). Th2 level was significantly increased compared to that of morphine at 24 h, 48 h and 72 h after surgery ($P<0.05$). The alanine aminotransferase (ALT) level and aspartate aminotransferase (AST) level in the oxycodone hydrochloride group were significantly decreased compared to that of the morphine group at 24 h after surgery. AST level in the oxycodone hydrochloride group was significantly increased compared to that of the morphine group at 72 h after surgery ($P<0.05$). The total bilirubin (TB) level was significantly increased in the oxycodone hydrochloride group compared to the morphine group at 24 h, 48 h and 72 h post-surgery ($P<0.05$). Compared with morphine, oxycodone can increase the level of Th1 and Th2 immune cells in peripheral blood in puerperas undergoing cesarean section, and has an increased effect on liver function, without causing abnormal liver function.

To the Editor,

Delivery by cesarean section has substantially increased in recent years, even in women with low-risk pregnancies. Cesarean section is not without risk for the mother compared to normal birth, as the puerpera is more likely to suffer serious complications, some related to decreased immunity (1). This may be due to surgical stimulation and anesthesia that can affect the immune function of the patients.

Oxycodone hydrochloride is mainly used for the treatment of moderate to severe cancer pain. Some studies have demonstrated that apart from this main utilization, oxycodone can be successfully used also for postoperative analgesia (2). The mechanism by which oxycodone determines its analgesic effect is related to μ -opioid receptor activation. Study has demonstrated that the use of morphine after cesarean section may cause moderate to severe itching

*Key words: oxycodone hydrochloride; cesarean section; immune cells; biochemical indicators**Corresponding Author:*

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and pain. Compared with morphine, oxycodone hydrochloride can significantly reduce the degree of itching and pain in pregnant women (3). Zare et al. found that oxycodone and morphine were effective in reducing pain in patients undergoing laparoscopic hysterectomy, however, to achieve the same analgesic effect, the amount of oxycodone was significantly lower than that of morphine. The analgesic effect of oxycodone was higher than that of morphine within 1 hour after analgesia (4). In a study of postoperative analgesia after hysterectomy, Kim et al. indicated that intravenous oxycodone had a shorter duration of action, only 2 h after surgery, and had a lower rate of analgesia (5). Therefore, the dose and time of oxycodone hydrochloride are very important for analgesic effects (6).

Stress reactions, such as surgical trauma, not only change the patient's biochemical indicators, but also have a certain impact on immune function. Study has shown that surgical analgesia using strong opioid drugs such as morphine can activate platelets, inhibit the level of T cells to some degree, and thus inhibit cellular immune function (7).

This study aimed to investigate the effect of oxycodone on the immune function and biochemical parameters in puerpera undergoing cesarean section.

MATERIALS AND METHODS

Experimental materials

Cesarean section was performed on 60 pregnant women with normal liver function in Taizhou Hospital of Zhejiang Province from April 2016 to March 2018. The puerperas were randomly divided into two groups, with 30 cases in each group: Group A - oxycodone hydrochloride group (Onomami Co., UK) and group B - morphine group (Shenyang First Pharmaceutical Co., Ltd., Shenyang, China). The venous blood of the two groups was extracted immediately after undergoing operation and 24, 48 and 72 hours after the operation. Two mL of venous blood was collected using a heparin sodium anticoagulant tube; 1 mL of venous blood was taken immediately for detection of Th1 and Th2 cell levels; 1 mL of venous blood was centrifuged at 3,500 g for 10 min in a JZ1612-1 high speed benchtop centrifuge (Shanghai Jinzhong Scientific Instrument Factory, Shanghai, China). The separated

serum was frozen at -20 refrigerator, and the biochemical indexes were measured at the same time. All subjects in the study had signed informed consent and the study was approved by the Ethics Committee of Taizhou Hospital of Zhejiang Province, China.

Numeric rating scale (NRS)

The NRS scoring method describes the pain using a number between 0 and 10: 0 signifies no pain and 10 means the most excruciating pain; 1-4 is mild pain that does not affect sleep. 5-6 is moderate pain, which affects sleep, but still being able to fall asleep; 7-9 means severe pain, which leads to waking up or being unable to sleep because of pain.

Postoperative analgesia

DDB-I-A injection pump (Aipu Scientific Instrument Co., Ltd., Jiangsu, China) was used for administration of postoperative analgesia to patients included in the study. Group A received 0.8 mg/kg oxycodone diluted with saline solution to a total amount of 100 ml in doses of 2 mL/h, and a booster dose of 2 mL/15 min. Group B received 0.8 mg/kg morphine diluted with saline solution to a total amount of 100 mL in doses of 2 mL/h, and a booster dose of 2 mL/15 min. In both groups, after surgery all the patients received analgesia. The uterine contraction pain numeric rating scale (NRS) scores at 1 h (T1), 2 h (T2), 6 h (T3), 12 h (T4), 24 h (T5), and 48 h (T6) after analgesia were recorded.

Th1 and Th2 cells detection

100 μ L of venous blood was added with an equal volume of serum-free FBS (Fetal bovine serum)-containing RPMI 1640 (Multisciences Hangzhou Lianke Bio, Zhejiang, China). 0.8 μ L of 250 $\times$ PMA (phorbol myristate acetate)/Ionomycin (Multisciences Hangzhou Lianke Bio, Zhejiang, China) and 0.8 μ L of 250 $\times$ Monensin/BFA (Brefeldin A) (Multisciences Hangzhou Lianke Bio, Zhejiang, China) were mixed and cultured in a 37°C incubator (SIM, USA) for 4-6 h in a chamber containing 5% CO₂ and shaken once every hour. In the test sample tube, 100 μ L of the cell mixture and 10 μ L of the cell surface marker antibody [human CD3, CD8 antibody (BD, USA)] were separately added and incubated at 4°C for 20 min in the dark. 100 μ L of FIX & PERM Reagent A (Multisciences Hangzhou Lianke Bio, Zhejiang, China)

was added and incubation was continued for 15 min at room temperature; 3 ml of staining buffer was added to each tube and centrifuged at 300 g for 5 min. After collecting all the cells, 100 μ L of Fix & Perm Reagent B (Multisciences Hangzhou Lianke Bio, Zhejiang, China) was added. At the same time, IFN- γ /IL-4 (BD, USA) fluorescent antibody and 3 ml of staining buffer were added to each tube. After centrifugation at 300 g for 5 min, it was resuspended in 0.5mL of PBS (Phosphate buffer saline) (Multisciences Hangzhou Lianke Bio, Zhejiang, China), and immediately analyzed by FACS Calibur Flow Cytometry (BD, USA).

Biochemical parameter detection

The determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TB) and lactate dehydrogenase (LDH) was performed by ES-200 automatic biochemical analyzer (Nanjing Haolanbei Biotechnology Co., Ltd., Jiangsu, China).

Statistical analysis

The data was statistically processed using SPSS 19.0 statistical software. Data is expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$). The comparison between the groups

was made by Student's *t*-test. A probability of $P < 0.05$ indicated that the data difference analysis between groups was statistically significant.

RESULTS

NRS score of uterine contraction at different time points after surgery

As shown in Fig.1, the NRS score of uterine contraction pain of puerperas after cesarean section was first decreased and then increased in both groups. There was no significant statistical difference in NRS scores at each time between the two groups ($P > 0.05$).

Changes in Th1 and Th2 cell percentage after analgesia in two groups

As can be seen from Fig. 2, the Th1 cell levels in THE oxycodone group increased at 24 h post-surgery. After 48 hours, it resumed the immediate level of surgery. The levels of Th1 cells in the morphine group decreased significantly at 24 h and 48 h after surgery, and remained at the lowest level at 48 h, and returned to the immediate level post-surgery at 72

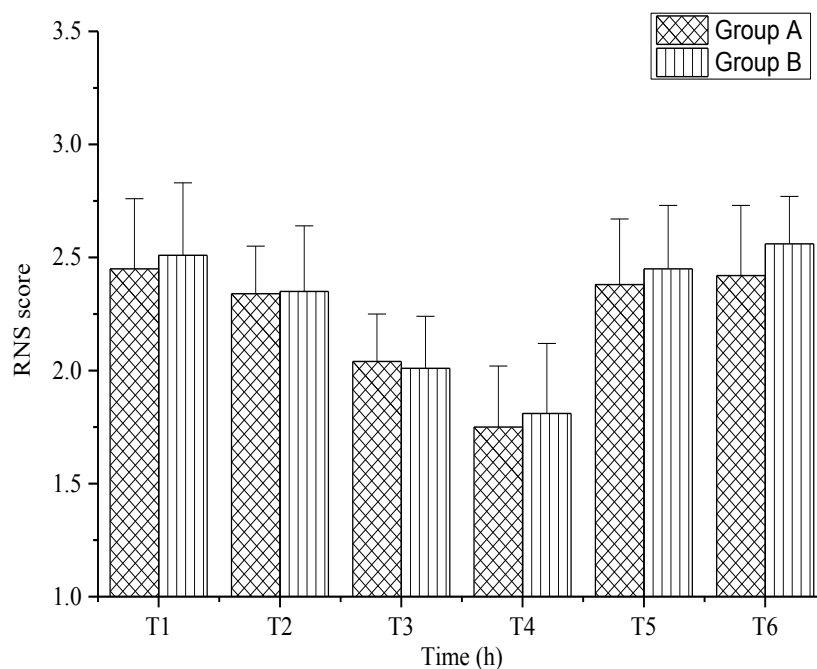


Fig. 1. NRS scores of uterine contraction pain in the two groups at different time (T1, T2, T3, T4, T5 and T6 are different time points after analgesia, which are 1 h, 2 h, 6 h, 12 h, 24 h and 48 h).

h. The Th1 cell levels in the oxycodone group was significantly higher than that in the morphine group at 24 h and 48 h after operation, and the two groups reached significant difference ($P<0.05$). At 72 h post-surgery, there was no difference of Th1 cells in the two groups. The level of Th2 cells in the oxycodone group was significantly higher than that in morphine group at 24 h, 48 h and 72 h post-surgery ($P<0.05$). The level of Th2 cells was the highest at 48 h post-surgery, and then decreased in each group. The level of Th2 cells in the morphine group increased at 24 h post-surgery, but then decreased at 48 h, and at 72 h returned to the level just after the surgery. The level of Th2 cells in the oxycodone group was higher than that in morphine group at 24 h, 48 h and 72 h post-surgery ($P<0.05$).

Changes in blood biochemical parameters at different time points after analgesia in two groups

As shown in Fig. 3A and B, the ALT levels in the oxycodone group continued to increase at different time points after surgery, while the morphine group increased first and then decreased. At 24 h, the ALT level of the oxycodone hydrochloride group was significantly lower than that of morphine group ($P<0.05$), but it was significantly higher than that of morphine group at 72 h ($P<0.05$). The ALT levels in

both groups increased. The changes in AST levels were similar to the changes in ALT levels. In the morphine group, the AST level increased and reached the highest value at 24 h after surgery, which was significantly different from the oxycodone group. At 48 h, the ALT levels were similar in both groups. At 72 h, the AST level in the oxycodone group continued to increase, while in the morphine group began to decrease ($P<0.05$).

As shown in Fig. 3C and D, TB level was highest at 48 h after surgery in the oxycodone group, and then began to decrease. In the morphine group, the TB content changed more slowly at various time after operation. At 48 hours, although there was a slight increasing trend, the increase was not obvious. The TB in the oxycodone group was higher than that in the morphine group at 24 h, 48 h and 72 h, and the difference was statistically significant ($P<0.05$). The LDH in both groups increased first and then decreased, but there was no significant difference in LDH levels between the two groups at each time point.

DISCUSSION

The NRS sedation scoring system is widely used in anesthesia, critical medicine and other

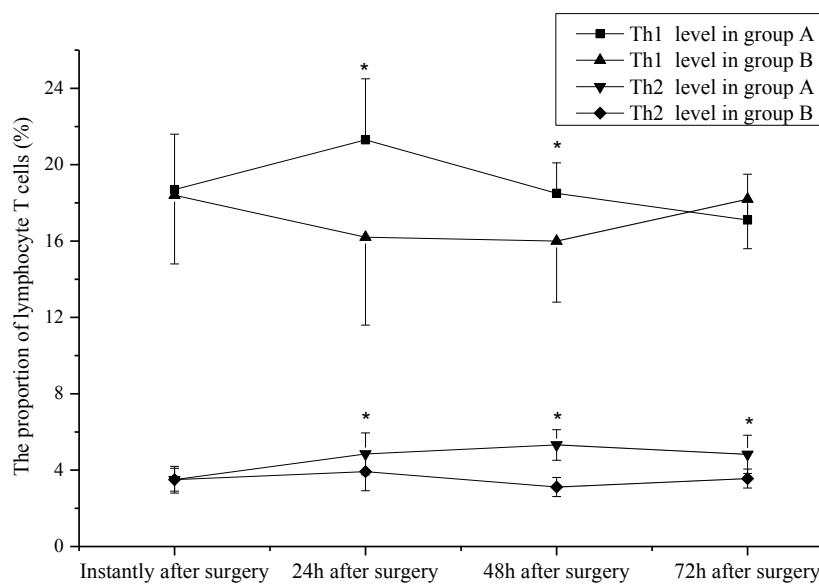


Fig. 2. Dynamic changes of Th1 and Th2 immune cells at different time points after analgesia. * $P<0.05$, group A compared with group B [Oxycodone hydrochloride group (group A) and morphine group (group B)].

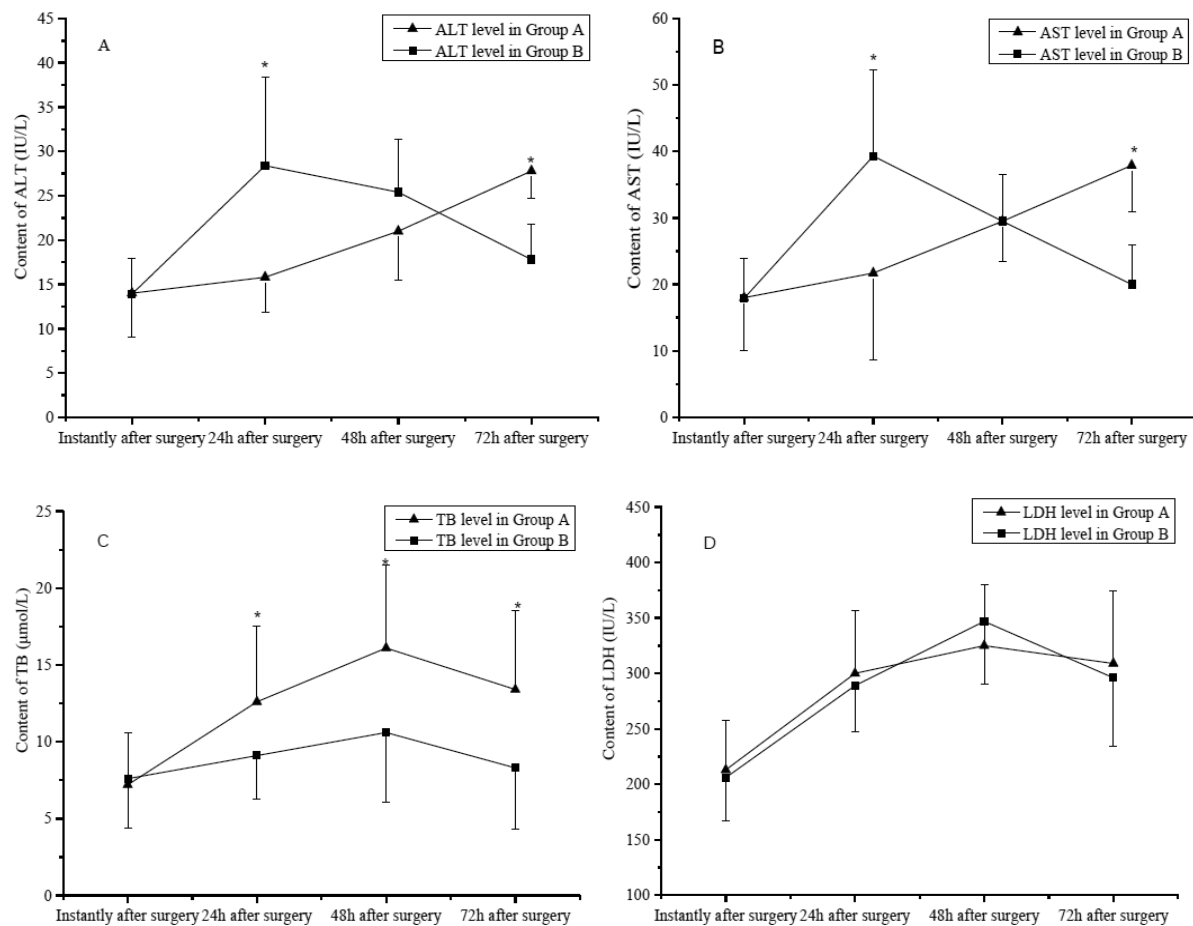


Fig. 3. Changes in ALT (A), AST (B), TB (C) and LDH (D) levels at different time points after analgesia [Oxycodone hydrochloride group (group A) and morphine group (group B)].

professional fields, as it is a common tool for sedation assessment. Gach et al. (8) found that morphine can inhibit the expression of pro-inflammatory cytokines by monocytes during T lymphocyte activation. In addition, studies found that [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-Enkephalin (DAMGO) (a selective agonist of μ -opioid receptor) can inhibit the proliferation of T lymphocytes (9, 10). In this experiment, it was also found that the agonist of κ -opioid receptor did not have this effect, suggesting that kappa opioid receptor (KOR) may not produce significant immunosuppressive effects.

ALT in the oxycodone group continued to increase at different times after surgery, while in the morphine group was first increased and then decreased. Both groups had increased ALT levels. The change in AST levels was consistent with the

trend of ALT. In the oxycodone group, total TB level was highest at 48 h after surgery and then began to decline. In the morphine group, the TB level changed gently at various time points after surgery. The LDH level in both groups increased first and then decreased after surgery, but without significant difference between the two groups at each time point. This indicates that the activation of the immune function may cause damage to the liver cells by the immune system, and is reflected in the increase of some indicators of liver function.

In summary, oxycodone can increase the percentage of Th1 and Th2 immune cells in peripheral blood of puerperas after cesarean section. In the postoperative analgesia of puerperas, liver function was greatly affected, but it did not cause abnormal liver function. This study was limited

to the effects of oxycodone hydrochloride on Th1 and Th2 in immune cells, but the situation of Treg cells and other cells such as Th17 is still unclear. Therefore, whether oxycodone hydrochloride promotes the secretion of TGF- β by Treg cells and plays a negative immunoregulatory function deserves further study.

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LETTER TO THE EDITOR

MECHANISM OF PLATELET-RICH PLASMA IN PROMOTING BONE DEFECT REPAIR

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The aim of work was to study the mechanism of platelet-rich plasma (PRP) in promoting the repair of lacunar bone defects in the lower extremities of rabbits. PRP was first prepared, and PRP and artificial bone were mixed into PRP gel at a ratio of 2:1. Forty-nine healthy New Zealand white rabbits were selected, and unified standard PRP was prepared using the blood collected from one of these rabbits. The other rabbits were randomly divided into two groups, a PRP group (the bone defect area was filled with PRP gel) and an artificial group (the bone defect area was filled with only artificial bone). At weeks 3, 5, 7 and 9 post-operation, the animals were sacrificed, and the bone defect repair was observed by gross observation, X-ray, pathological and immunohistochemical methods. The bone repair of each group was scored, and the barrier density value in the bone defect area and the proportion of positive staining of the immunohistochemical sections were recorded. The results showed that the wound healing of the two groups was good, and the radiological score and the value of refractive density were significantly different between the two groups at weeks 3, 5 and 7 post-surgery ($P < 0.05$). Both gross observation and HE staining showed that healing of the bone defect in the PRP group was better than that in the artificial group, and the proportion of positive staining of the immunohistochemical sections in the PRP group was higher than that in the artificial group at weeks 3 and 5 post-operation. In conclusion, PRP can promote repair and healing of bone defects. Vascular endothelial growth factor (VEGF) is an important stimulating factor for early bone regeneration.

To the Editor,

Repair and regeneration of bone defects are difficult problems in the field of orthopedics. Although autologous bone transplantation can achieve good clinical outcomes, the source of bone is limited and can barely meet the clinical needs (1). The traditional study of bone repair-related compound growth factor was mainly based on only a single growth factor. However, the repair and regeneration of bone defects

are complex physiological processes which often require a variety of growth factors to participate in the regulation to promote growth of bone tissues (2). Platelet-rich plasma (PRP) (8) is a platelet concentrate obtained from whole blood by centrifugation. When it is mixed with activators, such as thrombin or calcium chloride, the platelet is activated and its alpha particles will release many growth factors, which can promote cell proliferation and tissue regeneration (3). It has

Key words: PRP; bone defect; bone repair; VEGF

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been shown that PRP has a clear bone induction ability and can promote bone tissue repair (4). Balbo et al. (5) and Bernuzzi et al. (6) applied allogeneic platelet plasma to 132 cases of skin defect wounds, and Chen et al. (7) combined it with skin grafting in 17 cases of refractory lower limb ulcer. All of them had good clinical outcomes without obvious adverse reactions. In this study, a new type of bioactive bone graft material composed of RP composite artificial bone was used to repair lower limb lacunar bone defects of rabbits, and its ability was evaluated by comparing with the artificial group in different indexes.

MATERIALS AND METHODS

Animals

Forty-nine healthy New Zealand white rabbits (purchased from Jining Fuyida Animal Husbandry Co., Ltd.) aged 6 months (weight, 2.0-2.5 kg) were raised in separate cages with no limitation of gender. All animals were fed pellet feed, had free access to drinking water, temperature was maintained at approximately 20°C, relative humidity was about 50% and the experiment was carried out after adapting for one week. One of the rabbits was used to prepare a unified standard PRP. The remaining 48 rabbits were randomly divided into two groups, a PRP group (24 rabbits were implanted with PRP gel in the bone defect area) and an artificial group (24 rabbits were implanted with artificial bone in the bone defect area). This study was approved by the ethics committee of Hangzhou Normal University affiliated Hospital and all the animal experiments were in accordance with International law regarding animal studies. All the experimental operations and anesthetic use in the study are in line with the world regulations on animal protection and management.

Main materials and instruments

Main materials: anti-VEGF antibody (Hangzhou Jingjie Biotechnology Co., Ltd., China); injectable surgical grade calcium sulfate artificial bone (Baisai International Trade (Shanghai) Co., Ltd., China); 3.8% sodium citrate anticoagulant (Sichuan Mianzhu Hongji Pharmaceutical Co., Ltd., China), anesthetics (2% pentobarbital [Shandong Xinhua Pharmaceutical Co., Ltd., China] and lidocaine [Northeast Pharmaceutical Group Shenyang First Pharmaceutical Co., Ltd., China]);

penicillin (Tianjin Pharmaceutical Jiaozuo Co., Ltd., China); 0.1M phosphate buffer (PH = 7.0) (Shanghai Puzhen Biotechnology Co., Ltd., China), etc.

Main instruments: optical microscope; microtome; X-ray machine; centrifuge (Beijing Precise Instrument Co., Ltd., China); bone electric drill (bit d = 4mm) (Shanghai Zi'ai Medical Equipment Co., Ltd., China); centrifuge tube (15 mL and 50 mL), pipette, 96-hole plate (Nantong Lineng Experimental Equipment Co., Ltd., China); conventional surgical instruments: conventional surgical instruments package (scalpel, suture line, stitch needle, needle holder, tissue scissors, bone holder, wire saw, hemostatic forceps, periosteal dissector, hole towel, gauze, disposable medical syringe 5 mL, 10 mL and 20 mL, skin preparation knife, forceps, bone graft rod, iodophor disinfectant, and rubberized fabric).

PRP preparation

One rabbit was intravenously anaesthetized with sodium pentobarbital (30mg/kg), followed by blood extraction from its ventricle using a 20 mL syringe containing 3.8% sodium citrate anticoagulant. The blood sample was placed in a 50 mL sterile test tube and shaken evenly, then was sub-divided into four 15 mL centrifuge tubes. The secondary centrifugation method was adopted, where the constant temperature centrifuge was adjusted at 22°C with a centrifugal force of 400g and a centrifuging duration of 30 min. All supernatant, intermediate layer and ca. 0.2 mL erythrocyte layer were first extracted and transferred into another batch of 15 mL centrifuge tubes. After another centrifugation, the supernatant and intermediate layer were taken as PRP. PRP was separately loaded into sterile test tubes and stored at 4°C. In order to obtain the PRP prepared by a unified standard, allogeneic blood was used, and no animal blood matching was carried out limited by experimental conditions. In order to avoid the platelet immune reaction, HLA-I and HPA matching would make the application of allogeneic PRP safer.

Preoperative preparation and anesthesia

Before surgery, PRP and artificial bone were mixed into gel at a ratio of 2:1. The rabbits were anesthetized with 0.03 g/L pentobarbital sodium (30 mg/kg) at the venous site of the ear margin. 300,000U penicillin was injected intramuscularly before surgery to prevent infection and finally skin preparation was performed.

Animal models

A 2-cm straight incision was made along the long axis of the medial tibia on one side of the animal. The subcutaneous tissue and periosteum were separated layer by layer until the bone wall. A cavity-like bone defect with a diameter of 4 mm and a depth of 5 mm was made by drilling the tibia 1/4 cm above the knee joint. In the definition of bone defect, a smaller bone defect (usually less than 8 mm) may heal on its own for a healthy person; but if the bone defect is too large, or the defect is on a smaller bone, it is difficult to heal completely, therefore requires surgical intervention. The bone defect was made in the tibia the rabbits, and when it was converted into the same ratio as with the human body, it completely met the mode need of the bone defect which could not heal by itself. In the PRP group, the bone cavity was filled with the PRP-artificial bone gel, while in the artificial group, the bone cavity was filled with the artificial bone only, and the filling material reached the bone margin. After completion of the filling, the incision was sutured layer by layer. Penicillin (300 thousand U/d) was injected intramuscularly for 3 consecutive days, and the rabbits were maintained in single cages with regular diet, without external fixation.

Imaging examination

At weeks 3, 5, 7, and 9 post-operation, 6 animals whose anteroposterior and lateral plain films were obtained on both lower limbs after X-ray examination were randomly selected from each group. The projection conditions were as follows: current 30mA, voltage 220kV, duration 0.1s, and projection distance 1m. According to Lane's radiological scoring criteria, the bone repair of each group was scored, and the values of the radiographic density of the bone defect area were recorded. The density was set of the cortex of bone on the same plain film to 100, and the color of the plain film to 0 to measure the relative density of the bone defect area. In order to minimize the error, the average value was calculated at the upper, middle and lower points of the bone defect area.

Gross morphology observation

At weeks 3, 5, 7, and 9 post-operation, 6 animals were randomly selected from each group. In the ear vein, 0.03g/L pentobarbital sodium was used to anesthetize by 30mg/kg. After the animals were sacrificed by air embolization, the tissues were separated along the original incision to

the bone defect. The changes of implant surface, callus growth and surrounding tissue reaction were observed. The implant, together with autogenous bone (0.5 cm for each) at both ends of the bone defect, was removed and fixed in neutral formaldehyde. After decalcification, these specimens were embedded in paraffin.

Histological examination

After decalcification with 80% pure water and 20% nitric acid, the specimens of each group were embedded in paraffin, sectioned longitudinally with the defect as the center, and stained with HE. The formation of new bone was observed under a light microscope with three non-overlapping visual fields.

Immunohistochemical examination

After decalcification, the specimens of the two groups were embedded in paraffin. The defect area was taken as the center for longitudinal section, and stained with anti-vascular endothelial growth factor (VEGF) antibody. Three non-overlapping visual fields were randomly collected under a microscope. The proportion of positive staining was calculated by image analysis software: image-Pro Plus.

Statistical analysis

SPSS 22.0 software was used for statistical analysis. The results were expressed by $\bar{x} \pm s$ and measurement data were compared by *t*-test. $P < 0.05$ indicated significant difference.

RESULTS

General condition

During the first three days post-operation, the mental and dietary conditions of the rabbits in PRP group were worse, and there was less activity, but no signs of chills or fever were observed. On the fifth day post-operation, the mental state was improved, the diet returned to normal and the activity increased. During the observation period, there was no obvious redness, swelling, pyorrhea or fluctuation in the operated area of the PRP rabbits. The incisions were healed and the sutures fell off naturally, and no death was reported.

X-ray findings

At 3 weeks post-operation, the PRP group rabbits were filled with plenty of pale red soft fiber tissues in the bone defect, and the defect boundary was clear, with a small amount of fibrous callus. X-ray showed that the edge of the bone defect in each sample was blurred, external callus appeared and a small amount of artificial bone absorption was observed. The boundary between autogenous bone and implant in the artificial group was relatively blurred, and no obvious callus was found. X-ray showed that the edges of all of bone defects were clear with a small amount of callus, and the artificial

bone was still visible.

At 5 weeks post-operation, the bone defect area of the PRP group was pale red with a large amount of callus formed, and the defect boundary was unclear. X-ray showed that the edge of the bone defect in each sample was very blurred, but most of the artificial bone had been absorbed, accompanied by obvious callus shadows. X-ray showed that the margin of bone defects was still visible on the edge of the lacuna, with less callus and a small amount of artificial bone absorption.

At 7 weeks post-operation, the defect lacuna of the

Table I. The barrier density values and radiology scores of bone defect repair of PRP group and artificial group at different time points.

	Groups	3 weeks	5 weeks	7 weeks
Radiology scores	PRP group	1.43±0.52	2.78±0.43	9.55±0.52
	Artificial group	0.59±0.54	1.36±0.57	6.16±0.47
	P	0.013	0.004	0.001
Barrier density values	PRP group	34.03±1.74	54.89±1.43	61.78±2.37
	Artificial group	28.65±1.33	35.41±1.26	45.48±4.93
	P	0.015	0.001	0.011



Fig. 1. X-ray findings in the two groups.

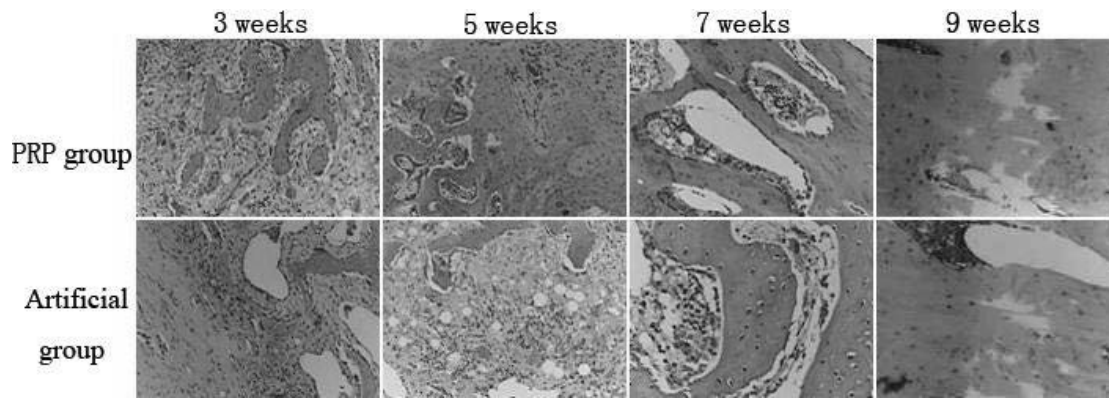


Fig. 2. Histological observation of the two groups.

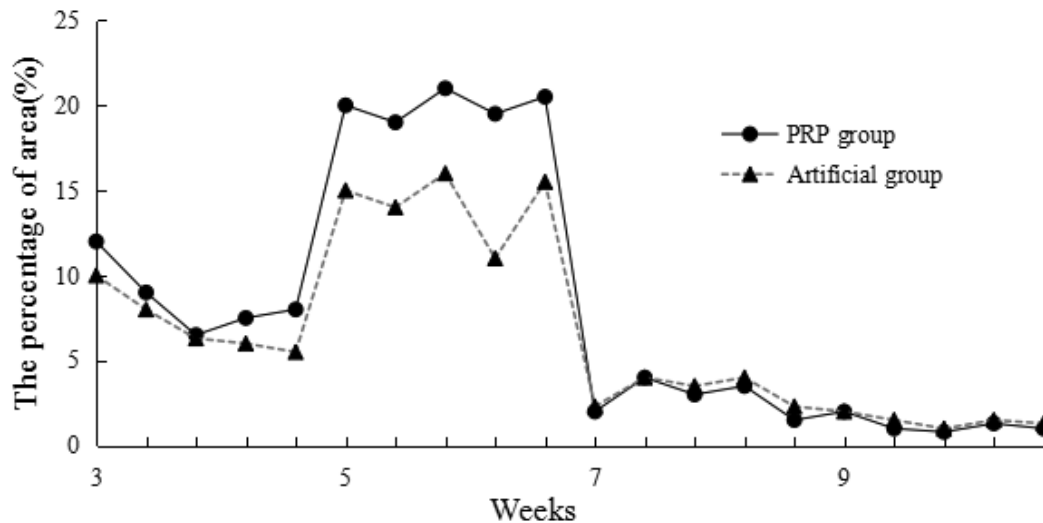


Fig. 3. Area percentage - time change polygon chart of VEGF in the bone defect area.

PRP group was completely filled and the boundary was indistinguishable. X-ray showed that the lacuna of bone defects had been completely healed, and a large amount of continuous callus grew on the defect surface of the rabbits in the artificial group, which blurred the boundary with the autogenous bone and was softer than that in the PRP group. It could also be seen that the contour of bone defects had basically disappeared and the area of callus was reduced.

At 9 weeks post-operation, bone healing was achieved in both the PRP group and the artificial group. No significant difference in color was found with the surrounding normal bone tissue. X-ray revealed that the two groups of bone defects were completely healed and moulded, as shown in Fig. 1.

As seen from to the data in Table I, the scores of the PRP group and artificial group were significantly different at 3, 5 and 7 weeks post-operation ($P < 0.05$). There was significant difference between the PRP group and artificial group in the value of bone defect repair barrier density at 3, 5 and 7 weeks post-operation ($P < 0.05$).

Histological observation

At 3 weeks post-operation, a large amount of fibrous tissue hyperplasia, capillary growth, active osteoblasts, and osteoid bone could be seen in the PRP group, and no implant materials were found under the microscope; the defect boundary of the PRP group showed an insignificant infiltration of

inflammatory cells and capillary growth, and some implant materials had not yet been fully absorbed and degraded. The osteoblast density in fibrous tissues was lower in the PRP group.

At 5 weeks post-operation, the PRP group displayed many island-shaped new bone tissues and osteoblasts, as well as abundant and dense capillaries, where the trabeculae tended to be connected into a network. In the artificial group, a large number of fibers were formed; osteoids were sheet and island-shaped; fibrous tissues were scattered in osteoblasts; and trabeculae were tiny and scattered.

At 7 weeks post-operation, the PRP group had a large number of trabeculae. These trabeculae were thicker. The density of newly formed bones was higher, and the degree of maturity was also higher. In the artificial group, trabeculae were connected and osteoblasts were active.

At 9 weeks post-operation, the PRP group and artificial group both showed massive new bone tissue formation. The coarse and dense trabecular bones were fused into one piece, and typical cortical bone could be seen, as shown in Fig. 2.

Immunohistochemical observation

At 3 and 5 weeks post-operation, the proportion of positive immunostaining in the PRP group was higher than that in the artificial group. After 7 and 9 weeks, the proportion of positively stained area in the PRP group was lower than that in the artificial group, as shown in Fig. 3.

DISCUSSION

Bone substitutes not only affect the biological behavior of cells, but also determine whether bone grafts can be well adapted and combined to repair bone defects (8). The results indicate that PRP can better promote repair of lacunar bone defects and accelerate repair of bone defects. This is related to a variety of high growth factors produced by PRP gel. These factors stimulate the chemotaxis and proliferation of osteoblasts and accelerate the formation of new bone (9).

When the organism is injured, platelets quickly attach to blood clots and release at least three growth

factors: Platelet-derived growth factor (PDGF), VEGF, Transforming growth factor (TGF)- β 1, β 2 and Epidermal growth factor (EGF) (10). VEGF has been proved to promote endothelial proliferation and induce angiogenesis through autocrine or paracrine binding to vascular endothelial surface receptors, providing a favorable microenvironment for local bone regeneration and metabolism (11). The study indicated that the PRP group was superior in promoting bone repair at the early stage; at the 7th and 9th weeks post-operation, the repair of bone defects showed a tendency of cartilage ossification, recanalization and shaping of medullary cavity so that the proportions of the positively stained areas are equal between the two groups.

To sum up, PRP has a good effect on promoting bone defect repair during the early stage, which is also featured by easy preparation and low cost.

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LETTER TO THE EDITOR

EFFICACY OF DIRECT-ACTING ANTI-VIRAL THERAPY ON CHRONIC, NAÏVE HEPATITIS C VIRUS PATIENTS OF PUNJAB, PAKISTAN: A CROSS-SECTIONAL STUDY

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The success of interferon-free regimens using new direct-acting antiviral (DAA) is a revolution and major breakthrough in the development of new therapeutic options against hepatitis C virus (HCV). Accumulating evidence suggest sustained virological response (SVR) with DAA in 95% of patients. To date, however, there are very few data related to efficacy of DAA in the Pakistani population. We aimed to investigate the efficacy of sofosbuvir-based regimen among Pakistani population. A total of 1,913 patients who attained SVR24 after being treated with sofosbuvir and ribavirin from August 2015 to March 2017 were enrolled in this study. We analyzed the demographic, clinical and virological data and screened all patients for HCV in March 2017 to evaluate the response rate. We found an overall response rate of 92.8%. In addition, we also observed lower response rates among older patients. It can be inferred that a large proportion of patients achieved SVR after treatment with sofosbuvir-based regimen.

To the Editor,

The estimated current global burden of hepatitis C virus (HCV) is 130-170 million, with an alarmingly high risk of liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC). HCV-related complications cause approximately 350,000 deaths annually. Over the last 15 years, prevalence of HCV infection has increased from 2.3% to 2.8% (1). Pakistan has one of the highest rates of HCV with about 10 million of its population known to be infected, with genotype 3 being the prevalent genotype which is difficult to treat compared to other genotypes (2). Chronic HCV patients develop liver cirrhosis within 25-35 years which can result in severe complications including hepatocellular carcinoma, esophageal variceal bleeding, jaundice, and ascites. Moreover,

co-infections with other viruses (HBV and HIV), diabetes, alcohol abuse, and iron overload are also important factors that play a role in the progression of disease.

Until 2011, the application of interferon alpha (INF- α) and ribavirin were considered to be the standard treatment against HCV, but they had poor outcome, detrimental side-effects, low efficacy and higher relapse which necessitated the development of a more effective therapeutic strategy. Recent emergence of interferon-free direct-acting antiviral (DAA) regimen represents a major breakthrough that has completely changed the background of HCV therapeutics. Sofosbuvir, a nucleotide HCV polymerase inhibitor is known as the backbone of DAA combinations due to its pangenotypic activity,

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high antiviral potency, favorable pharmacokinetics, high barrier to resistance, excellent tolerability and safety profile. Accumulating evidence shows that sofosbuvir and ribavirin combination for 12 and 24 weeks is highly efficacious for genotype 2 and genotype 3, respectively. These DAA agents target the genes involved in HCV replication viz. NS3 protease, NS5B polymerase and NS5A protein and such Sofosbuvir-based regimens have been proved as potent and highly effective therapeutic options for chronic HCV patients (3).

However, very little is known about the relapse after DAA mediated viral clearance and its response in Pakistani population. This study was therefore conducted to investigate the response rate of sofosbuvir and ribavirin in Pakistan.

MATERIALS AND METHODS

This prospective cross sectional study was conducted from August 2015 to March 2017 in the Department of Diagnostics, Centre for Applied Molecular Biology (CAMB), University of the Punjab Lahore Pakistan. Questionnaires were used to obtain information on the participants lifestyle and a written informed consent was signed by all the subjects involved before the collection of samples.

Inclusion and exclusion criteria

All subjects included in the study were naïve patients who had received 24-week DAA therapy (sofosbuvir and ribavirin), were non-cirrhotic and had not received any other treatment prior to DAA regimen, while recurring patients were excluded from the study.

Sample collection and processing

Blood samples were taken of 1,913 subjects and were processed for serum extraction followed by determination of serum HCV RNA levels using ROTOR-Gene Real Time PCR System, by QIAGEN (lower limit of detection 33 IU/ml). Sustained virologic response was assessed in routine clinical practice during and after the treatment in March 2017.

Statistical analysis

Frequencies and percentages were given for qualitative

variables. Diagnostic statistics were calculated using Microsoft office Excel 2007.

RESULTS

The total number of samples used in the study was 1,913. Genotype 3 was confirmed in all patients. We found an equal prevalence of HCV in both genders i.e, 50.9% of patients were males whereas, 49.1% of females were found infected with HCV. The majority of patients were middle-aged (41-50 years) whereas, the prevalence was least observed in the elderly population as well as the young population of under 18 (Fig. 1).

We found an overall response rate of 92.6% of the total 1,913 patients, 1,772 of whom were successfully treated with sofosbuvir and ribavirin whereas, only 144 (8.4%) did not show response towards DAA therapy.

Table I illustrates the number of responded patients and their respective percentage. In the group aged 11-20, there were 22 patients of whom 21 responded well, including 61.90% male and 38.10% female. In 21-30 age group, a total 216 samples were included of which 202 (58.42% male, 41.58% female) cleared the virus and became non-detected while the remaining 14 (42.86% male and 57.14% female) remained detected throughout the course period of 24 weeks. In the subjects aged 31-40 years, there were 393 samples of which 370 (55.40% male and 44.60% female) became non-detected while the other 23 (60.87% male and 39.13 % female) remained detected. In the group aged 41-50 years, of the 590 samples 540 (41.30% male and 58.70% female) responded well and became non-detected while 50 samples (64% male, 36% female) remained detected. In the 51-60 age bracket from the total of 484 samples, 449 (49.22% male, 50.78% female) were non-detected and 35 (71.43% male and 28.57% female) remained detected. In the age group of 61-70 years, from the 177 samples, 159 (55.35% male and 44.65% female) were non-detected and the remaining 18 (50% male, 50% female) were detected. In the 71-80 age group a total of 23 samples were included out of which 20 (65% male and 35% female) were non-detected and the remaining 3 (33% male, 67% female) were detected. In the current study it

can be seen that the percentage of responsive patients decreases with the increase in age (Table I).

DISCUSSION

To date, this is the first large observational study analyzing the response rate of sofosbuvir-

based regimen in naïve HCV patients of Punjab Pakistan. In concordance with other studies based on Pakistani population, our findings also showed an overall response rate of >90% with no relapse (4, 5). In two different studies of 3a-HCV patients treated with sofosbuvir and ribavirin >90% response rate and less than 10% relapse rate has also been

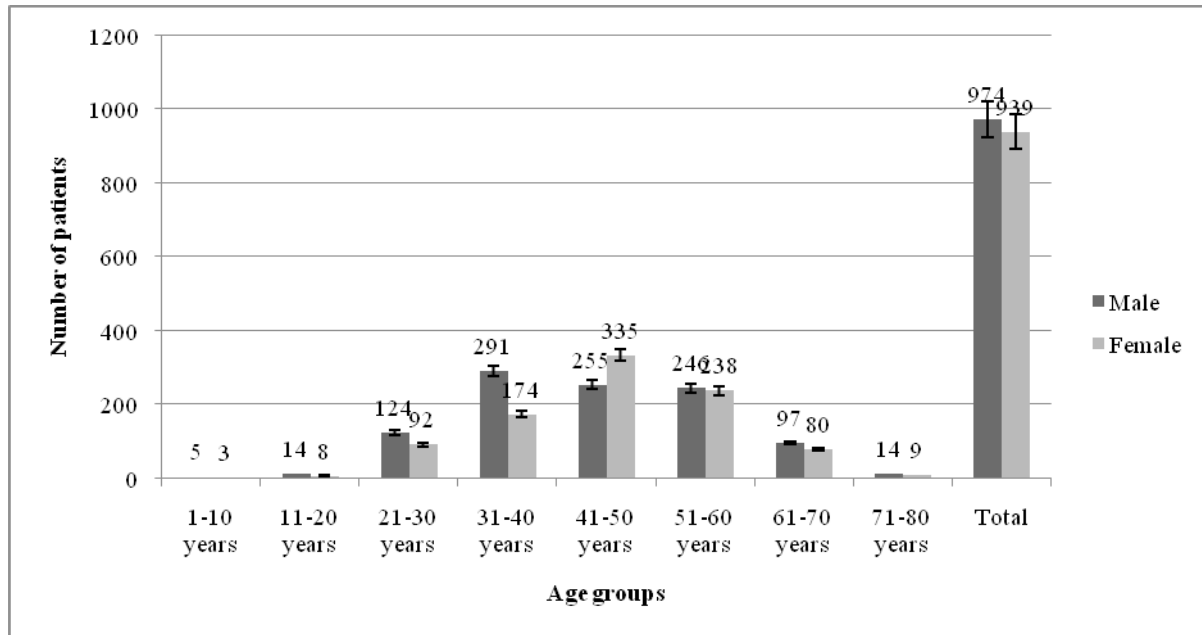


Fig. 1. Gender- and age-wise distribution of prevalence of HCV in 1,913 recruited subjects.

Table I. Treatment response in recruited patients after receiving 24-week sofosbuvir-based regimen.

Sr #	Age	Responders			Non-responders		
		Male		Total	Male	Female	Total
	1-10	5 (62.5%)	3 (37.5%)	8	0	0	0
	11-20	13 (61.9%)	8 (38.1%)	21	1 (100%)	0	1
	21-30	118 (58.4%)	84 (41.6%)	202	6 (42.86%)	8 (57.1%)	14
	31-40	205 (55.4%)	165 (44.6%)	370	14 (60.87%)	9 (39.1%)	23
	41-50	223 (41.3%)	317 (58.7%)	540	32 (64%)	18 (36%)	50
	51-60	221 (49.2%)	228 (50.8%)	449	25 (71.4%)	10 (28.5%)	35
	61-70	88 (55.3%)	71 (44.6%)	159	9 (50%)	9 (50%)	18
	71-80	13 (65%)	7 (35%)	20	1 (33%)	2 (67%)	3
		886	883	1772	88	56	144

noted (6, 7). Sustained virologic response (SVR) in > 97% of Japanese patients treated with sofosbuvir and ribavirin has also been observed (8). Sarwar and Khan compared viral response in patients of hepatitis C with and without liver cirrhosis (9). In a similar type of study design, sofosbuvir and ribavirin with or without pegylated interferon has shown excellent SVR12 in genotype 3 non-cirrhotic and cirrhotic patients (10).

In contrast to our observation, non-responsiveness has been noted in cirrhotic as well as interferon-treated patients. This might be due to the fact that patients included in our study were non-cirrhotic and had not received any prior treatment. Instead, one of our patients developed cirrhosis and ascites about one year post-treatment. Some workers confirmed high incidence of occurrence and recurrence of hepatocellular carcinoma (HCC) after interferon-free direct-acting antiviral (DAAs) use (11, 12) which is also non-consistent with our findings showing excellent efficacy of DAA therapy. Taken together, some observation based on Pakistani population regarding HCV recurrence and development of cirrhosis after DAA-mediated HCV eradication, it is important to carry out further research in this area for better treatment options.

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LETTER TO THE EDITOR

EFFECTS OF DEFICIENCY OF ACTIVE VITAMIN D ON BONE MARROW ABLATION OF FEMUR IN MICE

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The aim of this study was to investigate the effect of active vitamin D deficiency on bone marrow ablation (BMX) repair in mice. Six-week-old $1\alpha(\text{OH})\text{ase}$ knockout [$1\alpha(\text{OH})\text{ase}^{-/-}$] mice and control wild type mice were used to generate the BMX model. Serum Ca, P and PTH (parathyroid hormone) concentration were determined at 0 week (before operation), 1 week (1W), 2 weeks (2W) and 3 weeks (3W) post-operation. Callus formation and absorption 1W, 2W and 3W post-operation were observed by imaging and histochemistry. The morphological changes and osteoblast/osteoclast content in callus were determined by immunohistochemical analysis. The expression of gene and protein associated with bone resorption were determined in callus tissue to evaluate the impact of vitamin D on bone formation. The results showed that $1\alpha(\text{OH})\text{ase}^{-/-}$ mice suffered from low Ca, low P and hyper PTH due to deficiency of active vitamin D, and the symptoms persisted in the process of BMX and subsequent bone repair. In control mice, new calluses were observed in the medullary cavity at the first week, then part of the callus was absorbed in the second week, and most of the callus was absorbed in the third week. In the experimental group, new calluses were observed in the first week and reached its peak in the second week, and most of these calluses were mineralized in the third week. Meanwhile, the cortical bone became thinner and callus mineralization was decreased in $1\alpha(\text{OH})\text{ase}^{-/-}$ mice compared to the control mice. In conclusion, active vitamin D deficiency causes hyper PTH in mice. Hyper PTH promotes trabecular bone formation and decreases bone turnover; increases cortical bone formation and bone resorption while improving bone turnover.

To the Editor,

Bone injury is a common traumatic disease. Active vitamin D [formula of $1, 25(\text{OH})_2\text{D}_3$] is an active metabolite of vitamin D that affects bone development and mineralization (1). Studies have shown that the interaction between active vitamin D and PTH is essential in maintaining the homeostasis of calcium metabolism (2, 3). However, deficiency of active vitamin D blocks calcium and phosphorus absorption in the body, which further

results in osteoporosis, rickets and osteomalacia (4)., $1\alpha(\text{OH})\text{ase}$ can be expressed in several organs of the body (5), so it was previously difficult to create active vitamin D deficiency model in normal animals. More recently, the application of gene knockout technology successfully established an active vitamin D deficiency mouse model (6), the $1\alpha(\text{OH})\text{ase}$ knock out mice [$1\alpha(\text{OH})\text{ase}^{-/-}$]. It has been reported that $1\alpha(\text{OH})\text{ase}^{-/-}$ mice exhibit hypocalcemia, hypophosphatemia and hyper

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parathyroid hormone (PTH). In particular, active Vitamin D is undetectable. The skeleton of $1\alpha(\text{OH})\text{ase}$ mice exhibit widening of cartilage growth plate and resistance to calcification (7). This indicates that deficiency of active vitamin D affects the bone development of the body. However, the effect of active vitamin D deficiency on bone repair is still unknown. Therefore, in the current study, we propose to explore the mechanism of vitamin D deficiency on bone repair by determining bone formation and bone resorption in BMX mice.

MATERIALS AND METHODS

Animals

Thirty-five six-week-old SPF wild-type male Balb/c strain (control mice) and 35 six-week-old $1\alpha(\text{OH})\text{ase}/-$ mice fed by The Second Affiliated Hospital (Zhejiang University School of Medicine, Hangzhou, China) were used in the current study. These experimental mice were generated by in-house mating of heterozygote $1\alpha(\text{OH})\text{ase}/+$ mice on Balb/c background (The Second Affiliated Hospital, Zhejiang University School of Medicine). The mice were maintained in a 12-h light/dark cycle, temperature of $25\pm 2^\circ\text{C}$ and relative humidity of $50\pm 5\%$. The standard feed allocated to the animal center was given and they had free access to drinking water. This study was approved by the ethics committee of The Second Affiliated Hospital (Zhejiang University School of Medicine, Hangzhou, China). All animal experimental protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals.

Main instruments

Model 805 X-ray camera (Faxitron X-Ray, USA); 1072 scanner and analysis software and imaging software (Sky Scan Antwerp, Belgium); TP1020 automatic dehydrator; EG1150H embedding machine; EG1150C cooler; RM2235 slicer; HI1210 spreading machine (Leica, Germany); SZ2-ILST stereomicroscope; CX31 forward microscope; BX51 forward fluorescence microscope; DP70 CCD image acquisition system (Olympus, Japan); Power Pac Basic Power Supply type protein electrophoresis apparatus (Eppendorf, Germany); Mini-PROTEAN3 vertical electrophoresis cell; Mini Trans-Blot Cell type transfer electrophoresis cell; BS224S precision electronic balance;

ELGA ultra-pure water machine (Sartorius, Germany); My Cycler gradient electrophoresis cell PCR instrument (ABI, USA); Smart Spec Plus Spectrophotometer nucleic acid protein analyzer (Eppendorf, Germany); BI7300 real-time fluorescence quantitative PCR instrument (ABI, USA).

Major reagents

First antibody (polyclonal sheep anti-collagen I antibody); second antibody (biotin-bound rabbit anti-goat Ig G antibody) (Santa Cruz, USA); development reagent (biotin-horseradish peroxidase synthase kit); 3,3'-diaminobenzidine color reagent kit (Vector, USA); bovine serum albumin (Roche, Switzerland); protease K (Merck, Germany); hematoxylin; naphthol; xylene; neutral gum; hydrogen peroxide; trihydroxymethyl aminomethane (Tris); hematoxylin, eosin (Sigma, USA); Naphthol AS-MX phosphate; Chemiluminescent Substrate kit; Protease inhibitor cocktail tablets; Methyl green (Sigma, USA); Ethylene glycol phenyl ether and Magnesium chloride (Shanghai source leaf Biotechnology Co., Ltd., China); Fast Garnet GBC (Sigma, USA); Trizol and M-MLV reverse transcriptase (Invitrogen, USA); DEPC water (Ameresco, USA); Oligod(T)15 primer; dNTP Mix; Ribonuclease inhibitor; SYBR Green Jump Start Taq Ready Mix (Dalian TakaRa Company, China); 2.5 mM dNTP mixed liquor, Taq enzyme, DNA Marker, agarose, bromoethidium (Hangzhou Bor Technology Co., Ltd., China).

Identification procedure of animal genotypes

350 μL rat tail solution and 10 μL protease K (10 mg/mL) were added to a 1.5 mL EP tube, centrifuged at 170 rpm at 55°C , and incubated for 3 to 4 h until the rat tail solution was dissolved. Each tube was added with 350 μL solution (phenol: chloroform: isoamyl alcohol) (25:24:1), oscillated for 15 s, stationary at room temperature for 3 min, centrifuged for 15 min at 12,000 rpm. The upper colorless aqueous liquid (200 μL) was transferred to a new EP tube and 400 μL absolute ethanol was added. The mixture was turned upside down 20 times, and then was centrifuged for 5 min at 12,000 rpm. The upper layer of liquid was discarded, the pellet was dried for 10 min at room temperature and 100 μL sterile distilled water was added and then placed at 55°C bath for 1 h to dissolve DNA.

The PCR reaction system was: 250 ng DNA template 1 μL , $10\times\text{PCR}$ buffer 2.5 μL , 2.5 mM dNTP 2 μL , 25 mM

MgCl₂ 2 uL, 10 µM Primer Reverse 0.5 uL, 10 µM Primer Forward 0.5 uL, Taq polymerase 0.25 uL, H₂O 15.5 uL and Total volume 25 µL. PCR products were mixed with 4 uL sample loading buffer and 12 µL sample buffer was loaded on 2% agarose gel and the electrophoresis was conducted at 120V for 10 min.

Preparation of animal models

The mice were given 400mg/kgbw chloral hydrate by intraperitoneal injection until deep anesthesia. The right knee operation area was exposed, shaved and disinfected by iodophor. A 1-cm incision was made at the anterior of the right knee, the patella was pushed open, and the medullary cavity was destroyed by drilling the bone marrow cavity with needle No. 23 between the femoral condyles. The marrow was extracted by vacuum, and was flushed with 3 mL of warm saline. The patella was moved back to the normal position, and the surrounding ligaments and skin incision were sutured. After the mice were revived, they were kept in cage and maintained at 25±2°C. The mice were carefully monitored and were allowed to eat and drink freely.

Detection of serum concentration of Ca, P and PTH

Serum concentrations of Ca, P and PTH in 0W, 1W, 2W and 3W mice were detected by routine Ca, P ion kit and PTH radioimmunoassay kit (Nanjing Xinfan Biotechnology Co., Ltd., China).

X-ray photography and micro-CT scan reconstruction analysis

1W, 2W, and 3W post-operation, right femur X-rays were taken by an 805 small animal X-ray camera (Faxitron X-Ray Company). The conditions were 22kv, exposure for 4 min.

Micro-CT scan reconstruction analysis: After the radiological survey was taken, the femur specimens were isolated and fixed with 70% alcohol for 24 h, and analyzed with imaging software. The conditions were: 100kv, 98 µm, 0.9 degree rotation distance, 18.2 µm resolution, and the minimum contrast threshold detection.

Specimens fixed, decalcified, embedded and sliced

The right femur specimens of 1W, 2W and 3W mice were taken, and fixed in PLP (periodate-lysine-paraformaldehyde) for 24 h. The samples were soaked

and rinsed for 12 h in 0.01 M PBS solution containing 5%, 10% and 15% glycerol, respectively. The specimens were decalcified in EDTA-G (ethylenediaminetetraacetic acid-G) solution (EDTA 14.5g, NaOH 12.5g, and glycerin 15 ml dissolved in distilled water, adjusted pH=7.3, constant volume 100 ml, and preserved at 5°C) for 14 days. The decalcified specimens were soaked in 15 g sucrose+92.5 mL 0.01 M PBS+7.5 ml glycerol, 15 g sucrose+100 mL 0.01 M PBS, 50 mL solution, and 2+50mL distilled water for 12 h at 5°C. After dehydration, the femurs were paraffin embedded and sliced at 5 µm thickness.

Total collagen histochemical staining

The femurs of 1W, 2W and 3W mice were sliced, paraffin sections were routinely dewaxed, and the sections were stained with supersaturated picric acid straight red dye, counterstained with hematoxylin, dehydrated with ethanol gradient, rendered transparent with xylene and sealed. CCD image was collected under positive fluorescence microscope and analyzed by Northern Eclipse software. The percentage of the total collagen positive volume of the trabecular area and the volume of the trabecular area were calculated.

HE (hematoxylin-eosin) staining

Femur sections of 1W, 2W and 3W mice were taken and paraffin sections were routinely dewaxed. Then, hematoxylin-eosin staining was performed to dehydrate the transparent sealing section. Under the fluorescence microscope, DP70 CCD was used to collect images. Northern Eclipse software was applied for image quantitative analysis. Summary total gray (STG) = mean gray value * positive area; positive cell percentage = positive cell number/total cell number; positive area percentage = positive area/visual field area.

ALP (Alkaline phosphatase) staining

The paraffin sections of the right femur of 1W, 2W and 3W were collected and routinely dewaxed. 1% MgCl₂ -100 mM Tris-HCl buffer (PH9.2) was incubated overnight. Alkaline phosphatase stain was prepared and incubated at room temperature for 2 h. The section was rinsed with water, counter-stained with methyl green for 5 min, rinsed for 10 min with water and sealed with water-soluble sealing agents. Images were collected under

positive fluorescence microscope (DP70 CCD) and STG of positive area and positive products were quantified by Northern Eclipse software.

Tartrate resistant acid phosphatase staining

The paraffin sections were dewaxed with xylene, hydrated with gradient alcohol and washed with running water for 5 min. Slides were incubated with tartaric buffer for 20 min and then stained with Tartrate resistant acid phosphatase (TRAP) for 15 min. Slides were further counterstained with methyl green for 5 min and eventually were sealed with water-soluble sealing agents. Images were collected under positive fluorescence microscope (DP70 CCD) and positive osteoclast surface area/bone surface area were quantified by Northern Eclipse software.

Real-time quantitative RT-PCR assay

RNA was isolated from the callus of the right femur using the TRIzol method following the manufacturer's instructions. RNA concentration was measured by nucleic acid analyzer and RNA quality was determined by comparing the OD260/OD280 ratio. The extracted RNA (2 μ g) was then reverse-transcribed into cDNA with RT reagents.

The reaction system was: 5 $\times$ First-Strand Buffer 4 μ L, 0.1 M DTT 2 μ L, 40U/ μ L Ribonuclease inhibitor 1 μ L, 200UM-MLV reverse transcriptase 1 μ L, and 1 volume is 20 μ L. The reaction condition is: 37°C water bath for 55 min, and then placed at 70°C bath for 15 min. PCR reaction conditions were: cDNA Template 1 μ L, 10 $\times$ reaction buffer 5 μ L, 2.5 μ M dNTP 3 μ L, 25 mM MgCl₂ 3 μ L, 5U/ μ L Taq DNA polymerase 1 μ L, 10 μ M Primer forward/reverse 1 μ L, dH₂O 35 μ L in a 50 μ L volume.

The reaction products were collected and analyzed by agarose gel electrophoresis to verify the molecular weight of the RT-PCR products. The experimental results were analyzed with 7300 System SDS software.

Western blot

Protein from the callus of right femur of mice at 1W, 2W and 3W was extracted by Radio-Immunoprecipitation Assay (RIPA) buffer. Protein concentration was determined by BCA reagents. Protein lysates were further mixed with SDS loading buffer and an equal amount of protein was loaded onto SDS-PAGE gel. The samples were separated on SDS gel by running at 110V for 2 h, after which protein

was further transferred onto nitrocellulose membrane. The nitrocellulose membrane was blocked in PBS with 5% milk at 37°C for 1 h twice. The membrane was then incubated with primary antibody at 4°C overnight. After washing 3 times in 5% PBST, the membrane was further incubated with the second antibody for 2 h at room temperature. Membranes were extensively washed and exposed by ECL. The positive bands were collected on the scanner, and the experimental results were analyzed by image software.

Immunohistochemical staining

The femur sections of mice were harvested from the two treatment groups at three time points and embedded in paraffin. Tissue blocks were sliced into 5 μ m sections which were dewaxed with xylene and hydrated with gradient alcohol. Slides were digested with 5 μ g/mL bovine testicular hyaluronidase at 37°C for 30 min and incubated with 3% hydrogen peroxide for 20 min to inactivated endogenous peroxidase. The sections were further incubated with blocking buffer (10% normal rabbit serum) at room temperature for 10 min, followed by incubation with primary antibodies (Pyclonal sheep anti-Collagen I (Col-I) antibody; 1:400) overnight at room temperature. PBS buffer was used instead of primary antibody as negative control. Afterwards, sections were incubated with biotin labeled secondary antibodies (Rabbit anti-sheep IgG; 1:400) at room temperature for 45 min. Sections were rinsed in cold PBS, incubated in biotin-horseradish peroxidase solution (Vectastain Elite ABC kit) 37°C for 30 min and rinsed with PBS (5 min, 3 times). Finally, sections were developed in diaminobenzidine substrate (DAB) for 5 min and counterstained with hematoxylin. Slides were further dehydrated in gradient alcohol, cleared by xylene and mounted with resinous media. Images were taken under fluorescence microscope (DP70 CCD) and areas of positive staining were analyzed by Northern Eclipse software. The quantitative indicators included STG of positive area and positive products.

Statistical analysis

The data obtained were analyzed by one-way factor analysis of variance with SPSS 22.0 statistical software, the two-two comparison was conducted by q-test, and the difference was statistically significant ($P < 0.05$).

RESULTS

Changes of serum Ca, P and PTH concentration

Compared with control mice, the experimental mice showed hypocalcemia, hypoproteinemia and secondary hyper PTH before the operation (Ca: 1.20 ± 0.21 mmol/L, P: 1.75 ± 0.06 mmol/L, PTH: 72.61 ± 1.23 pg/mL), and these symptoms remained in the bone marrow removing model. The experimental group mice showed hypocalcemia, P and hyperPTH at 1W (Ca: 1.21 ± 0.20 mmol/L, P: 1.78 ± 0.08 mmol/L, PTH: 74.12 ± 1.35 pg/mL), 2W (Ca: 1.52 ± 0.18 mmol/L, P: 2.08 ± 0.13 mmol/L, PTH: 58.63 ± 3.62 pg/mL) and 3W (Ca: 1.11 ± 0.19 mmol/L, P: 1.92 ± 0.16 mmol/L, PTH: 69.35 ± 4.03 pg/mL) post-operation. In the process of bone repair after BMX, in the two groups (control group: 0W [Ca: 2.13 ± 0.16 mmol/L, P: 2.21 ± 0.11 mmol/L, PTH: 31.22 ± 1.43 pg/mL], 1W [Ca: 2.21 ± 0.14 mmol/L, P: 2.16 ± 0.08 mmol/L, PTH: 30.03 ± 0.82 pg/mL],

2W [Ca: 2.58 ± 0.11 mmol/L, P: 2.57 ± 0.09 mmol/L, PTH: 25.17 ± 0.97 pg/mL], 3W [Ca: 2.28 ± 0.23 mmol/L, P: 2.25 ± 0.16 mmol/L, PTH: 33.72 ± 1.89 pg/mL]), serum Ca and P concentrations in both groups remained unaltered during 1W, while these values were significantly increased at 2W and then dropped to preoperative level at 3W. In contrast, serum PTH concentration did not change significantly at 1W, but was decreased at 2W, and returned to preoperative level at 3W.

Callus changes during bone repair after BMX

As illustrated in Fig. 1, in the control group, a large number of new calluses were found in the medullary cavity and reached the peak at 1W. Calluses started to be absorbed at 2W, with most being absorbed at 3W. In the experimental group, a large number of new calluses were found at 1W and they continued to form and reached the peak at 2W. Most of the calluses were retained and mineralized

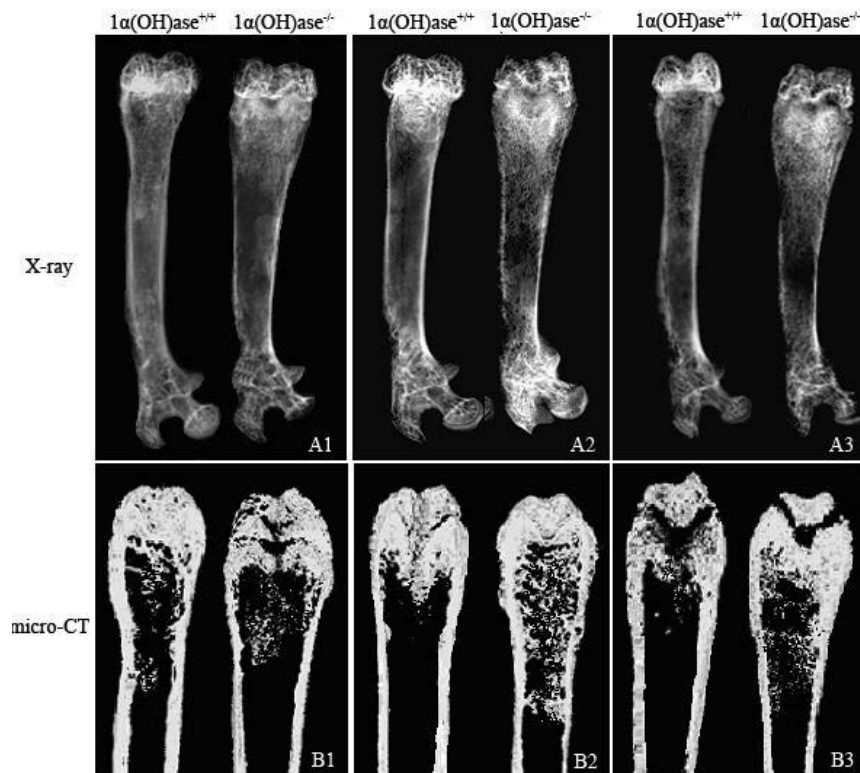


Fig. 1. Femoral X-ray and micro-CT three-dimensional reconstruction of mice at 1W, 2W, and 3W post-operation. **A)** X-ray; **B)** micro-CT three-dimensional reconstruction; 1:1W, 2:2W, 3:3W. $1\alpha(\text{OH})\text{ase}^{+/+}$ is the control group; $1\alpha(\text{OH})\text{ase}^{-/-}$ is the experimental group.

at 3W. At the same time, compared with the control group, the cortical cortex of the experimental group became obviously thinner and the mineralization of callus decreased. Collagen staining of femoral sections of 1W, 2W and 3W post-operation mice demonstrated similar results, further confirmed the imaging findings.

Osteoblast bone formation after BMX

As indicated in Fig. 2 and Table I, the number of osteoblasts per unit callus area, and the proportion of ALP positive area and Col-I positive area in the total callus area in the experimental group were significantly higher than in the control group. In the control group, the proportion of ALP and Col-I positive area in the total callus area reached the maximum at 1W but decreased significantly at 2W and 3W. In the experimental group, the proportion of ALP and Col-I positive area in the total callus area reached the peak at 2W and decreased at 3W. The expression of bone-related ALP, Col-I, Runx2, osteocalcin gene, Runx2, PTHR and insulin-like growth factor I (IGF-I) in callus tissue of the experimental group were higher than that of the control group at the same observation point.

Osteoclast bone resorption after BMX

Fig. 3 shows that the number of osteoclasts

per callus area in the experimental group was significantly lower than that in the control group at 1W, decreased at 3W and 2W, and the number of osteoclasts at 2W and 3W was higher than that in the control group at the same time. The proportion of osteoclasts in the total callus area at 1W, 2W and 3W was lower than that in control group at the same observation point.

Table II showed that the ratio of RANKL and OPG gene expression of osteoclasts in callus tissue of the experimental group was lower than that of the control group at 1W, 2W and 3W. The total collagen staining of the femur after BMX in mice showed that the cortex of the femur became thinner and the callus mineralization was less in the experimental group. Therefore, the cortical bone sections were further stained with ALP and TRAP and the image software measurement showed the positive area ratio of ALP staining in the cortical callus of the 1W, 2W and 3W mice, and the number of osteoclasts per unit callus area in the experimental group was significantly higher than those of the control group.

DISCUSSION

Many studies on active vitamin D showed that active vitamin D interacts with PTH and jointly maintains the homeostasis of calcium metabolism

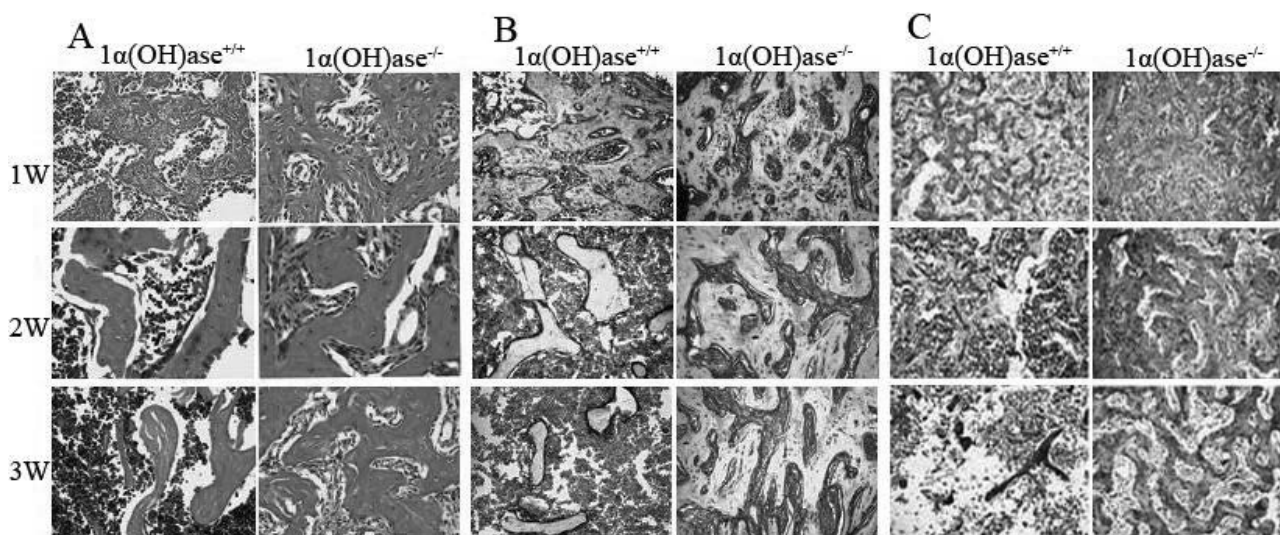


Fig. 2. A-C) HE staining, ALP staining, and Col-I staining micrographs (50x) of callus tissue sections of femoral medullary cavity in mice at 1W, 2W, and 3W. $1\alpha(\text{OH})\text{ase}^{+/+}$ is the control group; $1\alpha(\text{OH})\text{ase}^{-/-}$ is the experimental group.

Table I. The number of osteoblasts, and the proportion of ALP positive area representing osteogenesis, Col-I positive area to total callus area per unit area of BMX mice at 1W, 2W and 3W and expression levels of related genes and proteins in callus tissue.

		1W	2W	3W
HE staining (the number of osteoblasts in unit callus area /mm ²)	The control group	39.17±2.19	42.87±3.72	20.18±1.03
	The experimental group	53.72±4.19**	76.19±5.03***	67.93±3.04***
ALP staining (ALP positive area [% of tissue area])	The control group	14.02±1.02	4.87±0.04	2.13±0.06
	The experimental group	19.96±2.12**	24.37±6.27***	13.75±3.31***
Col-I staining (Col-I positive area [% of bone area])	The control group	0.28±0.03	0.12±0.02	0.03±0.01
	The experimental group	0.51±0.05***	0.55±0.07***	0.38±0.03***
Runx2 mRNA(% of Wild type control)	The control group	1.00±0.04	0.52±0.03	0.27±0.02
	The experimental group	2.38±0.08**	3.71±0.15***	2.11±0.12***
ALP mRNA(% of Wild type control)	The control group	1.00±0.05	0.45±0.02	0.38±0.02
	The experimental group	1.36±0.05*	1.19±0.07***	0.88±0.06***
Col-I mRNA(% of Wild type control)	The control group	1.00±0.05	0.45±0.02	0.37±0.03
	The experimental group	1.20±0.06*	1.13±0.07***	0.96±0.08***
OCN mRNA(% of Wild type control)	The control group	1.18±0.13	0.86±0.11	0.58±0.09
	The experimental group	2.10±0.14***	1.56±0.36**	1.82±0.17**
Runx2 protein (% of β -tubulin control)	The control group	0.65±0.02	0.37±0.04	0.29±0.08
	The experimental group	1.02±0.04**	1.23±0.02***	0.81±0.03***
IGF-1 protein (% of β -tubulin control)	The control group	0.79±0.03	0.58±0.02	0.36±0.03
	The experimental group	0.83±0.04	0.79±0.03*	0.67±0.04**
PTHr protein (% of β -tubulin control)	The control group	0.72±0.08	0.53±0.02	0.51±0.02
	The experimental group	0.84±0.04*	1.08±0.05***	0.85±0.03***

Note: OCN mRNA: osteocalcin gene; IGF-I: insulin-like growth factor I. The mice in the experimental group were compared with those in the control group, *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$.

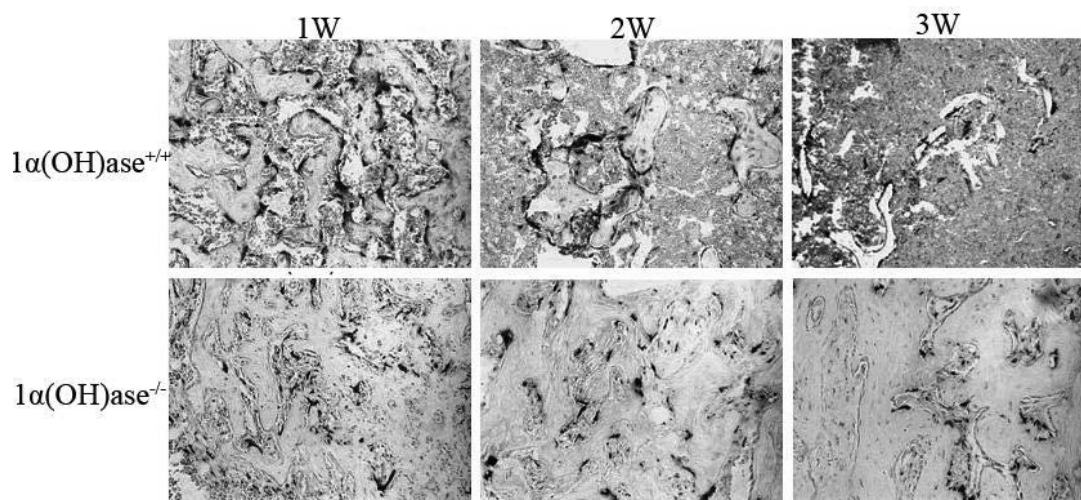
(8), and deficiency of active vitamin D is an important factor affecting bone development and mineralization (9). The present study showed that $1\alpha(\text{OH})\text{ase}^{-/-}$ mice have hyper-PTH due to deficiency of active vitamin D, with inconsistent roles in the formation and transformation of femoral trabecular bone and cortical bone.

Studies reported that $1\alpha(\text{OH})\text{ase}^{-/-}$ mice demonstrated hyper PTH which could cause the average volume of osteoclasts decrease (10, 11). The results suggest that, for $1\alpha(\text{OH})\text{ase}^{-/-}$ mice, trabecular bone resorption decreases, and trabecular bone turnover reduces due to the absence of direct stimulation of osteoclast resorption by active

Table II. The number of osteoclasts, the ratio of osteoclasts to total callus area, the level of RANKL/OPG in callus of BMX of mice at 1W, 2W and 3W, ALP positive area ratio and osteoclast count.

		1W	2W	3W
The number of osteoclasts in unit callus area /mm ²	The control group	39.28±4.17	23.31±3.82	14.19±2.18
	The experimental group	11.24±1.82***	31.19±3.02*	22.82±2.18*
The proportion of osteoclast area of total callus area (%)	The control group	7.36±1.14	7.92±0.34	6.14±0.52
	The experimental group	3.21±0.12***	3.68±0.16***	2.83±0.11***
Ratio of RANKL/OPG mRNA relative levels	The control group	1.02±0.04	0.78±0.03	0.62±0.03
	The experimental group	0.68±0.04**	0.62±0.03*	0.52±0.03*
ALP staining (ALP positive area[% of tissue area])	The control group	0.49±0.03	0.37±0.02	0.28±0.02
	The experimental group	16.83±0.12***	12.01±0.09***	11.87±0.07***
TRAP staining (Number of osteoclasts in unit callus area /mm ²)	The control group	0.91±0.11	0.82±0.08	0.62±0.04
	The experimental group	5.22±1.21***	6.93±0.83***	4.87±1.03***

Note: The mice in the experimental group were compared with those in the control group, *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$.

**Fig. 3.** TRAP staining micrograph (50x) of bone marrow callus tissue of mice at 1W, 2W and 3W. $1\alpha(\text{OH})\text{ase}^{+/+}$ is the control group; $1\alpha(\text{OH})\text{ase}^{-/-}$ is the experimental group.

vitamin D. After the operation, the cortex of femur became thinner and callus mineralization of mice in the experimental group decreased. During the process of cortical bone repair in $1\alpha(\text{OH})\text{ase}^{-/-}$ mice, osteogenesis and osteoclast activity were enhanced, and bone turnover was improved.

To sum up, the deficiency of active vitamin D causes hyper PTH in mice, and the repairing performance of trabecular bone and cortical bone is inconsistent during bone repair after femoral bone marrow ablation. Hyper PTH promotes the formation of trabecular bone in the medullary cavity, and reduces the absorption of new bone in the medullary cavity due to deficiency of active vitamin D, which reduces bone turnover and cortical bone formation, increases bone resorption, and improves bone turnover.

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LETTER TO THE EDITOR

**HUMAN WHARTON'S JELLY MESENCHYMAL STEM CELLS:
PROPERTIES, ISOLATION AND CLINICAL APPLICATIONS**

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Human Wharton's jelly mesenchymal stem cells (WJ-MSCs) exhibit CD29, CD79 and CD105 markers, characteristic for mesenchymal cell lines. Under the influence of the appropriate factors, WJ-MSCs can be dedifferentiated to osteoblasts, chondrocytes, adipocytes, myocytes, cardiomyocytes, glial cells and dopaminergic neurons. Wharton's jelly (WJ) is one of the potential sources of mesenchymal stem cells (MSCs) - obtaining these cells does not raise moral or ethical objections, because the umbilical cord (UC) is a regular waste material. The expression of the OCT-4 and Nanog proteins, which are characteristic for WJ-MSCs may indicate that these cells have retained some embryonic character. The collected data suggests that WJ-MSCs show increased division and telomerase activity compared to bone marrow MSCs (BM-MSCs). The published results showed no human leucocyte antigen (HLA) class II expression, with the possibility of HLA class I modification by WJ-MSCs, allowing for the transplantation of these cells both within the same and other species - which allows the use of human cells in animal models. The results of selected studies indicate that WJ-MSCs can be an essential element of regenerative medicine of the 21st century.

To the Editor,

Wharton's jelly (WJ) is the connective tissue that surrounds the blood vessels of the umbilical cord (UC). Its physiological function is to ensure the patency of the blood vessels of the UC. Cells isolated from WJ have a myofibroblast character - they have a morphology similar to fibroblasts, coupled with the presence of desmin and α -smooth muscle actin, as well as contractile properties (1). The studies carried out show that these

cells, according to the guidelines of the International Society for Cell Therapy (ISCT), are recognized as mesenchymal stem cells (MSCs) (2). Stem cells of WJ have a common origin with bone marrow MSCs (BM-MSCs). Mesenchymal stem cells of the bone marrow are cells that currently have the highest clinical use (3). However, the collection of BM-MSCs is invasive and painful and is associated with an increased risk of donor infection at the site of collection. The negative features

Key words: mesenchymal stem cells; WJ, properties; isolation; clinical application

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of this source of MSCs have contributed to the growing interest in the search for alternative sources of MSCs, which are non-invasive for the donor.

Wharton's jelly mesenchymal stem cells (WJ-MSCs) only start to show signs of aging after around 60 cell-cycles in culture (1). Despite their high proliferative potential and expression of markers characteristic of embryonic stem cells, they do not exhibit spontaneous neoplastic transformation which predisposes them for safe use in clinical trials (1).

Umbilical cord

The UC consists of three spirally twisted blood vessels (two arteries and one vein) enveloped in WJ connective tissue and covered by epithelium (4). WJ is a connective tissue, classified as mucoid. WJ consists of three zones: the sub-amnion, the intermediate WJ and the perivascular WJ. The cells included in the connective tissue of the UC exhibit features of both fibroblasts and smooth muscle fibres (1). In the literature, they are referred to as myofibroblasts. WJ cells are characterized by contractile properties, as well as the presence of desmin and L-smooth muscle actin. The least differentiated myofibroblasts are found in the under-amniotic part of UC, while the most diversified cells are closest to the blood vessels (3).

Properties of WJ-MSCs

WJ-MSCs are characterized by the expression of some surface antigens typical for mesenchymal stem cells: CD105, CD73, CD90, CD44, and CD51 (2).

The more primordial, multipotent character, more plastic than that of adult MSCs, characteristic for WJ-MSCs, increases their therapeutic usefulness. It was observed that WJ-MSCs have several features of embryonic stem cells (ESCs). The reduced expression of ESC-specific transcription factors, such as Oct4, Nanog and SOX-2, in WJ-MSCs may indicate that these cells retain some of the typical properties of the ESCs (1). Mesenchymal stem cells from WJ are characterized by higher divisional activity than bone marrow mesenchymal stem cells as well as high telomerase activity (4).

WJ-MSC isolation methods

The clinical application of MSCs requires a large

number of cells, which is why the most critical factor when choosing an MSC source is the ease of isolation and the proliferative potential of the obtained cells. The amount of MSCs obtained from WJ from 1 cm of the UC is about 3×10^4 cells. Of all the mentioned MSC sources the highest proliferative capacity is exhibited by WJ-MSCs (4).

Recently, many works focused on the development of efficient WJ-MSC isolation protocols. Currently, the most common isolation methods are explant culture of 1-3 mm pieces of the UC, and enzymatic digestion (5). The method of explant isolation allows to obtain 2.8 times more MSCs from 1 g of UC than enzymatic digestion. However, the doubling time of the WJ-MSC population obtained by the enzymatic method is much longer. MSCs obtained by explant culture have a longer lifespan than those isolated using enzymes, which can also induce cellular damage (5).

The presented information indicates that the WJMSC isolation using explant culture is more efficient and cost-effective, as well as more suitable for use in clinical trials.

The differentiation potential of WJ-MSCs

The results of studies published in recent years report remarkable plasticity of myofibroblasts derived from the WJ. ISCT states that one of the three significant MSC characteristics is their ability to differentiate into osteocytes, chondrocytes, and adipocytes *in vitro* (2). Experiments have shown that the surface of WJ-MSCs has an increased amount of CD146 marker compared to BM-MSCs, which is responsible for the chondrogenic, osteogenic and adipogenic potential (5).

When WJ-MSCs were exposed to 5-azacytidine for three weeks, they began to present the phenotype of cardiomyocytes. WJ-MSCs could also be successfully induced to differentiate into cells able to produce insulin, as well as endothelial cells *in vitro* (6). WJ-MSCs demonstrate the ability to differentiate into neuronal cells under strictly defined conditions: WJ-MSCs are capable of differentiating, among others, into cells of myogenic and hepatic lineages (7).

Immune properties of WJCs

The hypoimmunogenic properties of WJ-MSCs in both *in vitro* and *in vivo* models were confirmed experimentally. The lack of MHC-II expression on the surface of WJ-MSCs, as well as the low level of MHC-I prevents their lysis by natural killer cells. WJ-MSCs are characterized by a lack of immunogenicity, despite the low major histocompatibility complex (MHC)-I intake. This fact results from the cellular absence of CD40, CD80 and CD86 as well as a high level of inhibitors of the immune response: 2,3,4-dioxygenase of indolamine and prostaglandin E2 (PGE2) (6). These features allow for the implantation of WJ-MSCs on the path of allo- and xenotransplants. This also makes it possible to reference an experimental

animal model for future human applications (8). The immunosuppressive properties of WJ-MSCs have been confirmed in cellular co-cultures. The presence of WJ-MSCs reduced the proliferation of activated human peripheral blood mononuclear cells as well as rat splenocytes. With the non-aggressive nature of WJ-MSCs, matching the human leucocyte antigen (HLA) with the donor should not be required for a successful transplant.

Clinical applications

The first clinical trial using UC mesenchymal stem cells (UC-MSCs) was registered in July 2007. It concerned their use in the treatment of leukaemia and myelodysplastic syndrome. In November 2014,

Table I. A report of clinical trials of UC-MSCs on <http://www.clinicaltrials.gov/June 2018>.

Medical application	The number of trials	Start of studies	Phase	Sum of estimated enrollment
Diseases and dysfunctions of the nervous system (including autoimmune)	31	2011-2018	1,2, one of them 3	1445
Diseases of the cardiovascular system	10	2010-2018	1,2, one of them 3	844
Two main types of diabetes and complications	11	2009-2019	1,2	604
Liver diseases (including autoimmune)	17	2009-2018	1,2, one of them 3	1864
Diseases of the osteoarticular system (including autoimmune)	18	2011-2018	1,2, one of them 3	730
Haematopoietic system diseases (including cancer)	6	2007-2017	1,2	218
Autoimmune diseases of the gastrointestinal tract	4	2010-2017	1,2	182
Respiratory system diseases	9	2014-2016	1,2	226
Diseases of the genitourinary system	9	2012-2018	1,2	515
Disease of the immune system	9	2019-2018	1,2, one of them 4	302
Skin diseases	4	2012-2018	1,2	137
Sweat Gland Disease	1	2013	1	50
Metabolic Syndrome	1	2018	1,2	37
Lupus	5	2011-2018	1,2	115
Duchenne Muscular Dystrophy	3	2011-2014	1,2	26

51 trials that employed UC-MSCs were conducted (7) according to the clinical trial database (<http://www.clinicaltrials.gov/>), while in 2018 there were already 132 (Table I, search terms: umbilical cord mesenchymal stem cells or Wharton's jelly mesenchymal stem cells). The analysis of the data contained in Table I indicates that the largest number of clinical trials using UC-MSCs was carried out for the treatment of neurological, cardiovascular, and liver diseases as well as osteoarthritis, diabetes and autoimmune diseases. Most of the presented studies belong to the clinical phases I and II, very few are in phase III, with phase IV representing only one study regarding the use of UC-MSCs in the treatment of children with plastic anaemia. Currently, none of the presented studies has yet published results. However, growing interest in the clinical use of UC-MSCs results in many published observations.

UC-MSC for the treatment of cancer

WJ-MSCs are characterized by a lower expression of pluripotent genes than ESCs, as well as high expression of tumour suppressor genes. Moreover, WJ-MSCs secrete large amounts of cytokines and growth factors. These features explain why these cells do not tend to form teratomas after transplantation. It also explains their ability to contribute to the inhibition of tumour growth *in vitro* and tumour cells *in vivo*, as demonstrated in many preclinical studies (9). Numerous results indicate that WJ-MSC lysates and conditioned media inhibit the growth of breast adenoma, ovarian cancer, osteosarcoma, bladder tumours, and pancreatic tumours as well as lung tumours and lymphoma cells. Akimoto et al. demonstrated the inhibitory properties of UC-MSCs against Glioblastoma multiforme (GBM) both *in vivo* and *in vitro*. Tumour cells were induced to apoptosis through the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) (9). WJ-MSCs inhibit the proliferation of human breast cancer *in vitro* and, after intravenous administration, in the SCID mouse model. Studies have shown that WJ-MSCs inhibit the growth of breast cancer cells *in vivo* and *in vitro*, by inhibiting the cell cycle, apoptosis of tumour cells and inhibiting the enzyme protein kinase B (PKB) and phosphoinositide 3-kinase (PI3K). WJ-MSCs are characterized by their proangiogenic features

and have the ability to form functional vessels *in vivo*, which may accelerate the progression of some types of cancer (9). However, care should be taken in the implementation of WJ-MSCs in anti-cancer therapies, as they have already been noted to promote the development of kidney and oesophageal cancer (9).

UC-MSC for treatment of autoimmune diseases

Type I diabetes is an autoimmune disease that causes the destruction of B-cells of the pancreas by T lymphocytes. Immunomodulatory properties of WJ-MSCs indicate their potential efficacy in the treatment of this disease. Hu et al., in their study, evaluated the long-term effects of intravenous administration of WJ-MSCs to newly diagnosed patients. A double-blind study was conducted, with 29 patients randomly divided into two groups. The first group received WJ-MSCs in two intravenous administrations, while the second group received a saline solution. During the 24-month follow-up of the patients in the first group, no acute or chronic adverse events were observed compared to the second group. The results of the HbA1c and C peptide in the first group were significantly better than before or in the placebo group. The results obtained indicate that therapy with WJ-MSCs can safely restore the function of pancreatic B-cell islands in type 1 diabetes. However, the mechanism of this action is still unknown and needs further research (10).

Crohn's disease (CD) is classified as an inflammatory bowel disease (IBD). Its pathogenesis is multifactorial, and the unambiguous cause of its occurrence has still not been established. The hypothesis of an immunological, genetic and microbiological basis (intestinal dysbiosis) seems to be the most probable. Banerjee et al. focused on the diagnosis of intestinal tuberculosis (ITB) and CD. Both disorders have a very similar clinical picture and are difficult to differentiate. The results of the study indicate that the mesenchymal CD73 marker is expressed only in ITB. This discovery explains the ITB pathogenesis mechanism-evading host immunity through recruitment of high CD73 expression MSCs. The presented results indicate that MSCs with increased expression of CD73, including WJ-MSCs, may have therapeutic potential in the

treatment of CD (11).

Multiple sclerosis (MS) is an autoimmune disease characterized by chronic inflammation and demyelination of central nervous system neurons. The aetiology of MS is multifactorial, including environmental and genetic factors. Currently, there is no effective medicament for MS due to an incomplete understanding of its pathophysiology. Donders et al. showed that the systemic administration of WJ-MSCs at the beginning or during the course of disease temporarily alleviates autoimmune encephalomyelitis (an animal model of MS). The protective effect was parallel to the reduction of T-cell proliferation, confirming the immunomodulatory effects of WJ-MSCs *in vivo*. The results obtained indicate that WJ-MSCs may be a promising method of MS therapy (12).

CONCLUSION

The low availability of BM-MSCs, their low cellular proliferative potential and painful harvesting procedure, which requires an invasive method of material collection, contributed to the intensification of the search for alternative sources of obtaining MSCs. WJ-MSCs seem to be the most promising source of mesenchymal stem cells. These cells have many advantages: the acquisition procedure is not invasive and does not raise ethical dilemmas, and the cells have a very high proliferative potential, as well as the ability to differentiate into many cell lines different from the connective tissue (8).

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LETTER TO THE EDITOR

MEDICATION ADHERENCE AMONG ALBANIAN PATIENTS
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Non-adherence in patients with rheumatoid arthritis (RA) using disease-modifying antirheumatic drugs (DMARDs) may lead to increased disease activity and symptoms, poorer quality of life and increased costs related to the disease treatment. The aim of this study is to determine the level of adherence to the treatment protocols of a chronic disease such as rheumatoid arthritis in the Albanian population and to investigate the predictors of adherence to medication. This is a single-center cross-sectional study in which included 125 patients diagnosed with RA recruited from the University Hospital “Mother Teresa” in Tirana, Albania from January to December 2013. The study was based on self-reporting by patients through a standard questionnaire divided into two parts. The first part consisted of a face-to-face interview with the patients in the hospital setting and the second part aimed at evaluation of the adherence to the prescribed therapy and was filled in via a telephone interview 4 weeks after the patient’s discharge from the hospital (117 patients participated in the second part of the interview). The Morisky Medication Adherence 4-item Scale (MMAS-4) was used to measure patients’ self-reported adherence to their medications. Factors affecting adherence are evaluated through the values of χ^2 (chi-squared test of Pearson) for categorical variables and p (Student’s t -test) for continuous variables; 30.8% of full adherence to DMARDs was found in this study. Adherence to the medication was associated with education, income, number of comorbidities, use of biologic DMARDs, use of traditional medication and patients neglectful behavior toward medication. Three of the most important reasons reported by the patients as reasons for non-adherence to the medication were: the cost of the treatment; forgetfulness in taking the drug; and side effects of the treatment. The findings emphasize the need for intervention of the health authorities to improve the access to medicines for RA. Health professionals have a crucial role to inform and educate patients in order to support them in improving their adherence.

To the Editor,

Rheumatoid arthritis (RA) is a chronic inflammatory disease of the joints, the consequences of which are damage of cartilages and bones, associated with different levels of disability (1). The etiology of the disease still remains unclear, but an increased risk for RA has been associated with

genetic and environmental factors (2).

Medication adherence is defined as a complex behavior influencing drug therapy and related to different factors such as socio-economic, psychological, therapy, and the health care system (3). Non-adherence to disease-modifying antirheumatic drugs in rheumatoid arthritis and spondyloarthritis

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results in increased disease activity and symptoms and poorer quality of life. It has been reported that adherence rates to prescribed medicines in patients with RA are low varying from 30 to 80%. Adherence to medication can be evaluated in a variety of ways, including: patient self-report, physicians' report, through direct methods such as use of biomarkers, or through indirect methods such as pharmacy refill, tablet counts, electronic monitors and questionnaires (4).

To our knowledge no studies have evaluated the extent of medication adherence in patients with rheumatic arthritis and the factors that affect non-adherence in Albania. Previous studies reported some level of inadequacy with patients' behavior toward medication schedules in Albania, and therefore there is the risk that this population also have medication adherence problems in chronic diseases such as rheumatoid arthritis (5, 6).

The aim of this study is to determine the level of adherence to the treatment protocols of a chronic disease such as rheumatoid arthritis (RA) in the Albanian population and to investigate the adherence to medication schedules.

MATERIALS AND METHODS

The study was performed between January 1 and December 31, 2013, enrolling the hospitalized patients diagnosed with RA in the Service of Rheumatology at the University Hospital Center "Mother Theresa" in Tirana. This is the most frequented university hospital in Albania (as one-third of the Albanian population resides in Tirana) and serves as a reference center for the entire country. The inclusion criteria for enrolment in the study were: Patients aged 18–80 years diagnosed with RA (according to ACR/EULAR RA classification criteria 2010) (7) for at least 3 months, based on clinical records, taking at least one type of disease-modifying antirheumatic drug (DMARD) during the timeframe of the study. Patients diagnosed with life-threatening disorders and severe mental disorders were excluded from the study.

This is a single-center cross-sectional study in which 144 patients fulfilling the inclusion criteria were invited to participate. One hundred and twenty-five of these patients accepted the invitation at the first stage (response rate 125/144=87%).

The study was based on self-reporting by patients through

a standard questionnaire divided into two parts. The first part consisted of a face-to-face interview including general demographic data, social, economic and clinical history-related factors and was completed by the patients during their stay in hospital with the assistance of two interviewers. Prior to answering the questionnaire, the interviewers explained to each patient the intention of the study and the importance of their contribution, as well as assuring them of total anonymity of the data collected.

The second part aimed to evaluate the adherence to the prescribed therapy and was filled in via a telephone interview 4 weeks after the patient's discharge from the hospital. Out of 125 patients who participated in the study at the first stage, 117 took part in the second part (response rate 117/125=94%). The Morisky Medication Adherence 4-item Scale (MMAS-4) was used as a reference to measure the patients' self-reported adherence to their medications (8).

The questionnaire consisted of the following 4 questions having "yes" or "no" as response: (i) Do you ever forget to take your medicine? (ii) Are you careless at times about taking your medicine? (iii) Do you sometimes stop taking your medicines when you feel better? (iv) Sometimes, if you feel worse when you take your medicine, do you stop taking it?

To each "yes" response is assigned a score of 1 and to each "no" response is assigned a score of 0. The "0 score group" is considered as fully adherent to the medication and the "4 score group" is considered as the worst adherent to the medication. Patients having scores of 1–4 are considered non-adherent.

The questionnaire was further supplemented with items regarding potential affecting factors on adherence, which were classified into three groups: i) social, economic and demographic; ii) clinical history; and iii) neglecting behavior of the patients towards their health.

The collected data were analyzed using statistical program IBM SPSS Statistic – 16.0. Factors affecting adherence were evaluated through the values of χ^2 for categorical variables and p (Student's t -test) for continuous variables.

All patients gave their informed consent to participate in the study, and ethical approval was received.

RESULTS

One hundred and twenty-five patients were included in the study and responded to the

questionnaires at the first stage (of the 144 patients approached, with a response rate of 87%). Demographic, clinical and psychological characteristics of the study population are described in Table I. The average age was 52.04 ± 8.35 years and the ratio female: male was 111:14.

The analysis of demographic data included place of residence (urban/rural), level of education (<8 years; 8-12 years; >12 years), income level (low, medium, high corresponding to: less than the minimal state income, between minimal and maximal state income and more than the maximal state income, respectively), level of information regarding the disease (low, medium, high as reported by the patient's perception about his/her knowledge of the disease). Most of the patients come from rural areas (62.4%), belong to the medium education level (6-12 years of studies), have a low-income level (61.6%) and have little information regarding their disease (52.8%); 96% of the patients reported to have medical insurance.

According to the severity of the disease as described by the physician in the medical record of the patient, 17.6% had mild disease, 53.6% moderate and 28.8% had severe disease. The disease duration was 13.99 ± 10.98 years, and the length of time from the onset of regular drug treatment was 8.47 ± 8.02 years. The difference between the time of the onset of the symptoms and the beginning of the treatment was 5.21 ± 8.2 years.

Comorbidities were common among the participants as 72% reported to suffer from at least one other disease, with the number of diagnoses varying from 0–8 (the average value of 1.7 ± 1.602).

The majority of patients used at least one non-biologic DMARD to treat the disease, with only 2.4% use of biologic DMARDs. Methotrexate was the most prescribed DMARD (69.6%), followed by Leflunomide (57.6%), Hydroxychloroquine (6.4%) and Etanercept (2.4%); 94.4% of the patients used Glucocorticoids, 76% used non-steroidal anti inflammatory drugs (NSAIDs) and 32.8 % used traditional medications (*Urtica Dioica* L).

In order to identify the behavioral characteristics of the patients toward medication, they were asked to respond to questions related to their habits, and

the results show that 88 (70.4%) of them never missed a medical visit, but 39 (31.2%) stopped taking medication after improvement of the medical condition although without consulting their physician.

Four weeks after the patient's dismissal from the hospital, they were interviewed by telephone regarding their adherence to the drug treatment. A total of 117 (93.6%) patients were contacted (response rate $117/125=94\%$) and 6.4% of all patients did not respond to the questionnaire (they were not available to respond or they were no longer living in Albania).

The level of medication adherence was assessed through MMAS-4 based on the responses to the 4 questions of the questionnaire: 30.8% of the patients resulted as fully adherent to the medication and 5.1% were scored as the worst adherent. Overall 69.2% of the patients belonged to the non-adherent group.

The non-adherent patients ($n=81$) were also asked about their reasons for not using their drugs regularly. Three of the most important reasons reported were the cost of the treatment, forgetfulness in taking the drug, and the side effects of treatment, as shown in Table II.

After an extensive analysis of the data on the adherence through χ^2 (*chi-squared* test of Pearson) and p (Student *t*-test), the results are presented in Table III.

Education level and income level are related positively to the medication adherence as patients with more years of studies and those with good economic status tend to be more adherent to the medication regimen ($P=0.045$, $P=0.003$, respectively). Gender, age, place of residence, information about the disease and medical insurance did not show any statistically significant difference between the adherent and non-adherent groups ($P>0.05$).

In the group of factors related to medical history, only the number of comorbidities resulted significantly related to the medication adherence, showing that patients with less diseases are more adherent to the therapy ($P=0.015$).

Patients using biologic drugs and traditional medications tend to be more adherent to medication ($P=0.008$ and $P=0.020$, respectively). There was no

Table I. *Demographic, social, economic, clinical and psychological characteristics of the patients with RA (n=125).*

Characteristics	Values
Gender, female, n (%)	111 (88.8)
Age (years), mean $\pm$ SD	52.04 $\pm$ 8,35
Place of residence, n (%)	
Urban	78 (62.4)
Rural	47 (37.8)
Education level, n (%)	
< 8 years	8 (6.4)
8-12 years	113 (90.4)
>12 years	4 (3.2)
Income level, n (%)	
Low	77 (61.6)
Medium	42 (33.6)
High	6 (4.8)
Information about the disease, n (%)	
Low	66 (52.8)
Medium	40 (32)
High	19 (15.2)
Medical insurance, n (%)	
Yes	120 (96)
No	5 (4)
Severity of the disease, n (%)	
Mild	22 (17.6)
Moderate	67 (53.6)
Severe	36 (28.8)
Disease duration (years), mean $\pm$ SD	13.99 $\pm$ 10.98
Time lapse from the onset of regular drug treatment (years), mean $\pm$ SD	8.47 $\pm$ 8.02
Difference between onset of symptoms and beginning of treatment (years), mean $\pm$ SD	5.21 $\pm$ 8.2
Comorbidities, yes, n (%)	90 (72)
Number of diagnoses, mean $\pm$ SD	1,7 $\pm$ 1,602
DMARD used, non biologic n (%)	122 (97.6)
DMARD used, n (%)	
Methotrexate	87 (69.6)
Leflunomide	72 (57.6)
Hydroxychloroquine	8 (6.4)
Etanercept	3 (2.4)
Use of other drugs, n (%)	
Glucocorticoids	118 (94.4)
NSAIDS	95 (76)
Traditional medications (Urtica Dioica L)	41 (32.8)
Total number of drugs used, mean $\pm$ SD	6.78 $\pm$ 2.07
Do not miss the medical visit, yes, n (%)	88 (70.4)
Stop taking drugs after clinical improvement, yes, n (%)	39 (31.2)

Table II. *Reported reasons for non-adherence in the non-adherent group of patients (n=81).*

<i>Reasons for non - adherence</i>	<i>% of the patients</i>
Cost of treatment	46.9
Forgetfulness in taking the drug	39.5
Side effects of the treatment	34.6
Simultaneous taking of different drugs	17.3
Lack of the faith that the drug would improve the health status	14.8
Lack of the knowledge about the disease	13.6
No available pharmacy near the habitation	12.3
The thought that the drug is expired	9.8
Lack of information by the doctor	7.4
Medical counseling	6.2
Different trademark names for the drug	3.7

Table III. *Associations between several characteristics and medication adherence in patients with RA.*

Characteristics	Chi-Squared	P value
<i>Demographic, social and economic factors</i>		
Place of residence	0.226	0.635
Age ($\leq 50 / > 50$ years)	0.484	0.487
Gender	0.406	0.524
Education level	6.798	0.045*
Income level	11.335	0.003*
Information about the disease (RA)	0.576	0.75
Medical insurance	0.682	0.437
<i>Clinical history related factors</i>		
Severity of the disease	0.689	0.709
Does the patient have accompanying diseases?	0.675	0.411
Comorbidities ($\leq 2 / > 2$)	5.943	0.015*
Disease duration	0.098	0.754
Time lapse from the onset of regular drug treatment ($\leq 5 / > 5$ years)	0.423	0.516
Difference between onset of symptoms and beginning of treatment	0.688	0.407
Number of drugs taken ($\leq 5 / > 5$)	0.044	0.834
<i>Type of drugs used</i>		
Leflunomide	0.078	0.781
Methotrexate	0.974	0.324
Hydroxychloroquine	0.02	0.889
Biologic drugs	6.928	0.008*
Corticosteroids	1.098	0.295
NSAIDs	0.423	0.516
Traditional medications	5.423	0.020*
<i>Patients behavior</i>		
Do not miss the medical visit	94.628	0.001*
Stop taking drugs after clinical improvement	10.597	0.005*

Note: * <0.05 . Adherence measured with the MMAS-4 is expressed as 0 score= full adherence.

significant difference in the type of other drugs used between the two groups of individuals.

Strong association was shown between patient behavior and medication adherence. Patients who reported not missing appointments with their doctors, are the fully-adherent patients ($P=0.001$), while patients who stopped taking their medication after improvement of the clinical conditions, are the most non-adherent ones ($P=0.005$).

DISCUSSION

The study revealed a low level of adherence to DMARD medication of Albanian patients with RA, as compared with previous studies that have reported medication adherence to DMARDs through self-reports of the patients at 50-99% (4). The three most important reasons for the lack of adherence were the cost of the treatment, forgetfulness in taking the drug, and side-effects of the treatment. Data from this study show that RA prevalence was 7.9 times higher in females, a higher prevalence if compared to the 3:1 ratio already reported in the international literature (9). The medication adherence was significantly higher among patients with a higher level of education and higher income level. This finding is in accordance with previous studies showing a positive relationship between education and income level with medication adherence (10). The number of comorbidities is related negatively to the level of adherence in this study, probably due to the fact that less comorbidities are related to less drugs taken, which seems also to be a factor significantly related to the medication adherence (11), but surprisingly in our study this relationship was not demonstrated. The positive relationship between the use of biologic drugs and medication adherence is a finding of this study, in accordance with previous similar studies (12). Interestingly, we also found a positive relationship between the use of traditional medications and medication adherence.

Our findings should be interpreted with caution in light of the fact that this study aimed to evaluate patients' adherence through self-reporting of the patients, which tends to overestimate the adherence. Another issue is the fact that the sample of patients

studied belongs to hospitalized patients, showing more severe disease as compared with the outpatient population with RA.

These findings emphasize the need for intervention of the health authorities to improve the access to medicines for RA, through inclusion of more DMARDs in the national reimbursement list. Health professionals have a crucial role to inform and educate patients in order to support them in improving their adherence.

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LETTER TO THE EDITOR

EFFECT OF GLUCAGON-LIKE PEPTIDE-1 ON AUTOPHAGY OF ISLET β CELLS IN TYPE 2 DIABETIC MICE WITH HIGH FAT DIET

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The aim of this study was to investigate the effect of glucagon-like peptide-1 (GLP-1) on islet β -cell autophagy in mice with type 2 diabetes induced by high-fat diet and low-dose streptozotocin (STZ). Twenty-four 6-week-old male C57BL/6J mice were divided into four groups: group A (control group; 8-week low-fat diet and 8-week normal saline treatment), group B (8-week high fat diet and streptozotocin intervention as well as 8-week normal saline treatment), group C (8-week high fat diet and streptozotocin intervention as well as 2-week GLP-1 intervention), and D (8-week high fat diet and streptozotocin intervention as well as 8-week GLP-1 intervention), with six mice in each group. At the end of 16-weeks, the mice were sacrificed, and blood and tissues were collected. The pathophysiological and morphological changes were evaluated by H&E staining. Immunohistochemical staining of proliferating cell nuclear antigen (PCNA) was performed. Autophagy in islet cells was accessed by immunofluorescence staining of LC3 and P62. Glucose tolerance test (GTT) showed that blood glucose was lower in group C than in group B at 60 min after fasting and glucose load ($P < 0.05$). Insulin tolerance test (ITT) showed that blood glucose in group C was lower than that in group B at 30 min, 60 min, and 120 min, respectively ($P < 0.05$). H&E staining showed that the hepatic steatosis, necrosis and inflammatory cell infiltration were significantly reduced in group D compared with group A, B and C ($P < 0.05$); mice in group B had the highest degree of islet morphological changes, cell necrosis, and inflammatory cell infiltration among the four groups ($P < 0.05$). The level of LC3 was higher in group D than in group B ($P < 0.05$). The P62 level was reduced in group D compared with that in group B ($P < 0.05$). In conclusion, GLP-1 intervention can change the proliferation and autophagy status of islet β cells in mice, and thus protect the function of islet β cells.

To the Editor,

In recent years, diabetes has been listed as one of the top ten causes of death in the world by the World Health Organization (WHO) (1-4). According to WHO data, patients with type 2 diabetes will reach 5.52 million by 2030 (4). The pathogenesis of type 2 diabetes is associated with decreased number and impaired function of islet beta cells (5, 6). At present, effective hypoglycemic drugs are mostly directed at the general symptoms with some side

effects, including weight gain, nausea, diarrhea and hypoglycemia (7). Therefore, exploration of new therapeutic approaches for type 2 diabetes remains a major task for researchers.

As a new type of hypoglycemic drug, glucagon-like peptide-1 (GLP-1) can combat diabetes through multiple mechanisms, such as promoting insulin synthesis and secretion, promoting islet β cell proliferation, and inhibiting islet β cell apoptosis (8, 9). Dyachok et al. (10) revealed that GLP-1 promoted

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insulin secretion by regulating intracellular Ca^{2+} concentration and protein kinase A (PKA) signaling pathways. However, as a natural peptide hormone, GLP-1 has a half-life of only 1 to 2 minutes, so it is not a practical agent for clinical use. As an analog of GLP-1, liraglutide overcomes the short half-life of GLP-1, therefore it is widely used in clinical practice. In this study, the effect of liraglutide on alterations in pancreatic islet β -cell proliferation and autophagy status was evaluated in a high-fat type 2 glycemic model. The study provides an experimental basis for elucidating the role of GLP-1 in maintaining islet β cells activity and improving its hypoglycemic function.

MATERIALS AND METHODS

Animals

Thirty healthy 6-week old male specific-pathogen free (SPF) C57BL/6J mice with an average body weight of 20 ± 2 g (Beijing HFK Bioscience Co. Ltd., Beijing, China) were selected in this study. All mice were housed with a 12-h light/dark cycle, and were allowed free access to food and water. The temperature was controlled at $22 \pm 2^\circ\text{C}$ and 40-60% humidity. The experimental mice were allowed 1 week to acclimatize prior to intervention. Animal care and experimental procedures were in accordance with Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the ethics committees of Zhuji People's Hospital, China.

Experimental methods

Group A (normal control): six mice were fed with low-fat diet for 8 weeks (10% fat, 20% protein and 70% carbohydrate) and administrated with normal saline intervention for 8 weeks (0.2 mg/Kg, once daily, hypodermic injection).

The remaining 24 mice were fed with high-fat (60% fat, 20% protein and 20% carbohydrate) diet for 8 weeks followed by streptozotocin (STZ) (50 mg/Kg, once per day by intraperitoneal injection) treatment for 8 weeks. Eighteen mice which had blood glucose levels > 16.7 mmol/L after 1-week intervention were chosen and were further divided into three groups (6 mice in each group):

-Group B (high fat diet for 8 weeks + STZ intervention

for 8 weeks + normal saline intervention for 8 weeks);

-Group C (high fat diet for 8 weeks + STZ intervention for 8 weeks + GLP-1 intervention for 2 weeks);

-Group D (high fat diet for 8 weeks + STZ intervention for 8 weeks + GLP-1 intervention for 6 weeks).

Streptozotocin (STZ) was provided by Beijing Solarbio Technology Co., Ltd., China. Liraglutide (GLP-1) (Novo Nord Pharmaceutical Co., Ltd., China): 0.2 mg/Kg, once daily, hypodermic injection. Normal saline: 0.2 mg/Kg, once daily, hypodermic injection. Body weight and food intake were measured daily by electronic scales (Type BSA224S, Sartorius, Germany). The total time for molding and experiment was 16 weeks.

Glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed at the 7th, 11th, and 15th week of the experiment. GTT: mice were given a bolus of glucose (1.0 g/Kg) by intraperitoneal injection after 12-hour fasting. Blood was collected from the tail vein at 0, 15, 30, 60, and 120 min after glucose injection, respectively, blood glucose levels were measured with a Roche blood glucose meter and supporting test strips (Roche, America), and the area under the curve (AUC) was calculated. ITT: mice were given a bolus of insulin (0.25 IU/Kg) by intraperitoneal injection after 2-hour fasting. Blood was collected from the tail vein at 0, 15, 30, 60, and 120 min after insulin injection, respectively; blood glucose levels were measured with a Roche blood glucose meter and supporting test strips, and the area under the curve (AUC) was calculated.

At the end of the experiment, all mice were anesthetized by intraperitoneal injection of 4% chloral hydrate (10 $\mu\text{L/g}$ body weight) (Beijing Huagongchang, China) and blood was collected by eye bleeding. Mice were sacrificed by cervical dislocation, and liver and pancreatic tissues were collected. All tissues were cut into three parts and prepared separately: i) the tissues were fixed in 4% formaldehyde (Beijing Huagongchang, China), and paraffin-embedded for subsequent morphological analysis; ii) tissues were embedded in OCT (Opti-mum cutting temperature) and sliced with a rapid freezing slicer (Thermo Fisher Scientific, America) for immunohistochemistry analysis; iii) tissues were quickly frozen in liquid nitrogen for relevant protein detection.

Liver and pancreatic tissues were embedded with paraffin and the tissue blocks were sliced into 4- μm thick sections. The sliced sections were placed on slides, baked

at 65°C for 10 min, deparaffinized with xylene (10 min, twice) and rehydrated in the gradient alcohol (100%, 95%, 85%, 80% and 70% ethanol) for 2 min at each concentration. H&E (hematoxylin & eosin) staining was performed, and the results were observed under an optical microscope (Olympus, Japan).

Statistical methods

All data are presented as mean $\pm$ standard deviation and were processed with statistical software SPSS 20.0. Data between two groups were analyzed by *t*-test. The data among multiple groups were compared by one-way ANOVA. Fisher's LSD method was used for the comparison between two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Changes in body weight of mice in the high-fat diet group

After high fat feeding, the mice showed an average body weight gain of 2.07g per week. However, the mice receiving STZ treatment showed a significant decrease in body weight. Before GLP-1 administration, the average body weight of mice in

groups B, C and D was 31.71 g and no significant difference among the three groups was observed ($P > 0.05$). Two weeks after GLP-1 injection, the mice in group C and group D had a significantly lower body weight than that of group B (25.61 g and 27.20 g vs 28.76 g, respectively). No significant difference was observed between group B and group D after 6 weeks of GLP-1 injection (28.64 g vs 28.40 g respectively).

Results of GTT and ITT in mice of the high-fat diet groups (group B, C and D)

GTT results (Fig. 1, A and B) showed that there was no significant difference in blood glucose among the three groups at baseline ($P > 0.05$). After 2 weeks of GLP-1 intervention, blood glucose in groups C and D was lower than that in group B at 60 min ($P < 0.05$). The results of ITT (Fig. 1, C, D, E) showed that there was no significant difference in blood glucose between the three groups at baseline ($P > 0.05$). After 2 weeks of GLP-1 intervention, the fasting blood glucose of group C was significantly lower than that of group B at 30 min, 60 min, and 120 min, respectively ($P < 0.05$). After 6 weeks of intervention, the blood glucose levels in group D were

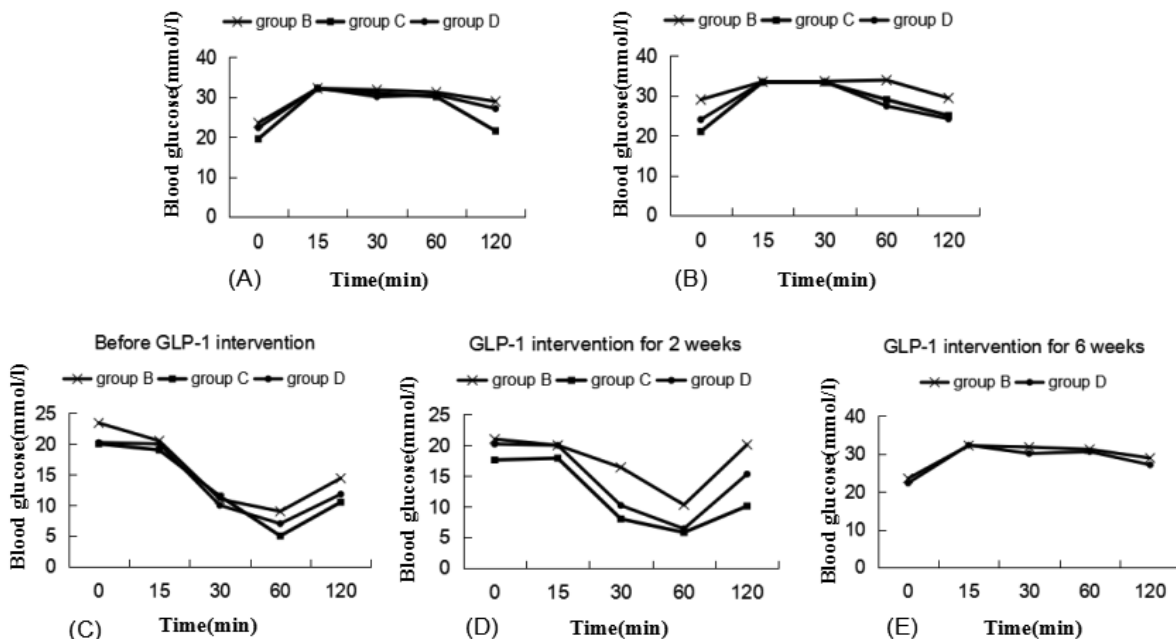


Fig. 1. Results of glucose tolerance and insulin tolerance test before and after GLP-1 intervention.

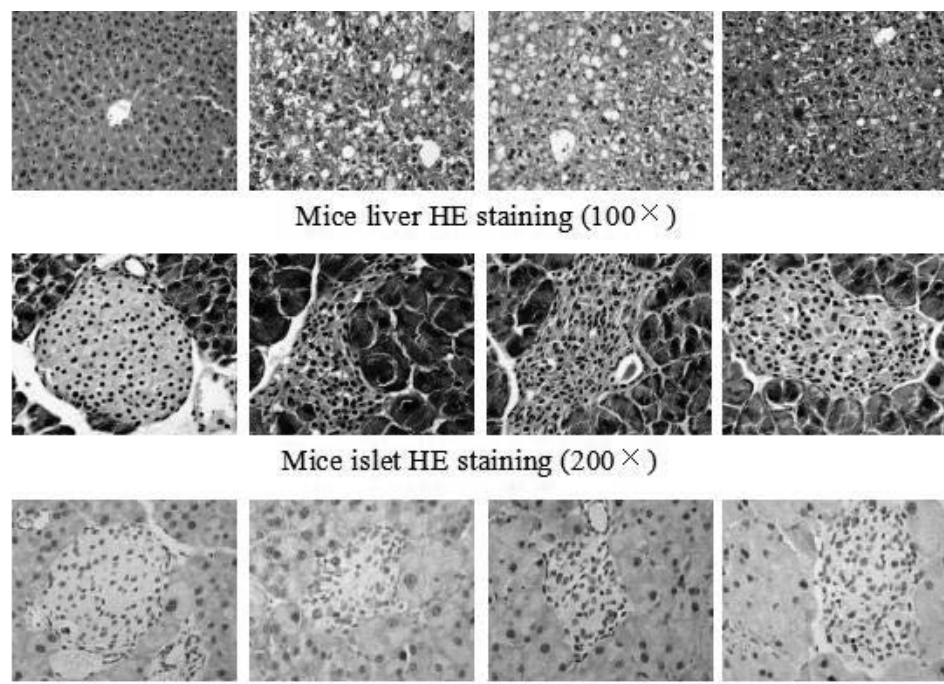


Fig. 2. Results of H&E staining in liver and pancreas of experimental mice and the expression of islet PCNA detected by immunohistochemistry.

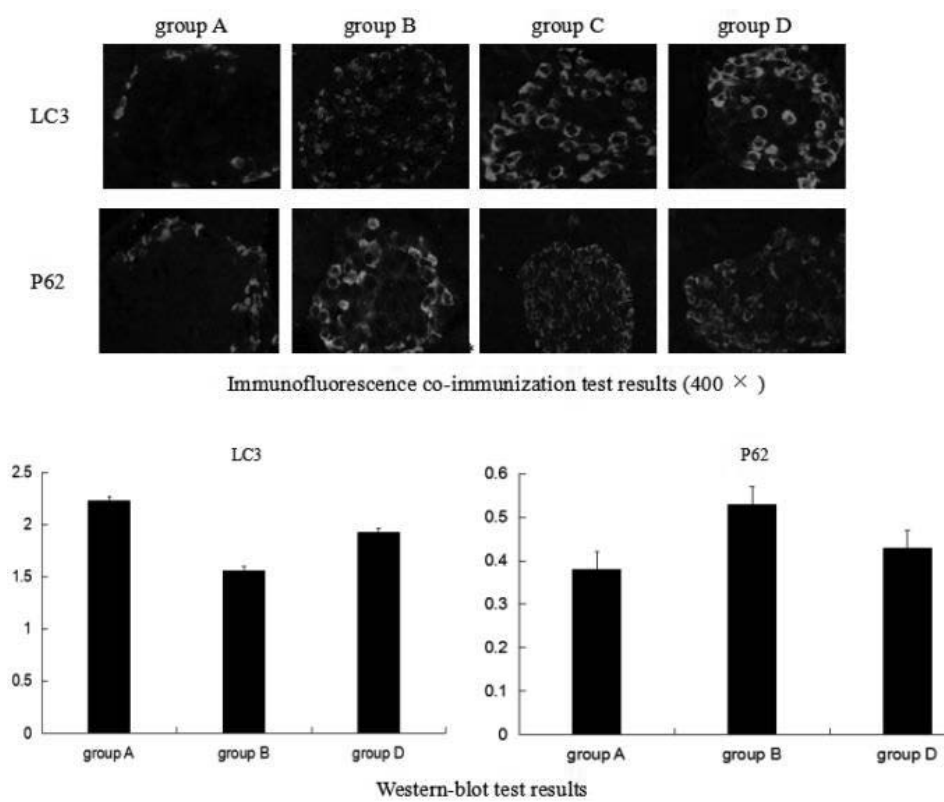


Fig. 3. LC3 and P62 expressions in islets.

lower than those in group B, but the difference was not statistically significant ($P > 0.05$). These results indicate that short-term (2 weeks) administration of GLP-1 could improve the islet function, but the benefit may not be sustained after an extended treatment (6 weeks) in the mouse model.

HE staining of liver and pancreas

The results of liver staining showed that the liver cells of group A were marshalling with no lipid droplets and few inflammatory cell infiltrations; the liver cells of group B showed diffuse steatosis, increased cell volume, and more fat vacuole formation than group A, with increased inflammatory cell infiltration and hepatocyte necrosis. Compared with group B, liver cells in group C showed less diffuse steatosis of hepatocytes, lipid droplet vacuoles in cytoplasm and inflammatory cell infiltration. Compared with groups B and C, group D showed less fatty degeneration and inflammatory cell infiltration (Fig. 2). The pancreatic staining results showed that the pancreatic islets in group A were of clumpy morphology without fissure between cells, the cells were round, and the islet adenoid cells were clearly arranged. The islets of groups B, C and D showed various morphological changes, including irregular islet edge, inflammatory cell infiltration and islet cell necrosis. The islets of group B had the most significant morphological alterations (Fig. 2).

The islet value

The results of immunohistochemistry showed that a small number of PCNA-positive cells were scattered in the islets of the four groups, and groups A and D had slightly higher expressions than those of groups B and C (Fig. 2).

Changes of the islet autophagy markers

Immunofluorescence staining revealed that the P62 expression in pancreatic islets was significantly higher in group B than in group A ($P < 0.05$); after GLP-1 administration, P62 in group C and D decreased to different degree, with significant difference between group D and group C ($P < 0.05$) (Fig. 3). Western blot results showed a significantly lower expression of LC3II/I protein in islets of group

B than that in group A ($P < 0.05$); after administration of GLP-1, the islet LC3II/I expression in group D was significantly higher than that in group B ($P < 0.05$), indicating that autophagy was activated to a certain extent after administration of GLP-1 (Fig. 3). These results indicated that autophagy might be activated after administration of GLP-1.

DISCUSSION

The type 2 diabetes model used in this study was established by a high-fat diet and repeated injections of small doses STZ according to the previous report (10). After 2 weeks of GLP-1 intervention, fasting blood glucose levels and blood glucose levels at 60 min in group C were lower than those in group B ($P < 0.05$), suggesting that GLP-1 could decrease blood glucose levels in mice. The results of ITT showed that after GLP-1 intervention, the blood glucose level of group C was lower than that of group B at 30 min, 60 min and 120 min, respectively ($P < 0.05$). The blood glucose level of group D was lower than that of group B ($P < 0.05$). These findings suggested that GLP-1 can improve the function of islets to a certain extent.

HE staining results showed that the islets and liver had a certain degree of steatosis, inflammatory cell infiltration and cell necrosis in the type 2 diabetes mouse model. This may be attributed to the high-fat feeding and STZ treatment (11). Under the high glucose state, the expression of PCNA was slightly increased after GLP-1 intervention, indicating GLP-1 might promote islet cell proliferation. Under the high glucose state, after administration of GLP-1, the expression of LC3 increased and the expression of P62 decreased, indicating that GLP-1 can activate autophagy under high glucose state. In fact, GLP-1 not only promotes the synthesis and secretion of insulin, but also promotes the proliferation of islet cells and inhibits their apoptosis. During this process, autophagy was also activated, which may contribute to the therapeutic effect of GLP-1 on diabetes (12).

In summary, GLP-1 can change the proliferation and autophagy status of mouse islet β cells, thus protecting the function of islet β cells. These results provide an experimental basis for clinical prevention and treatment of islet β cell apoptosis and diabetes.

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LETTER TO THE EDITOR

**GALACTOMANNAN TESTING IN THE TREATMENT OF AUTOIMMUNE DISEASE
COMBINED WITH INVASIVE FUNGAL DISEASE**PC. LI¹, XS. YANG² and WY. LI²

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Invasive fungal disease (IFV) has a high incidence among autoimmune disease patients who have poor immunity. Diagnosis of IFD is difficult in the early stage. Conventional imaging technologies are poor in sensitivity, specificity and detection rate, therefore, an effective method is needed for improving the diagnosis of autoimmune disease complicated with IFD. To study the benefits of galactomannan testing in the diagnosis of IFD, 256 patients who were diagnosed with autoimmune disease in our hospital between December 2014 and December 2016, and who developed pulmonary infection in the process of immunosuppressant treatment, were selected. They were classified into 2 groups: autoimmune disease patients with IFD and those with non-IFD, based on the diagnostic criteria and treatment principles of invasive pulmonary fungal infection. Among the patients with autoimmune disease in combination with IFD, there were 12 confirmed cases, 24 clinically diagnosed cases and 49 suspected cases. The 36 confirmed and clinically diagnosed cases were used as the observation group, while 171 patients with autoimmune disease in combination with non-IFD were placed in the control group. Galactomannan testing was performed on both groups. The results demonstrated that the level of galactomannan in the patients in the observation group were significantly higher than those in the control group ($P<0.05$). Receiver operating characteristic (ROC) curves revealed that galactomannan testing had high diagnostic value for autoimmune disease combined with IFD. The area under ROC curve was the largest when galactomannan value higher than 0.7 was taken as the positive breakpoint, and the sensitivity and specificity at this critical value were 87.1 and 92.6%, respectively. Therefore it can be concluded that galactomannan testing has important application values in the diagnosis of autoimmune disease in combination with IFD.

To the Editor,

Autoimmune disease refers to tissue and organ dysfunction induced by immune response produced by the immune system due to environmental or genetic factors (1). Studies on the pathogenesis of immune diseases have resulted in improvements in the quality of life and survival period of patients. However,

complications may appear during the process of treatment, among which infection is the most common (2). Infection is one of the main causes of death from systemic lupus erythematosus, rheumatoid arthritis and polymyositis/dermatomyositis (3). Pulmonary infection ranks first among the different kinds of infections (4). Invasive fungal disease (IFD) tends

Key words: galactomannan; autoimmune disease; invasive fungal disease

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to have a higher incidence; it is the major cause of death in patients with immunosuppression, and severely affects the quality of life and prognosis of patients with autoimmune disease (5).

The fatality rate of patients with IFD who are not timely treated is between 0.3 and 0.8% (6). Therefore, it is of great significance to make early diagnosis of autoimmune disease combined with IFD. Traditional diagnostic methods such as histopathology, imaging, fungi culture and microscopic examination do not easily achieve early diagnosis and treatment of IFD due to the defects of invasiveness, long duration and poor sensitivities. In recent years, galactomannan testing has been widely applied because of its non-invasiveness, high sensitivity and high specificity, and has been one of the early diagnostic criteria for fungal infection. Several studies have been carried out on the values of galactomannan testing in diagnosing hematological malignancies and transplant operation-induced IFD (7). However, studies on its values in diagnosing autoimmune disease in combination with IFD are limited. The present study was carried out to investigate the clinical values of galactomannan testing in the early diagnosis of autoimmune disease in combination with IFD.

MATERIALS AND METHODS

Research subjects

A total of 256 patients (98 males and 158 females) admitted to the Department of Rheumatology and Immunology and Department of Respiration and Intensive Care Unit between December 2014 and December 2016, who were diagnosed with autoimmune disease, and who developed pulmonary infection after immunosuppressive treatment, were selected. The patients received immunosuppressive treatment, developed pulmonary infection during the treatment, and satisfied the diagnostic criteria of pulmonary infection described in the Guidelines for the Diagnosis and Treatment of Community-acquired Pneumonia formulated by Respiratory Branch of Chinese Medical Association (8). They were divided into 85 cases of autoimmune disease in combination with IFD (9) comprising 12 confirmed cases, 24 clinically diagnosed cases and 49 suspected cases; and 171 cases of autoimmune disease combined with non-

IFD. The 12 confirmed and 24 clinically diagnosed cases were placed in the observation group, while the 171 cases of autoimmune disease in combination with non-IFD served as the controls. This study was approved by the ethics committee of our hospital, and the included patients signed informed consent.

Galactomannan testing was carried out on all the patients in the two groups. Peripheral blood (2 mL) was collected from each patient using a drying tube, and centrifuged at 3000 rpm for 5 min. Serum samples, standard serum and positive and negative controls were taken. Platelia Aspergillus double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kits (Bio-Rad Laboratories, USA) were used in line with the manufacturer's instructions. The serum samples were first fully mixed with galactomannan antigen. After incubation, the mixture was added to an ELISA plate containing galactomannan antigen, followed by incubation and washing. Then, enzyme-labelled antibody was added. After incubating and washing three times, 3, 3', 5, 5'-tetramethyl benzidine substrate was added to induce chromogenic reaction. The absorbance value (A) of each well was read in a microplate reader. Indices (I) were calculated using the standard serum threshold provided by the kit and conversion formula (absorbance index = A of the detected serum/A of the standard serum). The detection result was determined as negative if the value of galactomannan was lower than 0.5, and as positive if the value of galactomannan was higher than 0.5.

Statistical analysis

Measurement data are expressed as mean $\pm$ standard deviation (SD), and comparison between groups was performed using *t*-test. Comparison between enumeration data was performed using *chi*-squared test. Since the values of galactomannan in the serum of patients in the two groups were abnormally distributed, they were expressed as median (interquartile range) and processed by Mann-Whitney test. Data were analyzed using SPSS ver. 21.0. Differences were considered statistically significant at $P < 0.05$.

Receivers operating characteristic (ROC) curves were drawn to determine the optimal threshold, sensitivity, specificity, positive predictive value and negative predictive value of galactomannan testing in the diagnosis of autoimmune disease combined with IFD.

RESULTS

Comparison of general data between the two groups

The differences between the observation and control groups in gender, age and basic diseases were not statistically significant ($P>0.05$, Table I).

Comparison of the galactomannan test results between the two groups

The percentage of serum galactomannan in the observation group was significantly higher than

that of the control group [86.1% (31/36) vs 5.8% (10/171)], and the level of galactomannan in the observation group was significantly higher than that of the control group (0.41 $\mu\text{g/L}$ vs 0.11 $\mu\text{g/L}$) ($P<0.05$).

Analysis of ROC curves

The ROC curves of the galactomannan test results for the observation group were analyzed. The area under ROC was maximum (0.952) when galactomannan value higher than 0.7 was regarded

Table I. Comparison of baseline data between the two groups.

		Observation group	Control group
General data	Age (years) *	51.4 $\pm$ 17.3	51.1 $\pm$ 15.6
	Gender (male/female) *	14/22	67/104
Basic diseases (n) *	Rheumatoid arthritis	7	43
	Dermatomyositis	5	16
	Takayasu's arteritis	1	3
	Sjogren's syndrome	5	19
	Systemic lupus erythematosus	10	54
	Ankylosing spondylitis	0	4
	Mixed connective tissue disease	3	14
	Behcet's disease	0	2
	Microscopic polyangiitis	4	6
	Systemic sclerosis	1	10

Note: * indicated that the difference between the two groups had no statistical significance ($P>0.05$).

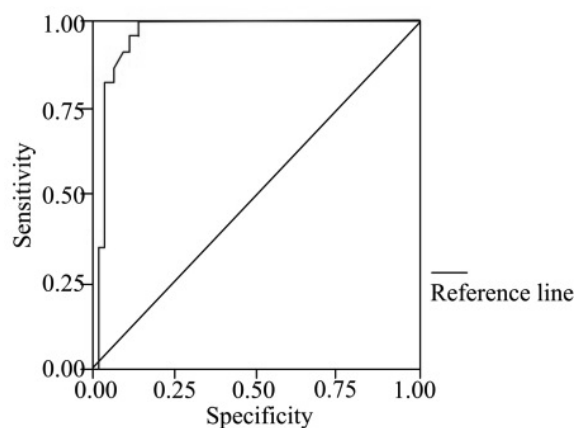


Fig. 1. ROC curve of the observation group.

as the positive breakpoint. The corresponding sensitivity and specificity were 87.1 and 92.6%, respectively.

DISCUSSION

Immunosuppressant treatment in patients with autoimmune disease can further aggravate the immunity impairment and significantly increase the risk of IFD (10). Delayed diagnosis and treatment of IFD may increase the risk of mortality. Therefore, clinical studies have focused great attention on early, rapid and accurate diagnosis and treatment of IFD. The galactomannan test relies on (1-3)- β -D glucan, one of the components of fungal cell walls. The sensitivity of galactomannan testing is not high under the effects of multiple factors, however, it can be used for early detection and rapid diagnosis of IFD. It can determine the categories of bacteria in the body of patients and, moreover, effectively enhance the detection rate of IFD.

Currently, the threshold of serum galactomannan in the diagnosis of IFV is controversial. Unified determination for break point has brought many problems. For example, the universal positive threshold of GM is higher than 0.5 in America (11), and higher than 1.0 or 1.5 in Europe. There are no relevant research data on the universal positive threshold in China. European and American experts have gradually agreed to define the break point of galactomannan as higher than 0.8 in one test or 0.5 in two tests, based on a substantial amount of clinical data (12). However, more evidence-based medicine research is needed to verify the reliability of the diagnosis. In this study, the ROC curve of the galactomannan test result was analyzed. The area under ROC was maximum when galactomannan value higher than 0.7 was taken as the threshold, and the positive and negative predictive values were 87.1 and 92.6%, respectively. These results are similar to the results obtained in another study (12). The results indicate that galactomannan testing has a high specificity in the diagnosis of autoimmune disease combined with IFD. Therefore galactomannan testing in combination with other diagnostic methods is beneficial for early diagnosis and timely treatment of autoimmune disease complicated with IFD.

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INSIGHT INTO “NUCLEAR-CYTOPLASMIC SHUTTLING” AS A DEVELOPMENTAL AND DIFFERENTIATIONAL CAPABILITY OF CELLS IN PRIMARY CULTURE MODELS

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Shuttling proteins are molecules that can facilitate transport through the nuclear envelope. A very large number of proteins are involved in this process that includes nuclear pore buildup, signal, receptor and enzyme proteins. There are many examples of proteins whose biological activity depends on nucleocytoplasmic transport. Very often they are largely responsible for the proper occurrence of cell division, maturation, development and differentiation. Thanks to the well mastered methods of *in vitro* cell culture, it is possible to trace the levels of protein expression and their distribution in cells. Advanced molecular techniques allow for precise determination of their displacement in time. Several studies are still being carried out, using primary cultures, to identify the factors that determine the maturation, development and differentiation of cells. In understanding of the detailed mechanisms controlling cell life, the key is not the level of expression of a specific protein, but its distribution in individual cellular compartments.

To the Editor,

The *in vitro* culture (IVC) systems are composed of primary cell culture and/or cell line models. Since the cell line systems are based on fully differentiated cells that are capable of displaying specific functions, the primary cell culture models also include cultivation of immature cells that have just achieved their developmental competence. The developmental competence of cells cultured in primary models is often characterized by a huge potency for growth. Moreover, these cells differentiate and/or mature

in vitro into several, sometimes distinct cell types with different functions from those of the primary cell. Our recent study on porcine model indicated a huge potency of ovarian granulosa cells and cumulus cells for real-time proliferation during short- and long-term cultivation (1). Since porcine GCs may change their morphological properties, as well as biochemical/metabolomic profile during proliferation *in vitro*, it is suggested that they may also differentiate into several other cell types during long-term culture. Recently, one of the biochemical

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changes identified in our research, during primary cultivation of porcine and human GCs, as well as epithelial cells, considered nuclear-cytoplasmic shuttling of proteins (2). These proteins include gap junction connection (GJC) molecules such as connexins (Cxs) and cyclin-dependent kinases, such as Cdk4, which are both recognized as markers of cellular metabolomic status, maturation capability, developmental competence and cell cycle control. The nuclear-cytoplasmic shuttling seems to be a compound process, which may be also defined as a specific “checkpoint” of cell differentiation. However, little data indicating association of this process with changes of cells’ capability for differentiation during growth in primary culture models is available. In this review article we present recent data related to cellular differentiation capability with special attention to nuclear-cytoplasmic shuttling. Numerous discoveries of proteins, which are constantly moving between cell compartments, are of great importance for progress in the development of new drugs.

“Nuclear-cytoplasmic shuttling” – main biochemical mechanisms

The division of cellular processes into those taking place in the nucleus and those taking place in the cytoplasm requires the transport of proteins through the nuclear envelope. The processes of importing and exporting substances between the nucleus and cytoplasm require the involvement of many proteins, and the recent research has shown that these particles are in continuous transport. Such shuttling proteins are of great importance for cell function and signal transmission (3). A significant role in nuclear-cytoplasmic shuttling is played by special channels in the nuclear membrane, called the nuclear pore complexes (NPCs). The nuclear pore complexes (NPCs) are built into the nuclear envelope and are designed to regulate the exchange of components in nuclear-cytoplasmic shuttling (4). The double-layer nuclear membrane contains many pores through which the nucleus communicates with the cytoplasm. These pores (NPCs) consists of multiple copies of 30 different proteins (named nucleoporins – NUPs) and are built into the membrane, being composed of three main elements.

The nuclear basket (about 100 nm length), which branches form the nuclear ring is located on the inner side of the nuclear membrane. On the outer side of the nuclear envelope there is a cytoplasmic ring with branching cytoplasmic fibrils (approximately 50 nm long). A central pore is contained between these two rings (5). Small molecules, that have a mass smaller than 40 kDa, usually diffuse across NPCs. These pores must have open aqueous channels with a diameter of approximately 9 nm, so that smaller particles can diffuse freely through the membrane. The transport of larger particles through the pores, on the other hand, requires an energy input. Typically, each protein has a nuclear localization signal (NLS) and a nuclear export signal (NES). There are many types of receptors that recognize different sequences. Sometimes the same amino acid sequence can support both import and export (6). The best-known NLSs are divided into classical (including monopartite and bipartite) and non-classical. Typically, NES sequences are rich in leucine. Export through leucine-rich complexes requires energy and has a saturation effect. Hence, protein export to the cytoplasm, in this case, takes place by the means of active transport (5). The transport proteins of the karyopherin family are responsible for the recognition and binding of these signal molecules to the cargo proteins and their transport through the nuclear membrane, being named nuclear transport receptors (NTRs). These protein receptors carry out active transport, mainly of proteins. In most cases, the whole process is based on the cooperation of the NTR and NPC and regulation through Ran GTPase. The NTRs comprise two families: karyopherin- α (importin α) and karyopherin- β (7).

Protein translocation as a basis for intracellular processes

The presence of nuclear membrane ensures control of gene transcription processes and is responsible for regulating the processes of maturation, differentiation and development of the cell. Therefore, some transcriptional activators are stored in the cytoplasm and some inhibitors are stored in the nucleus. Only their import or export from the nucleus, which is initiated by the appropriate

signals, starts the transcription mechanisms. Thus, the nuclear-cytoplasmic shuttling can be understood as one of the key mechanisms controlling the cellular processes. Many proteins have been recognized as nucleocytoplasmic shuttling proteins, such as: proteins involving transcription factors, cell proliferation regulators, translation initiation factors, hormone receptors, and RNA binding proteins (3, 6). This process, called nuclear-cytoplasmic shuttling, is aimed not only at carrying the cargo between the nucleus and the cytoplasm but also plays an important role in conveying information in the two most important compartments of the cell (6). The biological activity of some proteins is regulated by nuclear-cytoplasmic shuttling. These molecules are in constant motion, although at rest they accumulate mainly in the selected region, depending on the balance between imports and exports.

The presence of a nuclear membrane and the controlled passage of molecules through the relevant membrane complexes have an extremely significant effect on normal cellular divisions. An example is the export of protein phosphatase enzyme (Cdc25) to the cytoplasm, which prevents them from binding to the mitosis activators (cyclin-dependent kinases Cdc2), remaining in the nucleus, when the damaged DNA fragment needs to be repaired. In this way it is impossible for a cell with a damaged genome to start dividing (8). In contrast, apoptosis processes can also be controlled by nucleocytoplasmic transport. p53 protein is a transcriptional factor acting in the cell nucleus. Its role is to prevent the growth of damaged cells through various mechanisms, e. g. through apoptosis of this cell. In well-functioning cells there is a need to stop the p53 protein activity by exporting it from the nucleus to the cytoplasm by combining with NES (6). Not only the physiological cellular processes are controlled by protein translocations, but also stress factors can cause the changes in transmission of individual molecules. Recent reports also indicate a significant impact of heat stress on the blocking of HSPA8 nuclear exports, which significantly changes cell survival (9). HSPA8 protects cells during inflammation, which results from the production of reactive oxygen species by mitochondria. At the end of stress, this protein

is released into the cytoplasm under physiological conditions. The lack of export of this protein to the cytoplasm changes the ability of cells to survive after another heat stress. There are many examples in the available literature, that show that many proteins, including those that are enzymes (e.g. kinases), can circulate between the nucleus and cytoplasm. A necessary condition is the presence of signals from the nucleus, based on gradients, and the presence of appropriate NLS or NES. The above examples show the range of nuclear-cytoplasmic shuttling. They also indicate its great importance in the key life processes of the cell and, consequently, of the whole organism.

Nuclear-cytoplasmic shuttling in in vitro cell cultures as a path to biomedicine

Cell cultures, both primary and non-primary, offer great opportunities for research into the reactions of cells to various environmental factors. Thanks to this, we are able to find out which nuclear mechanisms are activated in the event of adverse conditions. For example, recent studies have shown that human bronchial epithelial cells (primary HBEC and BEAS-2B HBEC) have shown dynamic shuttling of SIRT1 when exposed to increased concentration of cigarette smoke extract (10). SIRT1 is an important cellular survivor protein under oxidative stress. It has been proven that it can shuttle between the nucleus and cytoplasm. However, with repeated decreased concentrations of cigarette smoke, shuttling was significantly smaller, which may indicate reduced antioxidant capacity in chronic pulmonary diseases caused by smoking. Research has shown that during myocyte cultivation in cultures, nuclear-cytoplasmic shuttling was important for differentiation. Shuttled HDAC5 histone deacetylase form the nucleus to the cytoplasm, while myoblasts were run to differentiate (11). During cell differentiation, calcium signals on the cell membrane activate protein kinases that connect phosphate groups to HDACs. These enzymes then dissociate from myocyte enhancer factor-2 (MEF2), revealing their NES. This leads to the export of nuclear deacetylase. As a result, MEF2 is free to activate the expression of muscular-specific genes and chromatin is less condensed. The obtained results show that HDAC nucleocytoplasmic

transport is involved in the processes of controlling the mechanisms of cell differentiation (11). In our study, the proliferation index of cumulus cells (CCs) was analyzed, in relation to expression and distribution of cyclin-dependent kinase 4 (Cdk4) and connexin 43 (Cx43), which are factors affecting oocyte maturation (2). We have shown that Cdk4 and Cx43 proteins, expression of which in oocytes was described in literature earlier, are expressed in pig CCs. This may prove that these proteins play a role in the maturation of not only oocytes, but also have a significant influence on the differentiation of the cumulus cells. Furthermore, after IVC, a significant transfer of both tested proteins from the nucleus to the cytoplasm was observed. The shuttling of these proteins from the nucleus to the cytoplasm may be associated with the CC expansion process (morphological changes during maturation of COCs) (2). Interesting results can be found in the work of Göttle et al., who studied the regenerative abilities and differentiation of oligodendroglia, which are important for the autoimmune nervous system diseases, such as multiple sclerosis (MS) (12). Physiological repair processes often fail, mainly through blocking the differentiation and maturation of the oligodendroglia. It has been shown that p57kip2 gene is a strong inhibitor of cell differentiation, for both the peripheral and central nervous systems. Although the way in which p57kip2 disrupts myelin formation is unknown, it is likely to regulate the fate of adult neural stem cells. Export of p57kip2 from the nucleus resulted in increased *in vitro* myelination. In turn, the accumulation of this protein in the nucleus caused the differentiation to be stopped. The Authors stated that the displacement of p57kip2 from the nucleus to the cytoplasm is crucial. Researchers have shown that the subcellular distribution of p57kip2 changes with the differentiation of oligodendroglial cells in rats, mice and humans, both *in vitro* and *in vivo*. Discoveries such as the fact that nuclear transport errors could be the basis for the development of cancer should provide a foundation for research into cancer treatment possibilities. Existing anti-neoplastic processes in the cell are avoided by cancerous cells thanks to the use of nuclear-cytoplasmic shuttling

processes. Turner et al. studied the nuclear export process, with particular attention to the export of CRM-1 receptor molecule and its relationship to leucine-rich nuclear export signals (5). They described the therapeutic ability of several CRM-1 inhibitors, e.g. leptomycin B, and their ability to reverse drug resistance by modifying nuclear export. In summary, blocking CRM-1 mediated transport from the nucleus may restore apoptotic pathways and cell sensitivity to chemotherapeutic drugs. If less toxic CRM-1 inhibitors are discovered, they could be used as individual drugs or in combination with chemotherapeutic agents.

All cellular processes depend on appropriate biomechanisms, which can only take place under proper conditions, such as concentration gradient or presence of relevant signaling molecules. The discovery of the transport of proteins through the nuclear envelope has created an enormous potential for research on cell metabolism and the errors leading to pathologies. It turns out that the key to the discovery of pathological mechanisms of many diseases is not only to determine the level of protein expression, but also to establish their subcellular localization. Thanks to the discoveries of recent years, the door has been opened for further research on cell metabolism, maturation and differentiation processes, as well as on the search for new drugs. Many diseases, especially cancer, viral and neurological diseases, seem to be incurable. The discovery of nuclear-cytoplasmic shuttling involvement in the formation of these diseases, as well as the possibility of modifying this type of transport, creates great opportunities for future medicine. However, substantial research is still required to make full use of the existing knowledge.

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LETTER TO THE EDITOR

MOLECULAR IDENTIFICATION OF BATS FROM PUNJAB-PAKISTAN

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Mitochondrial gene, COI has been identified as the standard barcode for molecular identification of many species. To date, no attempt has been reported for the molecular characterization of bats in Pakistan. The current study is therefore planned to design primers for the molecular marker COI through multi-species sequence alignment approach for Pakistani bat species. The COI sequences from major bat families were retrieved from NCBI and aligned on Mega 7 to find primers from most conserved regions. Based on the alignment results three primers with degeneracy were designed with one forward and two sets of reverse primers (PBCOIdF, PBCOIdR1 and PBCOIdR2). Touchdown PCR was carried out to amplify the COI gene from two major bats families i.e., Vespertilionidae, and Emballonuridae from the Punjab region. Primer pair (PBCOIdF and PBCOIdR1) yielded clean amplicons of size 579 and 576 bp for the bat specimens belonging to the Vespertilionidae family, whereas second primer pair (PBCOIdF and PBCOIdR2) gave good quality bands of size 673 and 664 for the Emballonuridae family. NCBI blast sequence analysis for specimens from Vespertilionidae and Emballonuridae revealed the identity score of 95% (*Pipistrellus tenuis*) and 97% (genus *Taphozous*), respectively. Group specific primers designed in the current study gave successfully satisfying results when applied to two large bat families. This work will certainly help to identify bats species across Pakistan and generate data regarding genetic diversity of this taxon parallel with other countries.

To the Editor,

Bats are a highly diversified group of mammals with over 1,100 species and 18 extant families (1). The huge ecological importance attached to this group, in terms of their role in pollination, seed dispersal, insect predators and as bio-indicators of environmental change (2), calls for special attention to explore this cryptic fauna. It is reported that diversity of this taxon was under-reported by 10% (1001 species) five years ago while molecular

dataset has now suggested an increased number of species in many bat taxa (3). Pakistan harbours rich bat diversity across the country with almost 50 species, 23 genera and 8 families, whereas there were expected to be more species (4, 5).

The molecular data to estimate diversity of this group is highly lacking. Recently, some efforts have been made to explore the varieties of bat species in different regions of Pakistan based on traditional taxonomic approaches (6). Hence, there is an urgent

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need to exploit the capacity of molecular techniques and improve the level of species identification of this group in the country.

New approaches using computational methods are being developed to identify sequences which are highly conserved across a variety of species termed as Multi-species Conserved Sequences (MCSs) (7). In recent years, many studies have been successfully incorporated to develop results for comparative genomes for a wide variety of mammalian species (8). The current study is presented as one of the first efforts towards developing primers for the molecular marker COI for local bat fauna and hence opening up the platform for future bat studies based on genetic tools in Pakistan.

MATERIALS AND METHODS

Ethical statement

This experimentation procedure on bats was approved by the institutional bioethics committee (D/503/UZ).

Specimen collection and processing for DNA extraction

Random bat specimens were collected from Kahna, Kasur (Latitude: 31.369555°; Longitude: 74.365162°)

and Derawar Fort, Bahawalpur (Latitude: 28.767951°; Longitude: 71.334216°) across Punjab, representing two large bat families i.e. Vespertilionidae and Emballonuridae, respectively. The captured specimens were euthanized and preserved below 0°C for further use. Reference specimens were morphologically identified. DNA was extracted from selected tissues of preserved bat specimens as described by Fan and Guley (2001) which was then quantified by measuring absorbance at 260/280nm. Data collection and experimental work was further processed as shown in Fig. 1.

Primer development

COI primers were designed with the aim to amplify the barcode gene across the broadest range of the Chiroptera order. Sequence data for the barcode region of COI was collected from the National Center for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov/>) covering a broad range of major bat families i.e., Vespertilionidae, Emballonuridae, Hipposideridae, Rhinolophidae, Molossidae. Eighty-seven COI sequences representing the above-mentioned bat families were aligned on Mega 7. The results obtained were then manually assessed for most conserved regions among all species for both forward and reverse primers. Based on

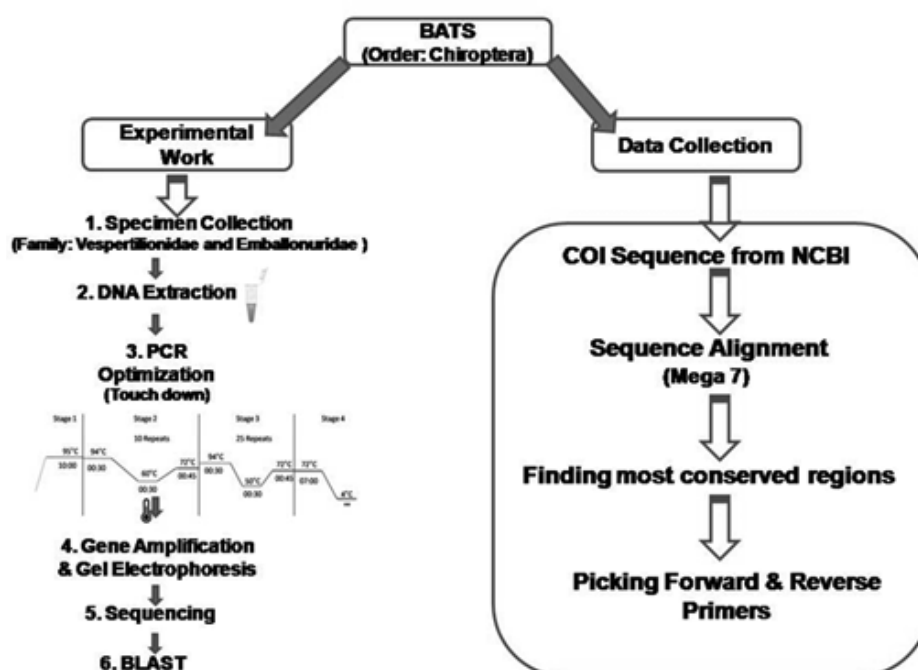


Fig. 1. Schematic representation of experimental approach.

the alignment results, three primers with degeneracy were designed with one forward and two sets of reverse primers for the barcode region of COI: PBCOIdF, PBCOIdR1 and PBCOIdR2, respectively (Table I).

Gene amplification

To enhance the primer specificity and amplification of target gene, touchdown PCR was set with the reaction conditions: initial denaturation at 95°C for 10 min; touchdown procedure consisting of 10 cycles of 30 s at 94°C, annealing for 30 s at temperatures decreasing from 60 to 50°C with 1°C decrease per cycle, 72°C for 45 s; then 25 cycles of 94°C for 30 s; 50°C for 30 s; 72°C for 45 s; final extension of 72°C for 7 min (Fig. 2). PCR was carried out in 25 µL reaction volume containing: 3 µL (15-50 ng/µL) template DNA; 0.5µL add a space in water bath of forward and reverse primer (0.1 mM) and 12.5 µLWizPure™ PCR 2X Master (W1401-2) and 8.5 µl ultrapure water for both set of primers (PBCOIdF and PBCOIdR1) and (PBCOIdF and PBCOIdR2).

Gel analysis

After amplification, PCR products were visualized on 2% agarose gel stained with ethidium bromide while portion was sent for purification and sequencing to Malaysia (ABI3730XL) using the forward primer (10pmol/µL) only.

RESULTS

PCR optimization

The results of PCR shown that target gene (COI) was successfully amplified for DNA extracted from bats belonging to the Vespertilionidae family with primer pair (PBCOIdF and PBCOIdR1) and bats belonging to the Emballonuridae family with primer pair (PBCOIdF and PBCOIdR2). The amplification results with primer pair (PBCOIdF and PBCOIdR1) produced clean amplicons for the bat specimens belonging to the Vespertilionidae family while the same primer pair did not yield any specific results for specimens from the Emballonuridae family. The second primer pair (PBCOIdF and PBCOIdR2) yielded good

Table I. Primers designed to amplify the barcode gene of bats belonging to different families in Punjab, Pakistan.

Sr. No.	Primer Name	Primer Sequence 5' to 3'	Region Amplified
1.	PBCOIdF	GCTGA <u>ACTAGGY</u> CARCCAGG	COI
2.	PBCOIdR1	CCAAAGAATCAGAATAR <u>GTGTTGRT</u> TATA	
3.	PBCOIdR2	ATTCCTATATATCCRA <u>ARGG</u> TTCTTT	COI

*The degeneracy added in primers is underlined.

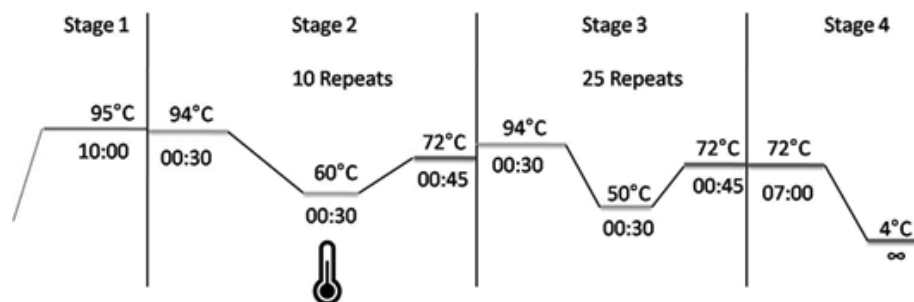


Fig. 2. Touchdown thermocycler programme.

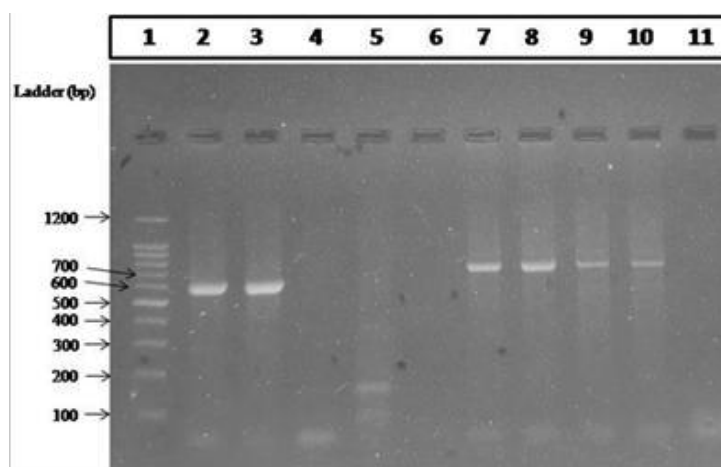


Fig. 3. Wells #2, 3, 4 and 5 in the gel represents amplified products of specimens from Vespertilionidae and Emballonuridae families, from Punjab Pakistan respectively with PBCOIdF and PBCOIdR1. Wells #7, 8, 9 and 10 represent amplified products of specimens from Emballonuridae and Vespertilionidae families, respectively with PBCOIdF and PBCOIdR2. Wells #6 and 11 represent gap and negative control, respectively.

quality bands for the Emballonuridae family but amplicons with non-specific products for the Vespertilionidae species (Fig. 3). The resulting data therefore revealed the efficiency of PBCOIdR1 for Vespertilionidae family and PBCOIdR2 for Emballonuridae family with the similar forward primer PBCOIdF working well for both selected bats families.

NCBI blast sequence analysis

Sequencing results had shown the amplicons size of 579 and 576 bp from the Vespertilionidae family and 673 and 664 from the Emballonuridae family. Blast search on NCBI for sequence similarity revealed the maximum score of 95% for two sequences of bats from Vespertilionidae specimens with partial COI sequence from *Pipistrellus tenuis* (Accession No: HM541294; HM541285; HM541298; GU684808; HM541299; HM541295; HM541293; HM541287; HM541278; HM541277; JF444030; JF444009; HM541279; HM541276; GU684809) with further classification of Family: Vespertilionidae; Genus: *Pipistrellus* and Species: *tenuis*.

The other two sequences from Emballonuridae showed the blast search with the highest score of 97% sequence similarity with 657bp partial COI fragment with *Taphozous* species (Accession No. JF442690) from Kenya with further classification of Family: Emballonuridae; Genus: *Taphozoinae*; Species: *taphozous*.

Analysis of sequence results was carried out with the aid of NCBI (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

DISCUSSION

Revealing cryptic diversity of species based on DNA sequence analysis has become a powerful tool in the field of systematics. Keeping in view the importance of biodiversity research and lack of data, especially based on molecular techniques in Pakistan, the present study was undertaken by selecting bats (Chiroptera) as an ecologically important species. COI was used as standard barcode region and sequences across varieties of bat families were assembled from the NCBI data bank to design primers through multi species sequence alignment. Briefly, three primer sets with degeneracy were picked i.e., one forward primer and two reverse primers to analyze their efficacy against wide families. The development of group specific primers (9, 10) yielded effective results from many workers. Concurrent with our study, Admassu et al. (11) designed earthworm specific primer pair using COI sequences of 11 different species to specifically amplify the target gene even in the presence of a wide range of prey species or amplified from predator DNA. Touchdown/step down thermocycler program was opted in this study as it is a better option while

initially optimizing the primers and confirming their suitability for required gene amplification (12).

Sequence similarity analysis revealed the following identity score for our experimental bat specimen. The specimens belonging to the Emballonuridae family had 97% similarity score with genus *Taphozous* and specimens from the Vespertilionidae family revealed the identity of 95% with *Pipistrellus tenuis*.

It can be concluded that the group specific primers designed in the present study proved to be successful for identification of species from two large bats families belonging to Punjab-Pakistan. Certainly this work will help to further identify bat fauna across Pakistan and can generate molecular data of this taxon parallel with other countries.

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LETTER TO THE EDITOR

COMPUTATIONAL AND *IN VITRO* ANALYSIS OF AN HBCD DEGRADING GENE *dehHZ1* FROM STRAIN HBCD-sjtuS.B. SHAH¹, A.C. KAUSHIK¹, F. ALI¹, L. HUANG¹, X. LU¹, L. SARTAJ²,
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Hexabromocyclododecanes (HBCD), an additive brominated flame retardant, is causing great concern as a result of its extensive use and increasing volumes in different environmental systems. In this study, the HBCD degrading gene *dehHZ1* was identified from strain HBCD-sjtu by homology modelling of enzyme DehHZ1 sequence with LinB from strain *Sphingobium indicum* B90A, an HBCD degrading enzyme. The three dimensional (3D) model structures of enzyme DehHZ1 were obtained. Structure and functional relationship of the enzyme DehHZ1 with compound HBCD was performed using active sites. The HBCD degrading ability of the transformant containing the gene *dehHZ1* was observed at 30°C, pH 7.0 and 200 RPM, in mineral salt medium (MSM) with 0.1 mM HBCD. An intermediate 1, 5, 9-cyclododecatriene (CDT) was recognized based on gas chromatography mass spectrometry analyses after HBCD degradation. These findings may provide new insights into the environment friendly route of HBCD degradation and its conversion into new metabolite.

To the Editor,

Hexabromocyclododecane (HBCD) is one of the commonly used brominated flame retardants (BFRs) which is a brominated aliphatic cyclic hydrocarbon. Producing animal developmental and thyroidal toxicity are one of its main effects (1). HBCD has been found mostly in biotic and abiotic samples. To determine whether it meets the standards of compounds in the Stockholm Convention and in the UN-ECE Protocol on POPs, HBCD is also under screening-level risk assessments (2-3).

Biological degradation of halogenated flame retardants occurs in different environmental and living samples (2). Microorganisms play key roles in degradation of these flame retardants. The structures

of HBCD and HCH (*Hexachlorocyclohexane*) are nearly identical. For degradation of HBCD, an HCH-converting haloalkane dehalogenase LinB was isolated from bacterium *Sphingobium indicum* B90A, which converted HBCD into pentabromocyclododecanols (PBCDOHs) (3). The *Pseudomonas* sp. HB01 also has an enzyme accountable for HBCD degradation which remains to be identified (4). In an earlier study, we isolated a *Bacillus* sp strain, HBCD-sjtu, which effectively degraded HBCD 0.5 mM in four days (5). Annotation of *linB*, *linA2* and eleven other dehalogenase encoding genes were also included in the HBCD-sjtu whole genome sequence. To date, very few bacteria are known to degrade HBCD (6). Hence, molecular

Key words: HBCD; degradation; dehHZ1; 1, 5, 9-cyclododecatriene

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studies involved in HBCD degradation are still in course.

In this article, we studied the predicted 3D model of enzyme DehHZ1 validated by different servers. An understanding of the structural details of enzyme DehHZ1 can be obtained by studying its 3D structure which would help in the design and development of novel drugs and to interpret the mechanism of enzyme DehHZ1. The intermediate was also identified after degradation of HBCD by the enzyme DehHZ1.

MATERIALS AND METHODS

Strain isolation

The HBCD-sjtu strain was isolated from a landfill in Shanghai, People's Republic of China (5). The whole genome of strain HBCD-sjtu was sequenced and deposited in GenBank under the accession number CP025122 (5).

Comparative homology modeling

DehHZ1 query sequence was aligned along with similar PDB structure of LinB (from *Sphingobium indicum* B90A), an HBCD degrading haloalkane dehalogenase, using BLAST to select more similar structure. The enzyme DehHZ1 model was built using aligned templates and loop modeling. The model refinements were applied using Modeller software. Modeling of protein DehHZ1 was validated from the alignment Z-Score and Q-Mean score. Model selection was based on optimized loop, side-chain conformations, Q-mean, Z-score, and Maximum deviation as well as Ramachandran plot. The homologue structures were identified through Blastp using the PDB database. The highest homology ranked was selected with sequence identity and query coverage. Due to low sequence to structure homology, threading method was used to model the 3D structure of DehHZ1. Threading was carried out using Modeller script, based on sequence-structure alignment technique of similar PDB protein structures.

Refinement and model validation

In order to get more accurate results, the model was minimized using AMBER force fields. The modeled structure was validated using SAVE server (6). Unknown residues were minimized using loop modeling and refinement from enzyme DehHZ1 models. The selection of optimized model was carried out based on Q-mean, Z-score values and Ramachandran plot (7).

Structural analysis of active site of enzyme DehHZ1 with compound HBCD

To obtain the functional relationship of structure, identification of enzyme DehHZ1 was made by similar structure from a protein data bank (PDB), as well tunnel prediction to identify how to reach HBCD on active site position and bind with enzyme DehHZ1. Cofactor server was used for the cross validation of predicted active site and prediction of active site and the function of target (4). Tunnel prediction was done by CAVER (8). Using this approach, HBCD dynamic behavior during binding with enzyme DehHZ1 can be identified.

DNA manipulation and plasmid construction

The sequences of the gene *dehHZ1* were prepared by Polymerase chain reaction (PCR). Restriction enzyme digestion, isolation of vectors, agarose gel electrophoresis, and other DNA manipulations were performed using standard protocols (9). The gene *dehHZ1* encoding dehalogenase of strain *Bacillus* sp. HBCD-sjtu was inserted into pUC19 between PstI and BamHI sites. The plasmid pUC19 containing gene *dehHZ1*, was transformed to Trans1-T1 competent cells. Furthermore, these Trans1-T1 competent cells were used for the degradation of HBCD.

Media and culture conditions for HBCD degradation

Culturing was carried out under optimal conditions using 10 ml (MSM medium) containing 0.1 mM HBCD and the transformant containing the gene *dehHZ1* at 30°C, pH 7.0 and 200 RPM for four days.

Analytical methods

A high-performance liquid chromatography (HPLC) (Agilent 1200 series) fortified with an Eclipse XDB-C18 column (column size, 250×64.6 mm; particle size, 5 µm; Agilent) was used for quantification of HBCD present in the culture medium. The mobile phase used in this experiment was a mixture of methanol- H₂O (95:5 v v⁻¹), at a flow rate of 0.3 ml min⁻¹ (5). Recognition of metabolite was made using the gas chromatography-mass spectrometry (GC-MS) system (GCD 1800C, Hewlett-Packard) equipped with a flame ionization detector and a 50-m-long J&W DB-5MS column (Folsom, CA, USA), at 140°C. The procedures performed were 70°C for 2 min, then 15°C/min to 240°C for 3 min, finally at 20°C/min to 300°C for 10 min.

RESULTS

Homology modelling of enzyme DehHZ1 with compound HBCD

Homology model of enzyme DehHZ1 protein was built using a modeller. Comparative protein modelling using PDB-BLAST find out the most probable template (4UAV-A, 3L5K-A and 3NAS-B) for *dehHZ1*, where sequence similarities with LinB were found 62.81% and query coverage was 0.91. The three-dimensional (3D) model of enzyme DehHZ1 was obtained by advance modelling. Out of five, one model was observed as a best model where QMEAN score was 1.64 observed in Fig. 1a.

Refinement and validation of enzyme DehHZ1

The top model was verified by ERRAT, Verify3D, Procheck, Whatcheck, Prove, Cryst modules of

SAVE server. We built five raw models of enzyme DehHZ1 and proposed only top 1 best model according to their values and Ramachandran plot in Fig. 1b.

Structural analysis of active site of enzyme DehHZ1

Significance of structure to function relationship of enzyme DehHZ1 is helpful in drug designing. Active site and tunnel prediction was done by Cofactor CAVER software, where Lys139, Leu190, Ser107, Asn167, Pro140, Asp7, Gly168, Glu163, Ser165, Asp9, Lys41, and thr187 residues were predicted of *dehHZ1* protein. For the cross validation of active site of DehHZ1, structure to function relationship was predicted using similar PDB crystal structure which were more similar to DehHZ1. Active site was predicted using PDB BLAST of NCBI Server. Structure analysis was done by docking with known ligand using AutoDock.

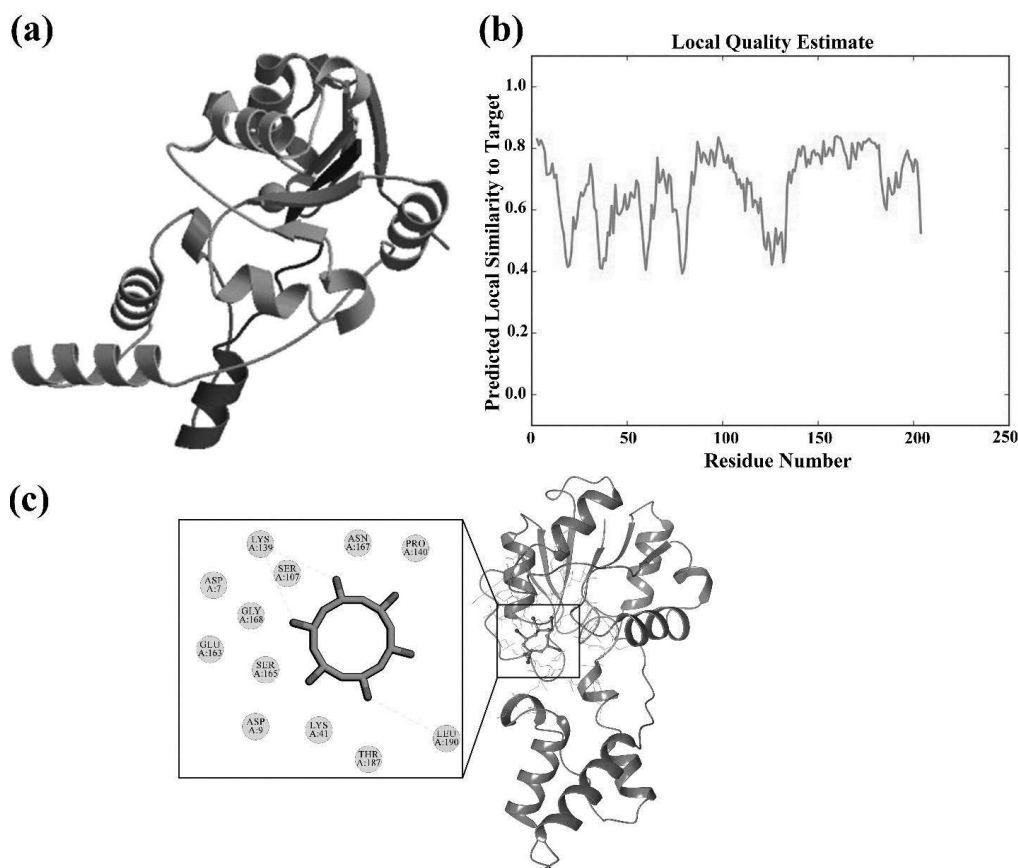


Fig. 1. *a)* Homology model of enzyme DehHZ1 protein sequence. *b)* Predicted local similarity estimate of enzyme DehHZ1 with its target. *c)* Molecular interaction of enzyme DehHZ1 protein with HBCD.

Molecular docking of enzyme DehHZ1

To perform Molecular docking studies, a molecule among the known ones in the active catalytic site of the enzyme DehHZ1 was chosen, and the molecular docking was carried out using Autodock suite software where enzyme DehHZ1 interacts with HBCD compound. The Lys139 and Leu190 residues showed H-bond interactions with predicted enzyme DehHZ1 protein (Fig. 1c).

HBCD degradation and metabolite identification by gene *dehHZ1*

The transformant containing gene *dehHZ1* were capable of degrading 0.1 mM HBCD (Fig. 2a). HBCD degradation by gene *dehHZ1* in MSM culture indicated dark white color of the medium which means successful HBCD degradation. As shown in Fig. 2b, the decrease in HBCD concentration suggests the degradation of HBCD. The structure of

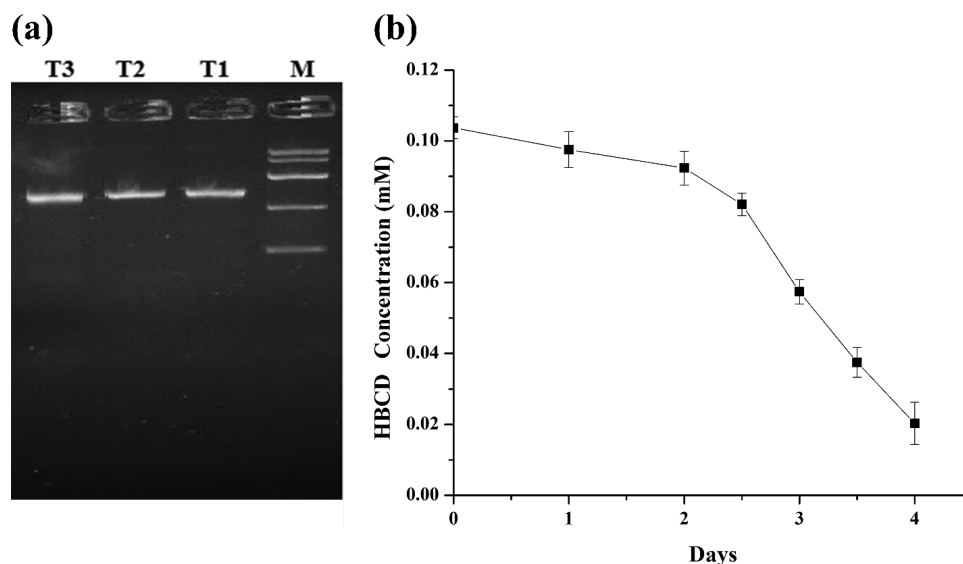


Fig. 2. a) Gel bands after transformation of gene *dehHZ1* into Trans1-T1 competent cells. b) HBCD degradation by gene *dehHZ1*, using HPLC.

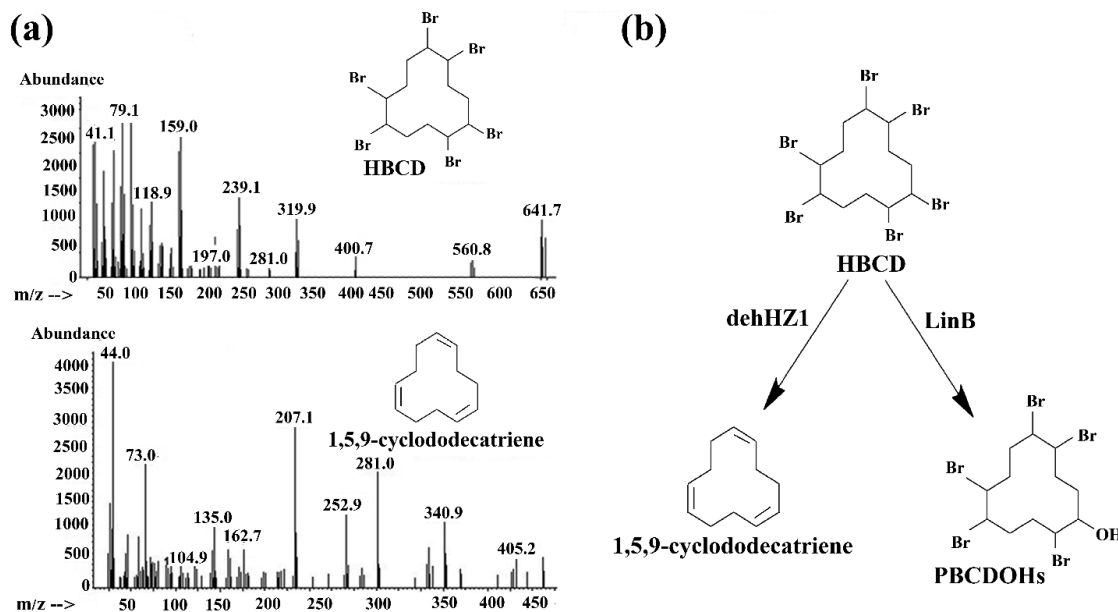


Fig. 3. a) Detection of the mass spectrum of HBCD and its metabolite 1,5,9-Cyclododecatriene using GC-MS. b) Biotransformation of HBCD in different metabolites by *DehHZ1* and *LinB*.

compound A (HBCD) and its mass spectrum 641.7, 560.8, 400.7, 319, 281, 239.1, 197, 118.9, 79, and 41.1 were determined, the structure of compound B (1,5,9-cyclododecatriene) and its mass spectrum 405.2, 240.9, 281, 252.9, 207.1, 162.7, 135, 73, and 44 were determined (Fig. 3a). The intermediate was identified by comparing their mass spectra with the standard GC-MS spectral library.

DISCUSSION

An understanding of the structural and functional features of enzyme DehHZ1 can be obtained from its predicted and optimized 3D model even though its crystallographic structure may not be present (10). Geometric and stereo chemical parameters were used for validation of the structure. A 3D-1D profile was created and an algorithm considering the atoms as hard spheres was used to estimate the volume of atoms in macromolecules. The algorithm computed a statistical Z-score deviation for the model from PDB-deposited structures. The results revealed the considerable accuracy of the model (11).

The homology model of protein DehHZ1 was constructed through this approach using more nearest and similar template structure (11). Molecular modelling was done to identify structure function and molecular interaction through docking approach (12). It is proposed that the enzyme DehHZ1 can degrade HBCD and convert it into metabolite 1, 5, 9-cyclododecatriene (Fig. 3b) (3). It is concluded that the microbial degradation was measured as a special methodology due to its good degrading efficiency and inexpensiveness, as it used living microorganisms for degradation of harmful substances.

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LETTER TO THE EDITOR

MECHANISM OF HIGH-FAT DIET-INDUCED NON-ALCOHOLIC FATTY LIVER DISEASE

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Nonalcoholic fatty liver disease (NAFLD) is gradually becoming one of the most frequent causes of liver diseases. The aim of this study was to establish a mouse model of nonalcoholic fatty liver disease induced by high fat diet, which could be used for studying the pathogenesis of NAFLD. Forty male C57BL/6J mice were randomly divided into normal and high-fat model groups. Compared with the normal group, after 5 weeks of high-fat diet, the model group showed significantly elevated serum TG, TC, fasting glucose, transaminase, hepatic TG, TC and TNF- α levels ($P < 0.05$). In contrast, hepatic adiponectin content was significantly reduced ($P < 0.05$) in the model group compared to the normal group. The morphological alterations determined by HE and Sudan III further showed diffuse hepatic steatosis and massive inflammatory cell infiltration in the model group. Immunohistochemistry staining further revealed significantly elevated CYP2E1 and TGF- β 1 expression and markedly decreased catalase and adiponectin expression in the model group compared to the control group ($P < 0.05$). This study confirmed that high-fat diet significantly increased liver CYP2E1 and TGF- β 1 expressions and decreased adiponectin and catalase expressions in NAFLD mouse model.

To the Editor,

Nonalcoholic fatty liver disease (NAFLD) is characterized by hepatic steatosis, which is becoming the most frequent liver disease worldwide (1, 2). NAFLD is directly associated with obesity and insulin resistance and subjects with an unhealthy lifestyle, such as Western type diet and physical inactivity, are more vulnerable to this disease (3, 4). The widely used NAFLD animal models are established by specific gene mutation, modified diet or treatment with drugs. In earlier studies, the ob/ob mice are most frequently used as NAFLD animal model (5). NAFLD models are also established by carbon tetrachloride injection, tetracycline induction or high sugar diet. High fat diet (HFD) is one of the most commonly used models to mimic human

NAFLD. The imbalance of liver lipid metabolism causes liver dysfunction. At present, the “second strike theory” is the main pathogenesis of NAFLD. The first strike is mainly insulin resistance (IR). Various factors, such as obesity, type 2 diabetes, and lipid metabolism disorders, can cause IR, which can aggravate fat accumulation in the liver (6). The second strike mainly is oxidative stress and lipid peroxidation caused by various factors (7). NAFLD is considered to be the major cause of end-stage liver diseases, which are directly associated with the development of hepatocellular carcinoma (HCC), liver failure, and death. However, the vital factors involved in the pathogenesis of NAFLD remain unknown. Therefore, the aim of this study was to establish an animal model of NAFLD and investigate

Key words: nonalcoholic fatty liver; cytochrome P4502E1; tumor necrosis factor- α ; transforming growth factor β 1; adiponectin; catalase

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the pathogenesis, which might provide scientific basis for the management of NAFLD.

MATERIALS AND METHODS

Animals

Forty male C57BL/6J mice (Experimental Animal Center of the Dalian Medical University), weighing 18-20 g, were maintained in a 12-h light/dark cycle at a temperature of $22\pm 1^{\circ}\text{C}$ and a relative humidity of $50\pm 10\%$. All animal experimental protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals (Certificate No. SCXK2016-2002) and were approved by the Hospital Ethics Committee of The Second Affiliated Hospital of Dalian Medical University.

After acclimatization for one week, 40 male C57BL/6J mice were randomly divided into 2 groups with 20 mice in each group. The normal group was fed on standard rodent chow diet and the model group was fed on Lieber-DeCarli (71% Fat by Calories). Liquid Rodent Diet consisted of the following ingredients: Casein (41.1 g/L), cystine (0.5 g/L), methionine (0.3 g/L), corn oil (48.5 g/L), olive oil (28.4 g/L), safflower oil (2.7 g/L), dextrin and maltose (25.6 g/L), choline (0.53 g/L), cellulose (10.0 g/L),

bio-glue (3.0 g/L). The Lieber-DeCarli Liquid Rodent Diet contained 71% fat, 11% sugar and 18% protein (Shanghai Beikaijie International Trade Co., Ltd.). Water and food were provided *ad libitum*. The NAFLD model establishment was evaluated by morphological changes as assessed by HE staining (2 mice were randomly selected and sacrificed every week from the end of the 3rd week).

Biochemical analysis

After 5-week treatment, all mice were sacrificed by cervical vertebra luxation and the blood was collected. Serum was isolated by centrifugation and serum AST, ALT, TC, TG and glucose levels were determined by ELISA kits following the manufacturer's instructions (Nanjing Jiancheng Biotechnology Nanjing, China). Liver tissues were homogenized in cold potassium phosphate buffer and centrifuged. The supernatants were used to determine the levels of TC, TG, TNF- α and adiponectin (Wuhan Boster Biological Technology).

Histological analysis

The histological changes of liver were determined by hematoxylin-eosin (HE) staining. The hepatic steatosis was evaluated under a light microscope.

Table I. Blood lipid (mmol/L), serum aminotransferase (IU/L) and blood glucose (mmol/L) in the model group and normal group at the 5th week.

Groups	N	TG	TC	AST	ALT	Blood glucose
Normal	16	0.71 $\pm$ 0.15	2.32 $\pm$ 0.63	48.70 $\pm$ 8.95	13.31 $\pm$ 4.47	7.78 $\pm$ 1.39
Model	16	1.06 $\pm$ 0.43 [#]	4.22 $\pm$ 0.62 ^{##}	67.53 $\pm$ 15.22 [#]	20.18 $\pm$ 5.77 [#]	11.22 $\pm$ 1.04 ^{##}

Data are represented as Mean $\pm$ SD. # $P < 0.05$ versus normal group, ## $P < 0.01$ versus normal group.

Table II. The levels of lipid (mg/g), TNF- α (pg/mg) and adiponectin (ng/mg) in the liver of normal and model group at the 5th week.

Groups	N	TG	TC	AST	ALT	Blood glucose
Normal	16	0.71 $\pm$ 0.15	2.32 $\pm$ 0.63	48.70 $\pm$ 8.95	13.31 $\pm$ 4.47	7.78 $\pm$ 1.39
Model	16	1.06 $\pm$ 0.43 [#]	4.22 $\pm$ 0.62 ^{##}	67.53 $\pm$ 15.22 [#]	20.18 $\pm$ 5.77 [#]	11.22 $\pm$ 1.04 ^{##}

Data are represented as Mean $\pm$ SD. # $P < 0.05$ vs normal group, ## $P < 0.01$ versus normal group.

Immunohistochemistry

Immunohistochemistry assay of CYP2E1, TGF- β 1, catalase and adiponectin of liver samples were performed based on the instructions of the manufacturer (Abcam Cambridge, UK). The immunohistochemistry staining results were determined as positive cells stained in brown or brown granules in the cytoplasm. Ten high-power fields ($\times 400$) were randomly selected from each section, and 100 hepatocytes were counted in each field.

Statistical analysis

All data were expressed as the mean $\pm$ standard deviation (SD) and subjected to analysis of one-way analysis of variance (ANOVA) followed by Dunn's post-hoc tests. P value <0.05 was considered significant.

RESULTS

Physiological parameters

No mice died after the 5-week treatment. The mice of the normal group were energetic, flexible, active and with neat fur, however, the mice in the model group displayed yellowish and untidy fur. Compared to the normal group, the urine volume of the model group was significantly increased, and the

stools were a grayish white color after 3 days high fat feeding. The bodyweights of two groups showed no significant difference during the 5-week treatment.

Biochemical analysis in serum

After the 5-week treatment, serum TC, TG and blood glucose levels were notably increased, compared to the normal group (Table I) ($P < 0.01$, $P < 0.05$, $P < 0.01$, respectively). Furthermore, ALT and AST levels in model group were markedly higher than that of normal group ($P < 0.05$), which indicated liver damage.

Biochemical analysis in liver

As shown in Table II, the levels of TC and TG in liver were also significantly increased after the with high fat diet ($P < 0.05$). In addition, the 5-week high fat diet significantly increased hepatic TNF- α and adiponectin ($P < 0.05$) in the model group, compared with the normal group.

Histological analysis

In the normal group, liver displayed clear lobule boundary, normal cell size, and neatly arranged

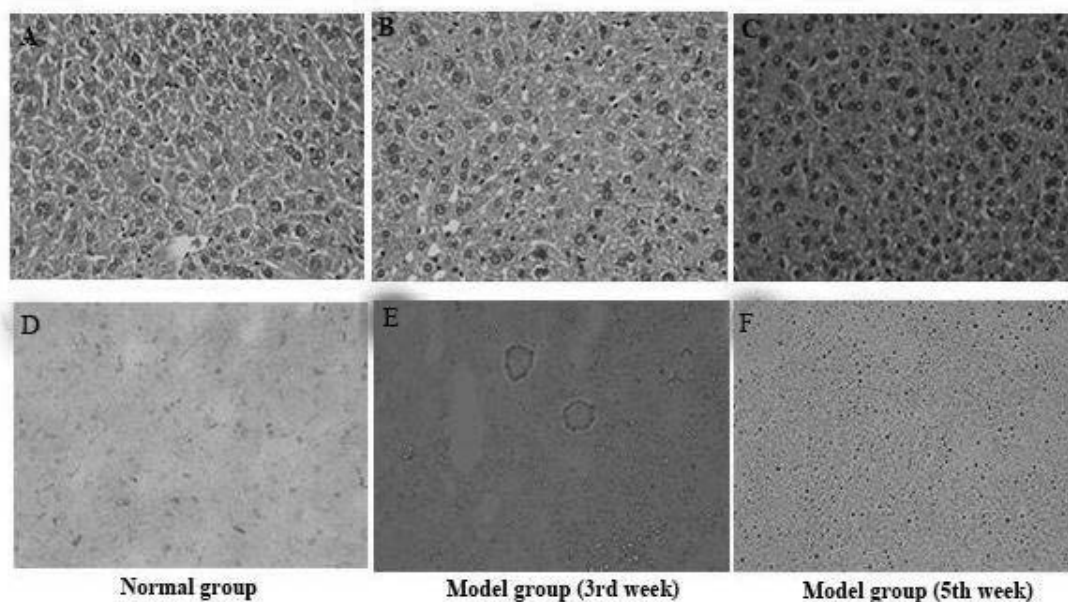


Fig. 1. Liver HE staining (A-C) and Sudan III staining (D-F) of normal group and model group (3rd week and 5th week) ($\times 400$). (A and D) normal group, (B and E) model group (3rd week), (C and F) model group (5th week)

cell structure, and no Sudan III positive staining was found (Fig. 1A and Fig. 1D). At the third week, in the model group, various sizes of lipid droplets appeared in the cytoplasm of the liver cells, and Sudan III staining showed a small amount of orange particles, indicating partial hepatocyte steatosis (Fig. 1, B and E). At the 5th week, mice in the model group showed hepatic cord disorder and hepatocyte edema, accompanied with diffuse hepatic steatosis and inflammatory cell infiltration. Sudan III staining showed large orange-yellow particles (Fig. 1, C and F). In addition, the model group showed severe inflammatory damage, and the inflammatory score was significantly higher than the normal group.

Immunohistochemistry

Immunohistochemistry staining results are shown in Fig. 2, the expressions of CYP2E1 and TGF- β 1 in NAFLD model group were significantly increased as compared to the normal group (Fig. 2, A-D). In addition, the expressions of catalase and adiponectin in the model group were dramatically lowered compared to the normal group (Fig. 2, E-H).

DISCUSSION

In the present study, the TNF- α level in the liver of the model group was significantly increased compared to the normal group, indicating the important role of TNF- α in the pathogenesis of NAFLD. TNF- α , which is mainly secreted by macrophages, T lymphocytes, fibroblasts and adipocytes, is the major regulator of inflammatory response and immune response. In the early stage of injury, TNF- α stimulates the expression of other pro-inflammatory mediators. In the pathogenesis of NAFLD, TNF- α is an important inflammatory factor involved in the “second strike” and plays an important role in IR. Previous study has reported that TNF- α affects hepatic fat metabolism and reduces insulin receptor tyrosine kinase activity through TNF- α receptors on hepatocytes, thereby aggravating IR (8). In addition, TNF- α can also activate the coagulation system and promote extracellular matrix synthesis, thereby promoting the development of non-alcoholic steatohepatitis (NASH) (9, 10). TGF- β 1 is a cytokine that promotes fibro-genesis and is closely related to the fibrosis

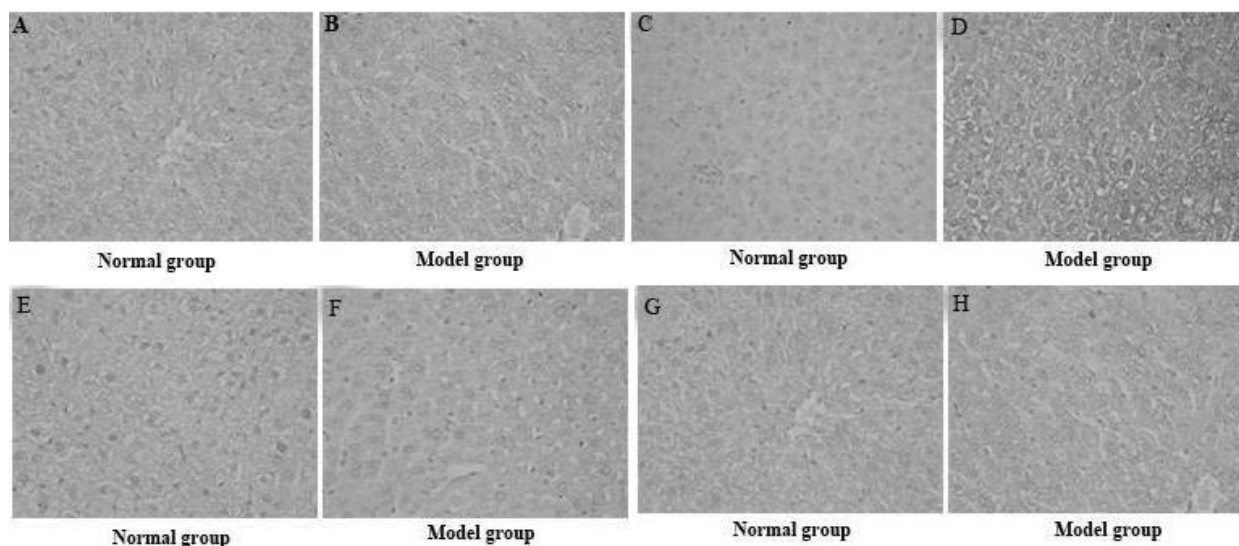


Fig. 2. CYP2E1 (A and B), TGF- β 1 (C and D), Adiponectin (E and F), Catalase (G and H) immunohistochemistry staining in liver of normal group and model group ($\times 400$).

formation. Recent studies have shown that TGF- β 1 expression increased in liver both in the early stages of NASH and cirrhosis. In the present study, we have confirmed that TGF- β 1 significantly increased in the liver of the NAFLD model group.

In summary, the NAFLD mouse model in this study simulates human diet, which has the advantages of short molding time, stable model and high reproducibility. The NAFLD model in this study is a promising animal model for studying the pathogenesis of NAFLD and screening new drugs.

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LETTER TO THE EDITOR

DELAYED-ENHANCEMENT 320 ROW VOLUME MULTIDETECTOR COMPUTED TOMOGRAPHY FOR THE ASSESSMENT OF REPERFUSED ACUTE AND OLD MYOCARDIAL INFARCTION IN A PORCINE MODELDL. GUO¹, ZW. GONG¹, T. ZHANG², L. XUE¹, JL. TONG² and XQ. LI¹¹*Department of Cardiology, the Fourth Affiliated Hospital of Harbin Medical University, Harbin, China;* ²*Department of Medical Imaging, the Fourth Affiliated Hospital of Harbin Medical University, Harbin, China**Received October 15, 2018 – Accepted November 21, 2018*

To evaluate the practical value of delayed enhancement 320 row volume multidetector computed tomography (DE-320 row volume MDCT) for reperfused acute and old myocardial infarction (MI) in a porcine model, 14 pigs underwent balloon-induced occlusion of the middle segment of the left circumflex coronary artery (LCX) for 90 min. The balloons were removed and reperfusion was performed. DE-320 row volume MDCT was performed 15 min after the injection of iodinated contrast. Delayed-enhancement magnetic resonance imaging (DE-MRI) was completed 15 min after injection of gadolinium-DTPA. The pigs were then divided into acute vs old MI groups. Six pigs in the acute MI group were sacrificed immediately and their hearts were harvested for triphenyltetrazolium chloride (TTC) pathology. The pigs in the old MI group were reared for another 6 months before undergoing repeat DE-320 row volume MDCT and DE-MRI prior to being sacrificed. On DE-320 row volume MDCT, the size of the acute and old myocardial infarction was $23.95 \pm 9.8\%$ and $16.93\% \pm 7.04\%$, respectively, which had a high consistency and correlation with DE-MRI and TTC pathology. The CT values of the delayed enhanced area were different between acute MI and old MI and were 132.5 ± 30.5 HU and 91.2 ± 18.3 HU, respectively ($P < 0.001$). The CT values of the delayed enhanced areas were different from that of viable myocardium (acute, $P < 0.001$, old, $P < 0.001$). The diagnosis of the infarcted segments by DE-320 row volume MDCT had good consistency with the results of TTC pathology (acute, kappa = 0.76; old, kappa = 0.64). DE-320 row volume MDCT can be an effective method for assessing MI.

To the Editor,

The degree of myocardial injury can predict the prognosis of patients. Myocardial function after ischemia-reperfusion can be restored by saving the viable myocardium. Because of the high medical costs and certain complications and mortality associated with Percutaneous Transluminal Coronary Angioplasty (PTCA) or Coronary Artery Bypass

Grafting (CABG), non-invasive examination of viable myocardium can be used to assess the benefit and risk of these patients with revascularization. MDCT has been widely used in the diagnosis of coronary heart disease, and, in particular, coronary artery stenosis. However, it is more important to distinguish viable from infarcted myocardium as such assessment has great prognostic significance.

*Key words: computed tomography; delayed-enhancement; myocardial infarction**Corresponding Author:*

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Currently, application of MDCT for the detection of infarcted *vs* viable myocardium is still in the research phase. Several studies have used MDCT to evaluate reperfusion in acute MI, but there are few reports on the use of MDCT for old MI. Since the latest 320 row volume MDCT has improved Z-axis resolution due to its fast scanning speed and large detector, we applied 320 row volume MDCT to assess acute *vs* old MI in a porcine model.

MATERIALS AND METHODS

Animal preparation

The use of experimental animals was approved by the Animal Ethics Committee of our university and the experiment was carried out by Large Animal Experiment Center of the First Affiliated Hospital of Harbin Medical University. Fourteen pigs weighing between 26 and 30 kg (mean 28.4 ± 4.7 kg) were included in the analysis. All animals underwent general anesthesia induced by intramuscular injection of 0.05 mg/kg atropine, 1 mg/kg midazolam, and 20 mg/kg ketamine. A 3% sodium pentobarbital solution was then slowly injected through the marginal ear vein for a total dose of 1 ml/kg, and the initial dose was half of the total dose. The pigs were placed in the supine position, connected to an ECG monitor, and underwent tracheal intubation and ventilator-assisted breathing (the ratio of oxygen to nitrogen was 1:2 in the gas mixture).

An arterial sheath was inserted through the femoral artery, and an 8-F guiding catheter was placed at the origin of the left coronary artery under X-ray guidance. Conventional coronary angiography was performed to determine the anatomy of the left coronary artery. Depending on the diameter of the left circumflex coronary artery (LCX), a balloon with a diameter of 2.5-3.5 mm was placed at the middle segment of the LCX to occlude the artery. The balloon was removed 90 min after occlusion and the infarcted myocardium was reperfused. The reperfusion was confirmed by coronary angiography. One pig died of ventricular fibrillation during balloon occlusion of the LCX. The remaining thirteen pigs were divided into acute *vs* old MI groups. Six pigs in the acute MI group underwent DE-320 row volume MDCT and DE-MRI after one day and were sacrificed after the scans. Their hearts were harvested for TTC histological staining.

Seven pigs were reared for a further six months after the initial DE-320 row volume MDCTs and DE-MRIs. One pig died during the breeding period. The remaining six pigs underwent repeat DE-320 row volume MDCT and DE-MRI after 6 months, were subsequently sacrificed, and their hearts were harvested for histological TTC staining.

The DE-320 row volume MDCT

CT examination began at 115 ± 9 min after LCX occlusion. For MDCT, a 320-row volume MDCT scanner was used (Aquilion One, Toshiba Medical Systems, Japan). The pigs were scanned in the supine position after anesthesia. Each animal's heart rate was controlled between 85 and 90 beats/min by intravenous injection of metoprolol tartrate. Images were acquired at a controlled heart rate of 85-90 beats/min during end-expiratory breath hold. The entire cardiac cycle was scanned with retrospective ECG-gating.

DE-320 row volume MDCT was performed 15 min after injection of non-ionic iodinated contrast medium at a dose of 1.5 ml/kg (Ultravist 370, Schering, Berlin, Germany) via the marginal ear vein and scanning for 3 heart periods. The scan mode was set as a volume acquisition, the coverage width was set to 160 mm, the tube voltage was 120 kV, tube current was 400 mA, the field of view was $160-180 \times 160-180$ mm, the detector collimation was $320 \text{ mm} \times 0.5 \text{ mm}$, reconstruction slice thickness was 1 mm, slice spacing was 0.2 mm, FOV 180×180 mm, reconstruction time phase was 60-80%R-R, Recon Process AIDR 3D Standard and the rotation time was 0.35 s. The contrast-enhanced scan used the SureStart contrast medium tracking technique. The scan was triggered when the CT value of the descending aorta reached 150 HU.

MRI

The MRI examination was started 25 ± 14 min after the 320 row volume MDCT, using a 1.5T whole body MR scanner (Intera, Philips Medical Systems, Best, The Netherlands). All animals were scanned in the supine position, and a five-channel cardiac coil was used. The image data were acquired during end-expiratory breath holding. The ECG-gated inversion-recovery gradient-echo sequence T1 was triggered by the R wave in the precordial lead, obtaining an 8 mm end-diastolic short axis view without intersection gaps 15 min after injection

of 0.2 mmol/kg Gd-DTPA via the marginal ear vein. The scan parameters included a TR: 2 cardiac cycles, TE: 4.0 ms, flip angle: 20 degrees, FOV: $350 \times 350 \text{ mm}^2$, inversion time: 250-300 ms, and matrix: 256×256 .

Histological examination

The pigs were sacrificed by intravenous injection of potassium chloride. The chest cavity was opened immediately to harvest each heart. The heart was cut perpendicular to the interventricular septum into 8 mm slices from the left ventricular apex to the bottom. The slices were placed in 2% 2,3,5-triphenyltetrazolin chloride (TTC) phosphate buffer in a water bath at 37°C for 30 min in the dark and fixed in 10% formaldehyde. Normal myocardium showed red staining and infarcted myocardium did not stain. Photographs of myocardial tissue slices were taken in order using a digital camera, and the images were analyzed using the Image-Pro Plus 6.0 image processing software.

Imaging analysis

Imaging analysis was completed by two experienced cardiovascular imaging physicians. DE-MDCT images and DE-MRI images were transferred to the workstation, respectively choosing the phase with no myocardial virtual shadow, internal papillary muscles in good shape and re-constructed. Volume data was imported to the Basic Vitrea 2 software for the three-dimensional reconstruction (multi Planar reconstruction, MPR), and re-constructed the heart short axis image perpendicular to the direction of ventricular septum. Short axis images of DE-MDCT matched the DE-MRI images and the TTC slices. All layers with delayed enhancement on DE-MRI and DE-MDCT images were analyzed.

On the DE-MRI images, infarcted myocardium was defined as highly enhanced areas with a signal intensity that was three standard deviations higher than the mean signal intensity of the normal myocardium. On the DE-MDCT images, infarcted myocardium was defined as enhanced areas with a CT value that was 20 HU higher than the mean CT value of the normal myocardium.

The infarct size was expressed as a percentage of the infarcted myocardium in the area of the left ventricular wall. It was calculated as follows: $\text{infarct size (\%)} = \frac{\text{the sum of the infarct area of each slice}}{\text{the sum of the infarct area of each slice} + \text{the sum of the area of normal}}$

$\text{myocardium}) \times 100\%$.

On the DE-MDCT images, region of interest (ROI) was delineated by hand to measure the CT values of normal vs infarcted myocardium and the left ventricular blood pool. The infarcted segments were determined by referring to the 17 segments of the short axis view of the heart recommended by the American Heart Association.

Statistical analysis

All data were expressed as mean $\pm$ standard deviation. Linear regression analysis and Bland-Altman analysis were used to study the correlations and consistency of infarct size as measured by DE-320 row volume MDCT, DE-MRI, and TTC pathology. Paired *t*-test was used to compare the infarct size as detected by DE-320 row volume MDCT and DE-MRI between the acute and old MI groups. The TTC pathology results were compared using independent samples *t*-test. The CT values of the normal and infarcted myocardium and the left ventricular blood pool were compared between the acute vs old MI groups using paired *t*-test. The CT values of the normal and infarcted myocardium and the left ventricular blood pool were tested with one-way analysis of variance followed by post hoc Bonferroni correction to adjust for multiple comparisons. The assessments of the infarct segments, obtained by DE-320 row volume MDCT and TTC pathology, were compared using *chi*-squared test and Kappa test. Taking TTC as the golden standard to evaluate the infarction segment sensitivity and specificity of DE-320 Row Volume MDCT. $\text{Sensitivity} = \frac{\text{both TTC pathology Infarcted and DE-320 Row Volume MDCT Infarcted}}{\text{both TTC pathology Infarcted}}$ $\text{Specificity} = \frac{\text{both TTC pathology Viable and DE-320 Row Volume MDCT Viable}}{\text{both TTC pathology Viable}}$.

RESULTS

Images of evaluable quality were obtained from both DE-320 row volume MDCT and DE-MRI. In the acute MI group, the infarct sizes detected by the DE-320 row volume MDCT, TTC pathology, and DE-MRI were $23.95\% \pm 9.80\%$, $23.55\% \pm 8.97\%$, and $22.8\% \pm 9.36\%$, respectively. In the old MI group, the infarct sizes for the same three methods were $16.93\% \pm 7.04\%$, $16.31\% \pm 6.02\%$, and $15.87\% \pm 6.16\%$, respectively. The infarct sizes by

the DE-320 row volume MDCT, TTC pathology, and DE-MRI were consistent with either acute or old MI group (Fig. 1). The infarct sizes by the DE-320 row volume MDCT correlated with TTC pathology (Fig. 2). The infarct sizes of old MI, as assessed by the three methods, were significantly reduced when compared to those of acute MIs ($P < 0.001$).

In the acute MI group, the CT values of the infarcted myocardium, blood pool, and normal myocardium (by the DE-320 row volume MDCT) were 132.5 ± 30.5 HU, 125.7 ± 21.1 HU, and 85.3 ± 18.6 HU, respectively. In the old MI group, the CT values were 91.2 ± 18.3 HU, 85.1 ± 14.6 HU, and 71.7 ± 11 HU, respectively. In both the acute and old MI group, the CT values of the infarcted and viable myocardium were significantly different (both $P < 0.001$). At the same time, the CT values of infarcted myocardium varied between the acute vs the old MI group ($P < 0.001$).

The TTC pathology results were used as the gold standard to assess the size of the infarcted segment as measured by DE-320 row volume MDCT. For acute

MI, DE-320 row volume MDCT had a sensitivity of 85% and a specificity of 93%, the Kappa value was 0.76. The old MI group had a sensitivity of 79% and a specificity of 90%, the Kappa value was 0.64 (Table I). Both evaluation methods exhibited good consistency.

DISCUSSION

Application of MRI for the detection of MI has been extensively reported (1-3). DE-MRI is an acceptable gold standard for distinguishing infarcted from viable myocardium (4, 5).

Several recent coronary angiography studies have been reported using 320 row volume MDCT (6, 7), however, no studies concerning the ability to detect viable myocardium using DE-320 row volume MDCT have been reported. The iodinated contrast used in 320 row volume MDCT has very similar pharmacokinetics and volume distribution to that of gadolinium used in MRI (8); therefore, the mechanism of enhancement of iodinated contrast in

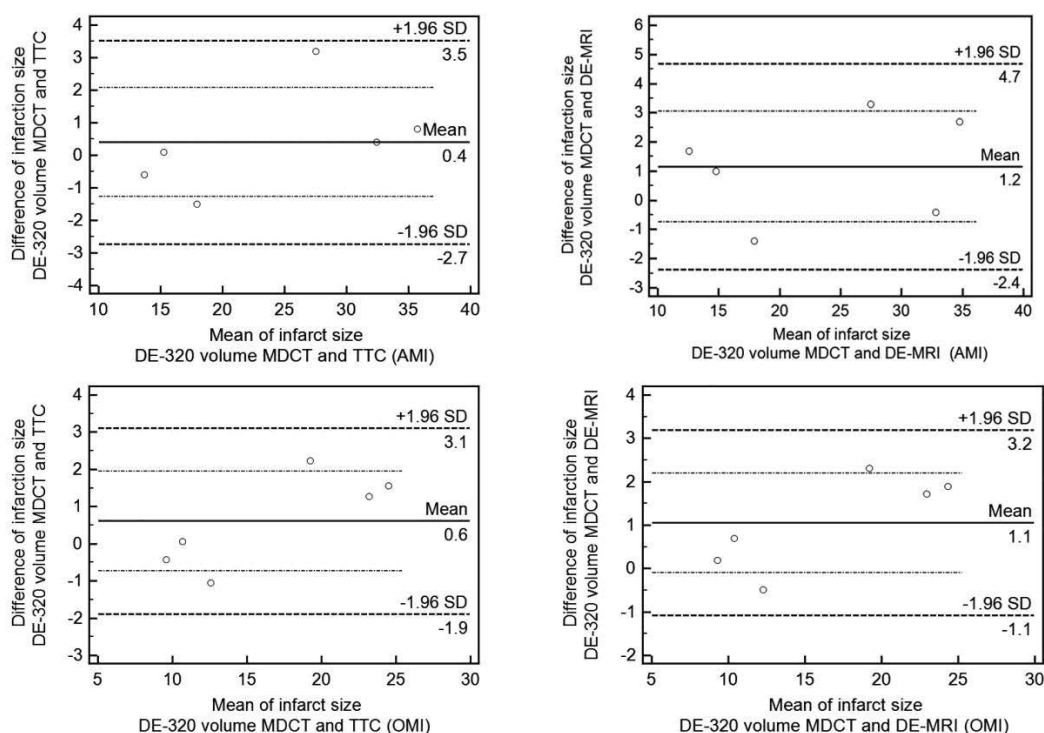


Fig. 1. Bland-Altman plots compare the size of acute MI vs chronic MI among DE-320 row volume MDCT, DE-MRI, and TTC staining.

MDCT could be similar to that of gadolinium (9). Some animal studies have shown good consistency between post-myocardial infarction myocardial viability (as detected by DE-MDCT), and DE-MRI and pathological results (10-12). Our study used DE-320 row volume MDCT to detect the infarct size on the day of the acute MI and 6 months after

the MI. The infarct sizes were $23.95\% \pm 9.80\%$ and $16.93\% \pm 7.04\%$, respectively. From TTC pathology, the infarct sizes at the two time points were $23.55\% \pm 8.97\%$ and $16.31\% \pm 6.02\%$, respectively. For old MI, DE-320 row volume MDCT and TTC pathology showed good consistency in detecting infarct size, and the CT values of infarcted

Table I. Evaluation of infarcted vs viable myocardial segments by DE-320 row volume MDCT for acute (old) myocardial infarction.

TTC pathology	Infarcted	DE-320 row volume MDCT Viable	Total	p	Kappa
Infarcted	17 (15)	3 (4)	20 (19)	0.000 (0.000)	0.76 (0.64)
Viable	5 (8)	77 (75)	82 (83)		
Total	22 (23)	80 (83)	102 (102)		

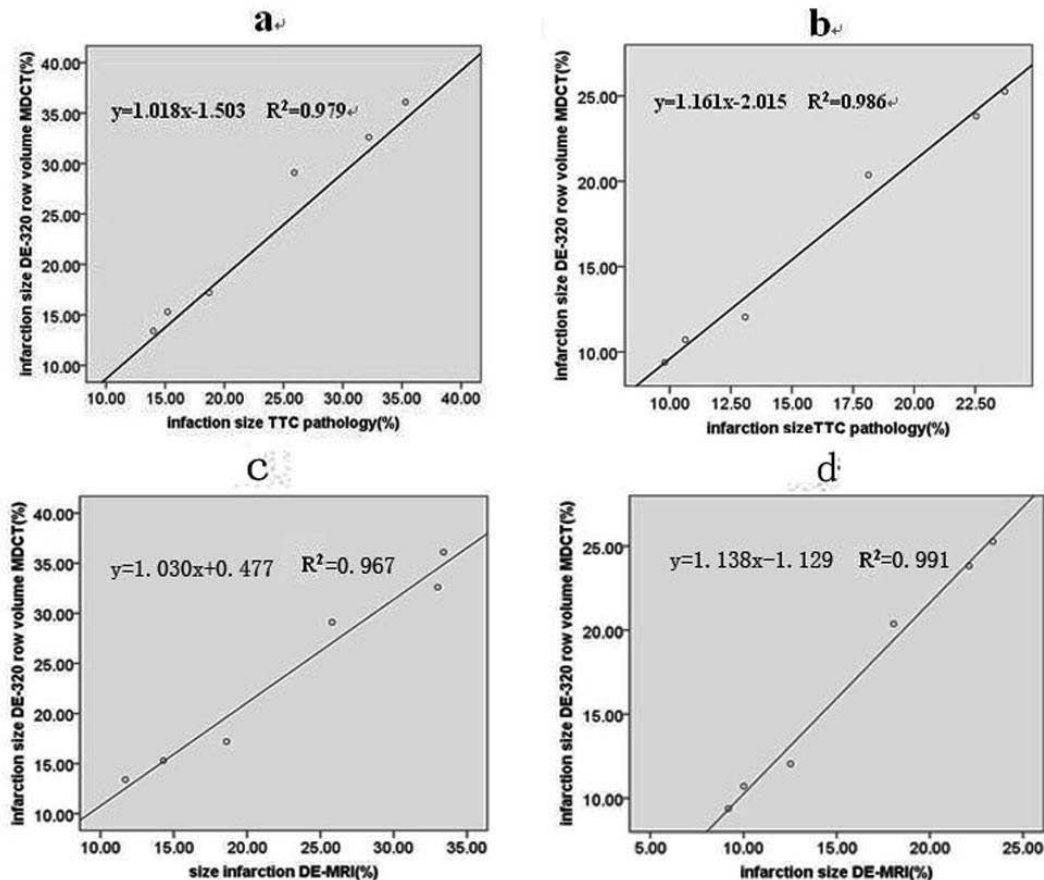


Fig. 2. The relationship between infarct size for AMI (a, c) vs CMI (b, d) by DE-320 row volume MDCT, DE-MRI, and TTC pathology.

myocardium on delayed scanning were significantly higher than that of non-infarcted myocardium in either acute or old MI.

In summary, DE-320 row volume MDCT can assess the infarcted myocardium in both acute and old MI.

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LETTER TO THE EDITOR

PROTECTIVE EFFECTS OF AQUEOUS EXTRACT OF WAMPEE FRUIT AGAINST ANTI-TUBERCULAR DRUG-INDUCED HEPATOTOXICITY IN RATS

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The fruits of wampee have been used traditionally as stomachic and vermifuge herbs. Previous research has shown that wampee fruits possess antioxidant and antialcoholism effects. Drug-induced liver injury is very common when using anti-tubercular drugs, and is considered to be linked to oxidative stress. This study was designed to investigate the hepatoprotective activity of wampee fruits against antituberculosis drug-induced hepatotoxicity. Hepatotoxicity was induced in rats by oral administration of a combination of isoniazid and rifampicin. An aqueous extract from the fruits of wampee (AEFW) at doses of 1.5 and 3.0 g/kg/d were evaluated for their potential hepatoprotective effects. The serum levels of hepatic alanine transaminase (ALT) and aspartate transaminase (AST) were estimated at the end of the experiments. An analysis of hepatic histopathology was carried out to assess injury to the liver. Western blot and immunostaining were used to determine the levels of protein expression. The results showed that AEFW (1.5 and 3.0 g/kg/d) was found to significantly decrease the levels of ALT and AST and reduce hepatocellular necrosis and liver fibrosis in treated rats, compared with the control group. Furthermore, Western blot and immunostaining analysis revealed that the extract can down-regulate the expression of MyD88 and NF- κ B p65, and inhibit NF- κ B p65 phosphorylation. Therefore AEFW can alleviate anti-tubercular drug-induced liver damage by enhancing anti-inflammatory effects.

To the Editor,

Tuberculosis (TB) is a serious infectious disease that affects one-third of the world's population (1). At present, the first-line anti-TB drugs, including isoniazid (INH) and rifampin (RFP), can result in drug-induced liver injury. Epidemiological reports have shown that cases of hepatotoxicity become

more frequent when the INH/RFP combination is employed (2, 3).

The pathogenesis of the combined INH/RFP-induced hepatotoxicity is unclear, but these anti-TB drugs which induce hepatotoxicity (ADIH) are considered to have similar mechanisms, increasing oxygen free radical generation and escalating

Key words: wampee, hepatotoxicity, antituberculosis drugs, NF- κ B

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the level of both oxidative stress (OS) and lipid peroxidation (4). Several antioxidants from herbal and natural compounds have been used and their mechanisms are associated with inhibiting the formation of reactive oxygen species (ROS) (5) and inflammatory responses (6).

Wampee (*Clausena lansium* (Lour.) Skeels) is characteristic cultivated fruit of southern China fruit. An extract of wampee fruit and some isolated compounds were studied for anticancer and anti-oxidant effects (7), and most reported the bioactivities were due to active components such as flavonoids, alkaloids and polyphenols to decrease oxidative stress.

Our previous studies showed the crude extract from wampee fruits could attenuate acute alcohol-induced liver injury (8). Therefore, the current study was to further investigate the effect of AEFW against ADIH.

MATERIALS AND METHODS

Collection of plant materials

The mature fruits from *Clausena lansium* were collected in June 2013 from Yunan, Guangdong province. The plant was identified by the corresponding author, and a voucher specimen was deposited in the herbarium of the Guangdong Key Laboratory for Research and Development of Natural Drugs, Guangdong Medical University, China. After collection, the fruits were washed repeatedly and air dried at room temperature.

Preparation of aqueous extract

The dried fruits of wampee were ground into a moderately coarse powder, and then extracted 3 times with distilled water (1:10) for 1.5 h at 90°C each time. The filtrates were combined, concentrated under vacuum, and lyophilized to give a brown red extract known as AEFW. The extract was stored in a dark bottle at 4°C until used.

Phytochemical screening

The AEFW was screened for the presence of secondary metabolites which have been previously related to antioxidant and anti-inflammatory activities. Thus, tests for alkaloids, coumarins, flavonoids,

phenols, terpenoids, steroids, glycosides and reducing sugars were performed using standard procedures (9).

Animals and treatments

Sprague-Dawley rats of either sex weighing 230–250g were procured from Guangdong Medical University Animal Center. All rats were cared for in accordance with all guidelines for the use and care of laboratory animals. The animals were housed under standard laboratory conditions on a 12-hour light/dark cycle, and fed with standard chow and water ad libitum.

Thirty-two rats were randomly divided into 4 groups with 8 animals in each (half male and half female). All the animals were treated for a period of 14 days: Group I rats received the same volume of saline solution, and served as a normal control; Group II rats (the model group) were treated with only a combination of two anti-tubercular drugs (75 mg/kg rifampicin and 75 mg/kg isoniazid); Groups III and IV rats respectively received 1.5 and 3.0 g/kg bw AEFW intragastrically, 6 h prior to the anti-tubercular drugs (9). On the 15th day, 16 h after the last administration of treatment, all animals were sacrificed by arterial exsanguinations while under deep anesthesia. The blood was collected by left ventricular arterial bleeding and the liver was dissected.

Plasma biochemical parameters

Blood samples were kept for 30 min in dry test tubes. The supernatant layer was centrifuged for 10 min at 3000 rpm to separate the serum. Alkaline phosphatase (ALT) and aspartate transaminase (AST) levels were detected using ALT and AST activity assay kit (Nanjing Institute of Bioengineering, China) (10).

Histopathological examination

The liver tissues were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin wax. Sections about 5 µm thick were made using a rotary microtome and stained with hematoxylin and eosin (H&E) and Masson. Morphological changes and fibrosis were observed in the liver samples using a light microscope.

Immunohistochemistry assay

De-waxed liver tissues, heated to facilitate antigen retrieval, were treated with 3% hydrogen peroxide to block endogenous peroxidase and with goat serum to

block non-specific binding sites. Then MyD88 monoclonal antibody (1:100), NF- κ B p65 monoclonal antibody (1:100), and Phospho-NF- κ B p65 (P-NF- κ B p65) polyclonal antibody (1:100) were added and the mixture incubated overnight at 4°C in a humid chamber. This was followed by horseradish peroxidase-conjugated marked secondary antibody. The peroxidase complex was visualized with 3,3'-diaminobenzidine (DAB) and, lastly, counterstained with hematoxylin, dehydrated and mounted. The positive expression of DAB staining (brown yellow) was observed under a light microscope.

Immunoblot analysis

Ten times volume of pre-cooled tissue homogenate were prepared by homogenizing the liver tissues, and centrifuged at 1500 rpm for 30 s. The Bicinchoninic Acid (BCA) method was used to measure the concentration of supernatant. The equivalent protein samples were loaded and separated by 10% SDS-polyacrylamide gel electrophoresis. Next, the gel-separated proteins were transferred onto a polyvinylidene fluoride membrane (PVDF). Next, the nonspecific sites of the membrane were blocked with 5% nonfat dried milk in 50 mL Tris-buffered saline containing 0.1% Tween-20 (TBST) for 30 min. Subsequently the specific primary antibodies MyD88 monoclonal antibody (1:1000, SAB), NF- κ B p65 monoclonal antibody (1:1000, SAB), P-NF- κ B p65 polyclonal antibody (1:1000, SAB) or anti β -actin antibody (1:2000, SAB) were added and the mixture incubated overnight at 4°C on a shaker. The samples were washed three times with TBST, secondary Horseradish Peroxidase-Linked (HRP-Linked) Sheep Anti-Mouse/

Table I. Phytochemical constituents of aqueous wampee fruit extract.

Phytochemical constituents	Results
Alkaloids	-
Flavonoids	+
Phenols	+
Steroids	-
Coumarine	+
Terpenoids	-
Glycosides	+
Reducing sugar	+

+indicates present; - indicates absent

Table II. Effect of aqueous wampee fruit extract on activity of alanine aminotransferase and aspartate aminotransferase.

Groups	ALT (U/L)	AST (U/L)
Normal	21.5 $\pm$ 4.8	60.4 $\pm$ 12.6
Model	48.9 $\pm$ 9.5**	143.1 $\pm$ 33.8**
Model+AEFW 1. 5	37.6 $\pm$ 8.3 #	106.5 $\pm$ 12.0##
Model+AEFW 3. 0	31.9 $\pm$ 6.6 ##	93.4 $\pm$ 15.4##

Data are presented as mean $\pm$ SEM. n=8 animals per group. **Significant at $P<0.01$, compared with normal group; #Significant at $P<0.05$, ##Significant at $P<0.01$ compared with model group. AEFW: aqueous extract from the fruits of wampee. ALT: aminotransferase; AST: aspartate aminotransferase.

Rabbit Ab was added, and the mixture incubated for 90 min at room temperature. After that, the blot was washed with TBST again three times. Subsequently, chromogenic reagent Electrochemiluminescence (ECL) was used to develop the PVDF membrane. Finally, the PVDF membrane was exposed to X-ray film in a darkroom. The strip densities were quantified using Image J software.

Statistical analysis

All data are expressed as means $\pm$ SEM and SPSS 13.0 software was used for computations. Differences between the mean values for individual groups were evaluated with one-way analysis of variance (ANOVA) with multiple comparison tests.

RESULTS

Phytochemical screening

Phytochemical screening of AEFW revealed the presence of different chemical constituents, including coumarins, flavonoids, phenols, glycosides, and reducing sugars (Table I).

Effects of AEFW on blood biochemical parameters

When the model group was compared to the control group, serum ALT and AST levels were significantly higher in the model group ($P<0.05$ and

Table III. Effect of aqueous wampee fruit extract on Myd88, NF- κ B p65 and Phospho- NF- κ B p65 expression in liver of rats with anti-tubercular drugs-induced hepatotoxicity.

Groups	Myd88 (%)	NF- κ B p65 (%)	P-NF- κ B p65 (%)
Normal	0.07 $\pm$ 0.01	0.15 $\pm$ 0.02	0.02 $\pm$ 0.01
Model	0.52 $\pm$ 0.07**	1.17 $\pm$ 0.21**	0.53 $\pm$ 0.07**
Model+AEFW 1.5	0.31 $\pm$ 0.04 #	0.31 $\pm$ 0.04 ##	0.25 $\pm$ 0.03 ##
Model+AEFW 3.0	0.12 $\pm$ 0.02 ##	0.19 $\pm$ 0.03 ##	0.13 $\pm$ 0.02 ##

The integral optical density (IOD) was quantitated by Image-Pro Plus 6.0 analysis. Data are presented as mean $\pm$ SEM, n=8 animals per group. **Significant at $P<0.01$, compared with normal group; #Significant at $P<0.05$, ##Significant at $P<0.01$, compared with model group. AEFW: aqueous extract from the fruits of wampee; P-NF- κ B p65: Phospho-NF- κ B.

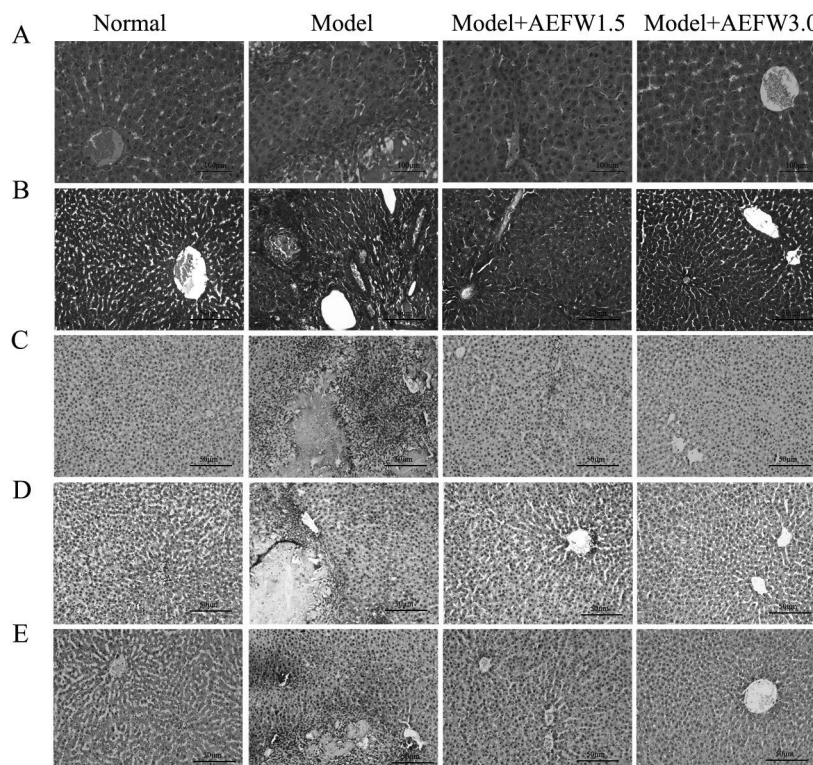


Fig. 1. Histopathological comparison of liver tissue of rats. **A)** H&E staining. Normal: normal lobular architecture and cell structure; Model: extensive liver damage with the presence of inflammatory cell infiltration and necrosis; Model+AEFW1.5: median inflammation and hepatocellular necrosis; Model+AEFW3.0: mild inflammation and hepatocellular necrosis. **B)** Masson and H&E staining. Normal: no significant collagen deposition; Model: substantial collagen deposition in the periportal and pericellular area; Model+AEFW 1.5: less collagen deposition; Model+AEFW 3.0: no significant collagen deposition. **C-E)** Immunohistochemistry staining of Myd88, NF- κ B p65, Phospho-NF- κ B p65 (P-NF- κ B p65): brownish yellow granules on cytoplasm presents positive cells. AEFW: aqueous extract from the fruits of wampee

$P < 0.01$, respectively). When the model group was compared to the AEFW group, the serum ALT in the 1.5 g/kg and 3.0 g/kg AEFW groups had significantly decreased by 23.1% and 34.8%, respectively. Serum AST had significantly decreased by 25.6% and 34.7%, respectively (Table II).

Histological studies

As shown in the H&E, AEFW treatment apparently ameliorated liver damage in a dose-dependent manner, as demonstrated by reduced

inflammatory cell infiltration and hepatocyte necrosis (Fig. 1A). The Masson staining results also showed that the collagen deposition in ADIH rats was greatly decreased after treatment with AEFW (Fig. 1B). These findings were in accordance with the levels found for serum transaminase.

Effects of AEFW on MyD88, NF- κ B p65 and P-NF- κ B p65 expression

Immunohistochemical analysis and Western blotting show that MyD88, NF- κ B p65 and P-NF- κ B

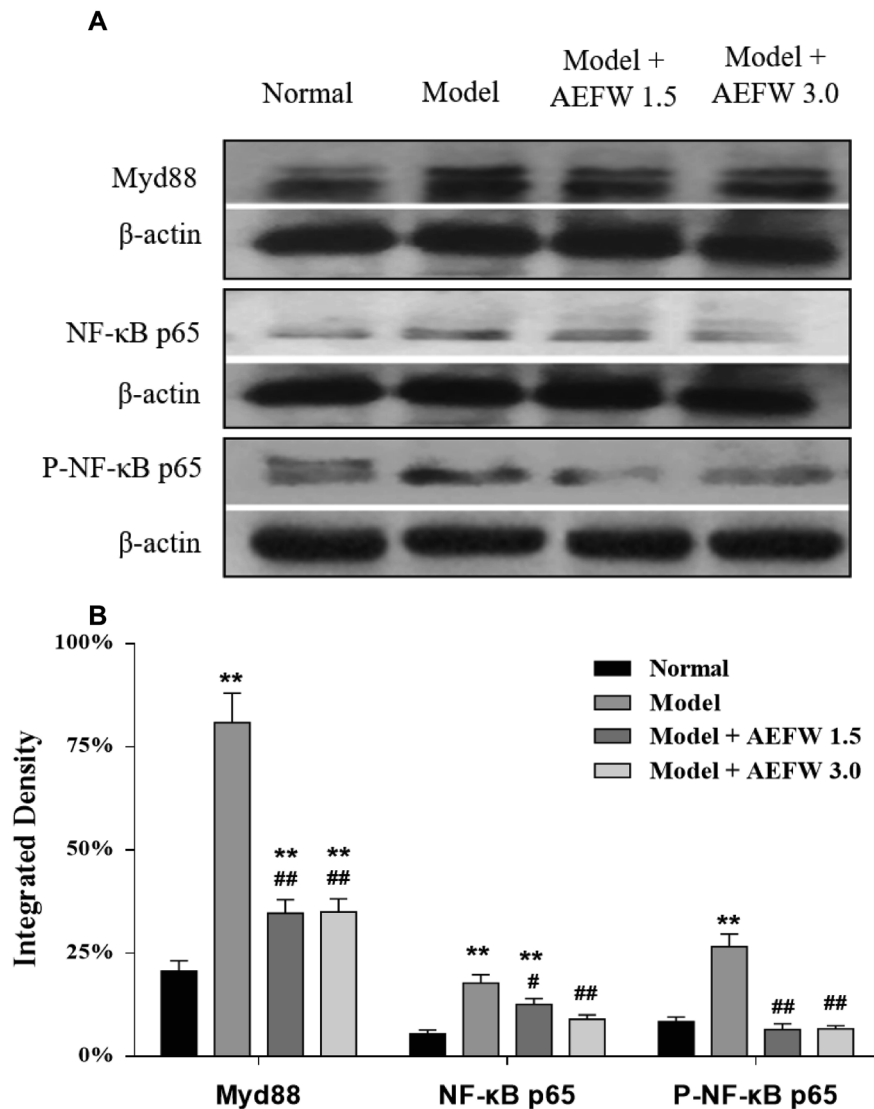


Fig. 2. Western blot of MyD88, NF- κ B p65 and Phospho-NF- κ B p65 of liver tissue in rats. Data are presented as mean $\pm$ SEM. $n=8$ animals per group. ^{**}Significant at $P < 0.01$, compared with normal group; [#]Significant at $P < 0.05$, ^{##}Significant at $P < 0.01$, compared with model group. AEFW: aqueous wampee fruit extract.

p65 expression were significantly increased in model rats, compared to the normal control rats ($P < 0.01$). However, AEFW treatment apparently decreased MyD88, NF- κ B p65 and P-NF- κ B p65 expression in a dose-dependent manner compared with the model group ($P < 0.05$) (Fig. 1C-F, Fig. 2, Table III).

DISCUSSION

Hepatotoxicity is the most common adverse effect of anti-TB drugs. The majority of ADIH events occur within the first 30 days when a combination of INH/RFP is used (34). In the present study, our results revealed that this combination remarkably increased the serum levels of liver enzymes (ALT and AST) and caused inflammatory cell recruitment and liver fibrosis, confirming its hepatotoxicity in rats. In this model, on administration of AEFW (1.5 and 3.0 g/kg) in the presence of an INH and RFP combination, the hepatic damage induced by ADIH was partly reversed. To further characterize the mechanism of AEFW in the rat model of ADIH, AEFW significantly decreased MyD88 and NF- κ B expression, inhibited NF- κ B phosphorylation, and consequently inactivated the downstream-related enzymes and cytokines.

In phytochemical screening, AEFW shows active chemical constituents, like flavonoids, phenols, glycosides and reducing sugars, which have been widely reported to be responsible for antioxidant activity (11-12). It is an important factor indicating that the mechanism of AEFW against ADIH could involve reducing oxidative stress.

This study demonstrated that aqueous extract of wampee fruit has hepatoprotective effects against anti-tubercular drug-induced liver damage. The mechanism is closely linked to suppressing the inflammatory response by inhibiting the activation of the MyD88-mediated NF- κ B signaling pathway. Aqueous extract of wampee fruit might be further developed as potential medicine for anti-tubercular drug-induced liver diseases.

Ethics approval

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Guangdong

Laboratory Animal Monitoring Institute and the National Laboratory Animal Monitoring Institute of China. The experiments were conducted according to the protocols approved by Specific Pathogen Free animal care of the Animal Center of Guangdong Medical University, and approved by the Academic Committee on the Ethics of Animal Experiments of the Guangdong Medical University, Zhanjiang, People's Republic of China, Permit Number: SYXK (Canton) 2015-0147.

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LETTER TO THE EDITOR

INTERDEPENDENCE BETWEEN AN EXPRESSION OF THE *ATG9A* GENE AND THE *BAX* GENE IN COLORECTAL CANCER

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Despite great progress in colorectal cancer (CRC) prevention, early recognition and treatment, a high frequency of morbidity and mortality of CRC patients is still observed even in developed countries. Molecular characterization of this tumor becomes a standard procedure allowing for application of personalized therapy. However, searching for new biomarkers and for new individual therapeutic strategies is increasingly desirable. In this study on molecular background of CRC, we focused on analyses of mRNA levels of autophagy gene *ATG9A* and pro-apoptotic gene *BAX*. Genes involved in autophagy, that is the catabolic and conservative cellular process, have been revealed as a promising new cancer biomarker as well as anti-cancer target. Under normal circumstances autophagy occurs at a low, basal level in most human cells, however in cancer cells its expression varies from down- to upregulation. Despite the fact that the complex link between autophagy and apoptosis is well documented, these interrelations have not yet been fully uncovered. The relative expression of mRNA values of *ATG9A* and *BAX* genes in colorectal cancer samples vs normal adjacent tissues was assessed by Real-time PCR with Universal Probe Library. The results of our study revealed a statistically significant correlation between the expression of *BAX* and *ATG9A* genes, showing that in CRC a higher expression of *BAX* gene is connected with lower expression of *ATG9A*.

To the Editor,

Colorectal cancer (CRC) belongs to the group of most frequent cancers in developed countries and is related to a high rate of cancer-deaths (1). Understanding the mechanisms underlying the individual susceptibility to CRC is vital for identification of key genes involved in cancer cell proliferation, differentiation and metastases formation and is therefore one of the main steps

in establishing of an optimal clinical treatment. Autophagy seems to be a promising process for seeking new biomarkers, therefore, may hasten our knowledge of potential therapeutic molecular targets in CRC treatment.

Autophagy is a highly conserved process enabling cell survival under adverse conditions (2). Briefly, during autophagy some parts of cytosol containing redundant proteins, lipids or organelles are engulfed

Key words: autophagy; apoptosis; colorectal cancer; relative expression; ATG genes

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by autophagosomes. Thereafter, macromolecules are digested by lysosomal acidic hydrolases and are finally recycled (2). Deregulation of autophagy has been proved to be linked to different pathologies, such as psoriasis, neurodegenerative disorders and cancer development (3). In cancerogenesis, the cross-talk of autophagy and apoptosis is well documented, hence in the present study focused on establishing the relationships between autophagy and apoptosis, relative expression of two chosen autophagy (*ATG9A*)/apoptosis (*BAX*) genes between colorectal cancer and adjacent normal tissue were compared.

ATG9A regulates autophagosome formation and is the only multispinning transmembrane protein in yeast and mammals (4-6). Physiologically *ATG9A* cycles between endosomes and trans-Golgi network (TGN) the natural reservoir of *ATG9* (6). During induction of autophagy, *ATG9A* is recruited and inserted into the outer bilayer of the phagophore, however, it is not integrated within mature/complete autophagosome membrane (5, 7). The pro-apoptotic protein *BAX* is involved in the activation of Caspase cascade which leads to cell death.

MATERIALS AND METHODS

The studied groups were precisely characterized in our previously published manuscript (8). Briefly, the study group consisted of 73 patients suffering from colorectal cancer. RNA was isolated from the tumor as well as normal corresponding tissue from each patient for Real-Time PCR analysis. Informed consent was obtained from all individual participants included in the study. The study design was accepted by Wrocław Medical University Ethics Committee (approval number KB-822/2012). The methods of molecular as well as statistical analyses were described previously. mRNA was extracted

from tumor specimens and noncancerous adjacent tissue, then, after quality control, the synthesis of cDNA was performed. We employed the RealTime ready Assay with Universal ProbeLibrary (Roche) with *ATG9A* gene (assay ID 132996) and *BAX* gene (assay ID 142318). For normalization of Real-time PCR results three reference genes were employed (8). For statistical analysis, the $2^{-\Delta\Delta C_t}$ method, Student's *t*-test, Spearman's correlation coefficient and Benjamini-Hochberg procedure were applied.

RESULTS

In this section, we show results obtained for *ATG9A* gene; results for *BAX* gene were published elsewhere (8). The relative expression level of *ATG9A* gene was as follows: in normal cells, Δ_N was 6.7145 with 95% CI [6.5362, 6.8928] and for tumor cells, Δ_T was 6.9464 with 95% CI [6.7041, 7.1887].

The $\Delta\Delta$ value and $2^{-\Delta\Delta C_t}$ (relative quantification) of *ATG9A* gene was: -0.2576 with 95% CI [-0.5580, 0.0428] and 1.1955 95% with CI [0.9708, 1.4722] respectively, with *p* value=0.08989 (no significant change).

Associations with differences in expression levels between tumor cells and healthy cells

We found a statistically significant correlation between an increase in the expression of *BAX* gene and decrease in the expression of *ATG9A* gene in tumor cells (Table I).

DISCUSSION

The *ATG9A* acts as a key regulator of inflammatory response (9). The double-stranded DNA (dsDNA) from pathogens, such as bacteria or DNA-viruses, may induce innate immune responses (via type I

Table I. Correlation between expression level of *BAX* gene and expression level of *ATG9A* gene.

Gene1	Gene 2	Spearman's correlation coefficient	p-value
<i>BAX</i>	<i>ATG9A</i>	R = -0.333	P = 0.004

interferon and pro-inflammatory cytokines) as a defense mechanism against infection (9). It has been shown that *ATG9A* is engaged in the dsDNA-induced innate immune response along with the stimulator of IFN genes (STING) (9).

Recent analyses of chromatin interactions which were based on genome-wide association studies (GWAS) revealed, that among others, the *ATG9A* is a new candidate gene in inflammatory bowel disease (IBD) (10).

It has been shown that *ATG9A* plays a vital role in autophagy induced by hypoxia after anti-angiogenic treatment of glioblastoma (GBM) (11). In all GBM cells only *ATG9A* was transcriptionally activated, indicating its crucial role in this type of tumor and it seems to be a promising target in GBM therapy (11). Thus, in primary GBM, targeting autophagy via *ATG9A* may lead to higher cell death index and also may sensitize tumor cells to anti-angiogenic as well as to immune therapies (11).

During the past decade, a correlation between autophagy and apoptosis has been found at a molecular level as numerous ATG proteins are engaged in apoptosis and *vice versa*, apoptotic genes are engaged in autophagy. Apoptosis may control autophagy and in turn, autophagy may control apoptosis (in both cases increasing or decreasing the process) (12). These connections seem to be important for the understanding of e.g. ineffectiveness of anti-cancer therapies, hence its elucidation may be helpful in introducing new therapeutic strategies.

To conclude, here we show for the first time the direct dependence between the expression of *ATG9A* (which encodes the only multispansing membrane protein from ATG protein family) and pro-apoptotic *BAX* mRNA expression in CRCs.

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LETTER TO THE EDITOR

IS THERE AN UNRECOGNIZED TYPE OF MASKED HYPERTENSION – “WEEKEND MASKED HYPERTENSION”?G. KORACEVIĆ^{1,2}, M. STOJANOVIĆ³ and D. LOVIĆ⁴*¹Clinic for Cardiovascular Diseases, Clinical Center Nis, Serbia; ²Faculty of Medicine, Niš University, Serbia; ³Institute for Treatment and Rehabilitation Niška Banja, Serbia; ⁴Clinic for Internal Diseases Inter Medica, Niš, Serbia**Received August 23, 2018 – Accepted December 3, 2018*

There is no reason to believe that excessive alcohol consumption during weekends substantially increases the risk of atrial fibrillation (“Holiday heart syndrome”) but it is not the case with the risk of blood pressure (BP) elevation. Practical implication of this “Weekend - Masked arterial hypertension (MAHT)” is to record 24-h ambulatory BP monitoring also during the weekend, in patients at high risk of MAHT, if recordings during workdays are normal.

“Holiday heart syndrome”

Excessive alcohol consumption is a well-documented risk factor for atrial fibrillation (AF) (1). The Framingham Study revealed elevated risk of AF following the chronic intake over 36 grams of ethanol per day (over 3 alcohol units daily) (2). Meta-analysis confirmed this for males and documented the AF threshold for females (>2 alcohol units per day) (3).

The latest meta-analysis (of 361,254 individuals) revealed that any ethanol intake increased the risk of arterial hypertension in men and confirmed the previous cut-off for women (4).

AF has been found in 15-20% of people with acute ethanol intoxication (5). As many as 60% of individuals with binge drinking can have an episode of AF (6). Supraventricular arrhythmias, most commonly AF, can occur in apparently healthy people usually during/after holidays or weekends (when ethanol is consumed excessively) which is how the

syndrome got its name “holiday heart syndrome” (6, 7). It was coined in 1978 by Ettinger and co-workers (8). This arrhythmia usually disappears after alcohol withdrawal without any residual cardiovascular disease (3, 9).

Multiple pathophysiologic mechanisms play a role in the genesis of the holiday heart syndrome, including sympathetic overactivity (1).

Excessive alcohol usage - known risk factor for arterial hypertension (AHT) and masked AHT (MAHT).

Not only AF, but also AHT are the recognized result of high ethanol intake (10-12). The definitive proof was published in a meta-analysis: a decrease in ethanol intake reduced BP in a dose-dependent fashion (13). Blood pressure (BP) increases particularly in the morning in alcohol-related AHT. Both systolic and diastolic BPs rise following binge drinking and systolic more so than diastolic (12, 14).

Key words: masked arterial hypertension; atrial fibrillation; alcohol; ambulatory blood pressure monitoring

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Additionally, excessive alcohol intake may promote resistant AHT as well as MAHT (15-17).

One of the pathophysiologic mechanisms of alcohol-induced BP elevation is the release of catecholamines (3). MAHT has been described as a result of sympathetic overactivity, similarly to the holiday heart syndrome (1, 18)

Alcohol-induced “weekend MAHT” — an analogy with holiday heart syndrome.

A recognized cause of MAHT is the exceeding alcohol consumption which takes place mostly during weekends/holidays (commonly in the form of “binge drinking”) — just as alcohol-induced AF (14, 16, 17). This weekend excessive ethanol intake can influence probably both BP (leading to MAHT in some individuals) and heart rhythm (leading eventually to AF) (14, 19). Importantly, such scenario is further supported by the fact that alcohol-induced AHT is the recognized risk factor for AF (7).

Therefore, excessive ethanol consumption may lead to AF both A) directly and B) indirectly - by inducing [M]AHT, which contributes to AF genesis. Thus, “Weekend MAHT” may facilitate the onset of holiday heart syndrome. Excessive ethanol intake, mental and/or physical stress and high heart rate contribute to the pathogenesis of both AF and MAHT (17).

Additional possible reasons for the “Weekend MAHT”

These can be: heavy (physical) work either at home or at an additional weekend job, high level of (psychic) stress at home or during the weekend work, large salty meals, etc. For example, the relationship between stress in the work-place and MAHT is well-documented (20).

In some individuals excessive alcohol intake, heavy work, stressful situations and their combinations may occur predominantly during weekends and they presumably can induce MAHT. Moreover, close analogy to the holiday heart syndrome makes the “Weekend MAHT” very probable. The best way to explore this possibility is to record ambulatory BP monitoring (ABPM) additionally during the weekend. In situations when MAHT seems likely to

be induced, but ABPM performed during workdays does not confirm it, the solution may be to repeat ABPM during the weekend, particularly if an individual drinks alcohol excessively.

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LETTER TO THE EDITOR

EXPLORATION OF THE INTERACTION OF IMMUNE FACTORS IN HIV/HCV INFECTION AND ITS IMPACT ON THE COURSE OF DISEASE

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The aim of this study was to explore the interaction of immune factors in acquired immune deficiency syndrome (AIDS) patients with hepatitis C infection and its effects on immune and liver function. Fasting venous blood was taken from hepatitis C virus (HCV) patients (HCV group), human immunodeficiency virus (HIV)-treated AIDS patients (HIV group), HIV-infected HCV patients (HIV/HCV group), and healthy controls (control group). Enzyme linked immunosorbent assay (ELISA) was used to detect immune factors (IL-10, IL-6, TNF- α) closely related to disease and intestinal microbial translocation products [Lipopolysaccharide (LPS), bacterial lipopolysaccharide binding protein LPS-binding Protein (LBP), soluble CD14 molecule (soluble CD14, sCD14), intestinal fatty acid binding protein (IFABP), and anti-endotoxin core antibody (EndoCAb)]. Flow cytometry was used to detect the number of peripheral blood CD4+ T cells. The immune factors closely related to the disease after HIV and HCV infection change in microbial translocation products, and correlations between peripheral blood CD4+ T cell counts and immunization, microbial translocation are compared. The results show that the concentrations of immune factors and intestinal microbial translocation products in the peripheral blood of the HIV/HCV group were higher than those in the HIV and HCV groups. HIV and HCV interact with the intestinal microbial translocation products LPS, LBP, sCD14, and IFABP. There were no significant correlations between immune factor and microbial translocation products in the HIV/HCV group and the number of CD4+ T cells in peripheral blood. In conclusion, after HIV is co-infected with HCV, the systemic immune system is activated, releasing a large number of immunostimulatory factors, further damaging the body. Due to the combined effect of HIV and HCV virus, the liver is further damaged, and the degree of damage mainly depends on the degree of HCV infection and microbial displacement.

To the Editor,

Acquired Immune Deficiency Syndrome (AIDS) is a fatal infectious disease caused by human immunodeficiency virus (HIV) infection and characterized by systemic severe immunodeficiency (1, 2). Hepatitis C is a viral hepatitis caused by hepatitis C virus (HCV) infection (3), which is mainly transmitted by blood transfusion, acupuncture, and

drug abuse. After the HIV virus enters the body, it mainly concentrates on the intestinal lymphoid tissue, multiplies and attacks CD4+ T cells, and releases various immunostimulatory factors, and the intestinal mucosal barrier is weakened (4). The mortality rate of HIV co-infection with HCV is higher than that of HIV-infected or HCV-infected patients. Although the prognosis of AIDS patients is improved after receiving

*Key words: AIDS; HIV; HCV; immune factor; intestinal microbial translocation product**Corresponding Author:*

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antiviral therapy, HIV and HCV viruses have complex interactions in the body (5), which makes the course of infection of the co-infected patients diverse and difficult to treat. Progression of liver fibrosis in HIV and HCV co-infected patients is faster than in patients with HCV alone, and increased metabolites of intestinal microbial translocation may play an important role in HIV/HCV co-infection (6, 7). The immune factor and intestinal microbial translocation of HIV-infected patients with hepatitis C infection and HIV- or HCV-infected patients were compared. The correlation between the number of peripheral blood CD4⁺ T cells and immune and microbial translocation in HIV-infected patients with HCV was analyzed.

MATERIALS AND METHODS

Patients

Thirty patients with HIV infection alone, 30 patients with HCV infection alone, 30 patients with HIV/HCV coinfection who were admitted to the anti-virus clinic of Wuwei District Center for Disease Control and Prevention from March 2016 to March 2018, and 30 healthy volunteers undergoing routine physical examination at the same center were enrolled in this study. Demographic information, such as name, gender, age, height, weight, and their medical history as well as epidemiological data were collected. Written informed consent was obtained from all participants prior to the experiment and all procedures were approved by the Ethics Committee of Wuwei District Center for Disease Control and Prevention.

Diagnostic criteria: Patients with HIV infection alone were diagnosed according to the Diagnostic Criteria for AIDS and HIV Infection (WS293-2008); patients with HCV infection alone were diagnosed according to the Diagnostic Criteria for Viral *Hepatitis C* (WS213-2008); patients with HIV/HCV coinfection were diagnosed according to the two criteria mentioned above.

All the patients were aged 20-60 years with expected survival of more than 2 years; the number of CD4⁺ T cells was 200~1000 / μ L; all HIV-infected patients had undergone anti-HIV treatment for 1~2 years; all patients had normal routine fecal test and fecal occult blood test. Subjects in the healthy control group had undetectable serum anti-HIV/HIV RNA and anti-HCV/HCV RNA.

Exclusion criteria: history of digestive tract disease and mental illness; infectious diseases such as tuberculosis,

hepatitis B virus infection, typhoid fever; serious damage of the heart, liver, kidneys and hematopoietic system; tumors; administration of antibiotics, microecological agents, sedatives, or sleeping pills in the previous 4 weeks; pregnancy or lactation; history of abortion in the past six months.

Sample preparation

Automatic microplate reader (Bio Tek, USA); PFE-5 automatic Elisa clippers machine (Changsha Pingfan instrument and Meter Co., Ltd., China); Bio-Tek Elx50 (Bio Tek, USA).

Five mL of venous blood were collected in a vacuum anticoagulation tube (Shenzhen Meirui Medical Technology Co., Ltd., China) on admission and centrifuged at 3000 r/min for 10 min. The plasma was collected and stored at -20°C. The numbers of CD4⁺ T cells in the patients of the HIV-infection alone group and HIV/HCV coinfection group were counted by flow cytometry. The concentration of IL-10, LPS, EndoCAb, IL-6, sCD14, TNF- α , IFABP and LBP was detected by ELISA (Shanghai Enzyme Biotechnology Co., Ltd., China). The serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TBil) were measured by an automatic biochemical analyzer (Dx C800) (Beckman, USA).

ELISA

The entire detection process was carried out in an Hfsafe-1200TE full-row biological safety cabinet (Shanghai Lishen Scientific Instrument Co., Ltd., China). The specimen was thawed and all reagents were allowed to reach room temperature before use. Fifty μ L of prepared standard and samples were added to wells, then 100 μ L of antibody labeled with HRP was added to each well. The plate was covered, and incubated in a 37°C incubator for 2 h, after which 50 μ L of stop solution was added to each well. The absorbance of each well was measured at a wavelength of 450 nm. Curve-fitting statistical software was used to plot a linear regression equation to the standards and then the results were calculated for the test samples.

Statistical analysis

Statistical analysis was carried out using the Statistical Program for Social Sciences (SPSS 19.0 for Windows; SPSS Inc., Chicago, IL, USA). Data were presented as the mean $\pm$ standard deviation. Difference of variables was

examined by Student's *t*-test. $P < 0.05$ (two-tailed) was considered statistically significant.

RESULTS

Comparison of immune factors in each group of patients

As shown in Table I, the average concentrations of plasma IL-10 in the HIV/HCV coinfection group, HIV group, HCV group and control group were 33.88 pg/mL, 28.46 pg/mL, 23.72 pg/mL and 9.82 pg/mL, respectively.

Comparison of related immune factors

As shown in Fig.1, patients with HIV/HCV coinfection had significantly higher IL10 levels than the other three groups ($P < 0.05$). Patients with HCV infection alone and HIV/HCV coinfection had significantly higher IL-6 and TNF- α levels than the other two groups ($P < 0.05$).

Comparison of microbial translocation products

As shown in Fig.1, patients with HIV infection

alone and HCV infection alone had significantly higher LPS and LBP levels than those of healthy controls ($P < 0.05$), indicating that the simultaneous infection of HIV and HCV had an interaction with the intestinal microbial translocation product LPS. Patients with HIV/HCV infection had significantly higher EndoCAB levels than the other three groups ($P < 0.05$). In addition, patients with HIV infection alone, HCV infection alone and HIV/HCV coinfection had significantly higher IFABP levels and lower sCD14 levels compared with the healthy controls.

Correlation analysis between the number of peripheral blood CD4+ T cells and immune and microbial factors

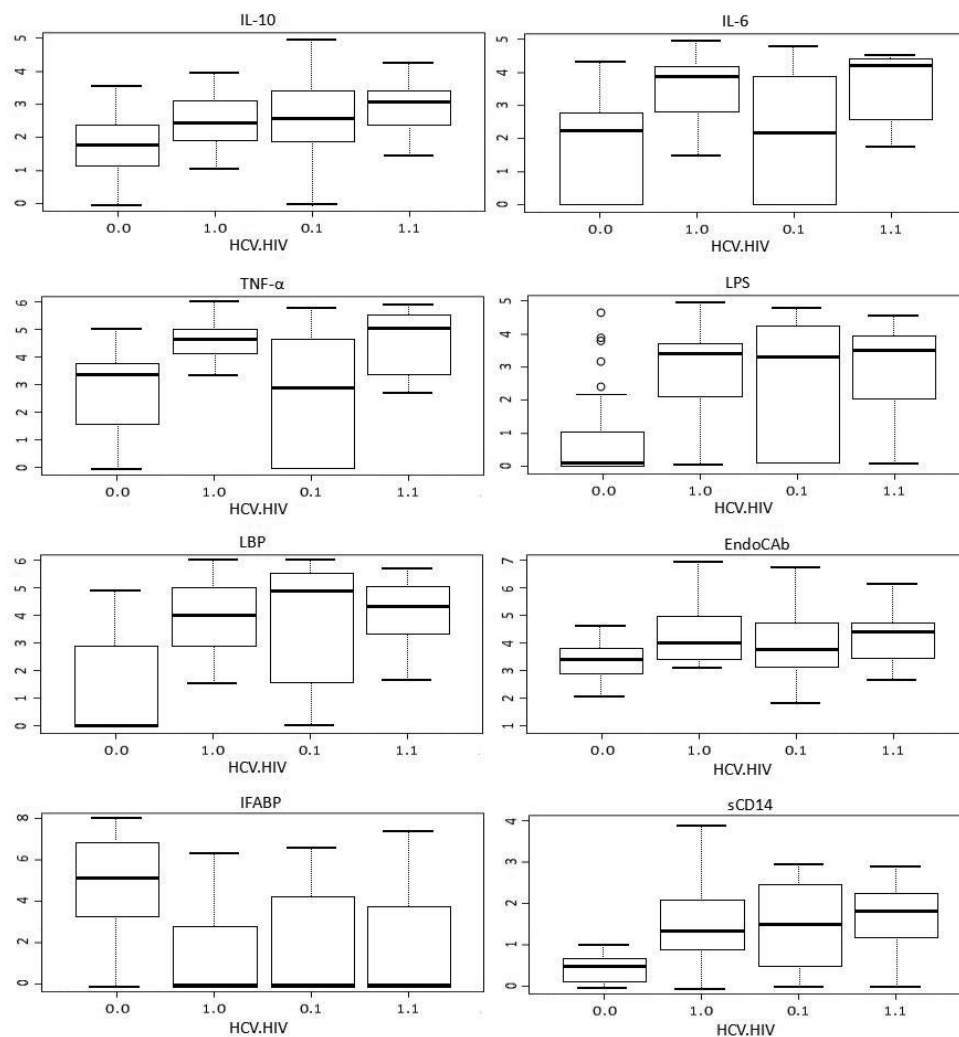
According to the CD4+ T cell counts, patients with HIV/HCV coinfection were divided into two groups, i.e., $200/\mu\text{L} \leq \text{CD4+T cells} \leq 350/\mu\text{L}$ and $350/\mu\text{L} < \text{CD4+T cells} \leq 1000/\mu\text{L}$. The immune factors and microbial translocation products were compared between the two groups, and no significant difference was observed ($P > 0.05$) (Table II).

Table I. Determination value of immune factors in plasma.

Category	Groups	Plasma concentration (pg/mL)	Category	Groups	Plasma concentration (pg/mL)
IL-10	HIV/HCV group	33.88±21.63	LBP	HIV/HCV group	42.32±15.76
	HIV group	28.46±20.16		HIV group	38.24±23.92
	HCV group	23.72±19.52		HCV group	36.64±18.66
	Control group	9.82±7.19		Control group	15.24±11.37
IL-6	HIV/HCV group	73.13±50.32	EndoCAB	HIV/HCV group	110.36±80.63
	HIV group	31.45±21.27		HIV group	102.15±84.92
	HCV group	52.74±37.93		HCV group	100.15±76.13
	Control group	15.12±10.64		Control group	41.23±31.22
TNF- α	HIV/HCV group	125.83±90.13	IFABP	HIV/HCV group	335.83±183.94
	HIV group	59.16±41.36		HIV group	95.03±58.28
	HCV group	125.32±80.35		HCV group	131.48±98.18
	Control group	30.97±23.74		Control group	801.43±372.19
LPS	HIV/HCV group	30.21±16.12	sCD14	HIV/HCV group	7.14±4.98
	HIV group	26.04±13.92		HIV group	6.68±4.23
	HCV group	29.31±14.43		HCV group	6.59±5.92
	Control group	7.25±5.15		Control group	1.63±0.47

Table II. Peripheral blood CD4+ T cells in the HIV/HCV group and Pearson correlation coefficient R^2 of each factor.

	IL-10	IL-6	TNF- α	LPS	LBP	EndoCAb	IFABP	sCD14	CD4+T
IL-10	1.00	0.56	0.63	0.45	0.77	0.86	-0.04	0.71	0.24
IL-6	0.56	1.00	0.87	0.72	0.75	0.58	-0.07	0.54	-0.11
TNF- α	0.63	0.87	1.00	0.73	0.92	0.51	-0.04	0.66	-0.13
LPS	0.45	0.72	0.73	1.00	0.75	0.34	-0.07	0.46	-0.12
LBP	0.77	0.75	0.92	0.75	1.00	0.71	0.01	0.66	-0.03
EndoCAb	0.86	0.58	0.51	0.34	0.71	1.00	0.02	0.56	0.22
IFABP	-0.04	-0.07	-0.04	-0.07	0.01	0.02	1.00	-0.07	0.11
sCD14	0.71	0.54	0.66	0.46	0.66	0.56	-0.07	1.00	-0.03
CD4+T	0.24	-0.11	-0.13	-0.12	-0.03	0.22	0.11	-0.03	1.00

**Fig. 1.** Significant difference test between groups of immune factors and intestinal microbial translocation markers (0.0: control group; 1.0: HCV group; 0.1: HIV group; 1.1: HIV/HCV group).

Analysis of liver function among the four groups

Alanine transaminase (ALT), aspartate transaminase (AST) and total bilirubin (TBil) were also compared among the four groups. The results showed that patients with HCV infection had significantly higher ALT levels than those of patients in HIV infection and the healthy control group. There was significant difference among HIV/HCV, HIV, and HCV groups ($P < 0.001$). The difference in AST between the HIV group and the healthy control group was also significant ($P < 0.05$). There was no significant difference in TBil among the four groups.

DISCUSSION

The decrease in HIV-specific immunity may be attributed to the inhibition of HIV-specific effector T cells. For example, IL-10 can inhibit immune responses to pro-inflammatory factors (8, 9). In our study, simultaneous infection of HIV and HCV did not influence the patient's immune factor IL-6. Although the IL-6 concentration in the HIV/HCV coinfection group was higher than that in the other groups, it could not be confirmed by the co-infection of HIV and HCV, which may be attributed to damage of the body by HCV. However, HCV infection may be the main cause of elevated TNF- α in patients with HCV infection alone and HIV/HCV coinfection. sCD14 promoted the release of TNF- α by LPS-stimulated macrophages in a dose-response fashion (10). This study suggested that simultaneous infection of HIV and HCV exacerbated the extent of intestinal microbial translocation and caused an increase in the serum sCD14 level. Following the gastrointestinal diseases caused by HIV infection, intestinal microbes were transferred to the systemic blood circulation. When immune responses were produced for the microbial translocation products such as LPS, a large amount of EndoCAB IgM and LPS was produced, reducing the damage to liver caused by LPS (11, 12). It was found that when AIDS patients were coinfecting with HCV, disorder of the systemic immune system was produced, the immunity was reduced, and the ability to clear LPS and LBP was also weakened. In addition, the degree of intestinal microbial translocation was closely related to the body inflammation caused by HCV infection,

and the liver damage was also aggravated after HCV infection. Moreover, the combined effects of HIV and HCV infection caused worse liver damage than HIV or HCV infection alone.

In conclusion, HIV and HCV coinfection could induce stronger immune responses than HIV or HCV infection alone, resulting in a greater release of immune factors, impaired intestinal barrier, increased intestinal microbial shift, and hence aggravated liver damage.

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LETTER TO THE EDITOR

EXPRESSION OF p16 AND p27 PROTEIN IN CERVICAL EXFOLIATED CELLS AND ITS RELATIONSHIP WITH HIGH RISK HUMAN PAPILLOMA VIRUS IN CERVICAL LESIONSH. MIAO¹, B. CAO², W. GE¹ and W. ZHAO¹*¹Obstetrics and Gynaecology, The Second Affiliated Hospital of Mudanjiang Medical University, Mudanjiang China; ²Physical Diagnostic Section, The Second Affiliated Hospital of Mudanjiang Medical University, Mudanjiang, China**Received July 24, 2018 – Accepted November 22, 2018*

This study aimed to investigate the expression of p16 and p27 protein in cervical exfoliative cells and explore the relationship between the expression of HR-HPV (High-risk human papilloma virus) and cervical lesions. One hundred-twenty cervical cytology specimens were selected as cases from patient cervical cancer screening. According to the double screening, results were divided into a double negative group, a single positive group, a double positive group [including Thinprep cytologic test (TCT) $\leq$ Low-grade squamous intraepithelial lesion (LSIL)] and an HPV positive group, [TCT $\geq$ High-grade squamous intraepithelial lesion and HPV positive group (HSIL)]. Flow cytometry was used to detect the expression of p16 and p27 protein in the specimens of the exfoliated cells of the cervix. The result showed that the expression of p16 in the TCT $\geq$ HSIL group was higher than other groups ($P < 0.05$), and the expression of p27 was lower than the other groups ($P < 0.05$). In group CIN3, the expression of p16 and p27 was $P < 0.01$ compared to the other groups. The positive rate of the A9 group in group CIN3 and the inflammatory and CIN1 group was statistically different ($P < 0.05$). Moreover, in the single positive groups (group A9, group A5/6, A7 group, and mixed-infection group), the infection rate of group A9 was significantly higher than that of the other groups. With the progression of cervical lesions, the expression of p16 increased and the expression of p27 decreased, and both expressions were associated with HR-HPV infection.

To the Editor,

The condition for cervical cancer occurrence and development requires multiple factors and multiple gene involvement, from the development of precancerous lesions for cervical cancer, generally after cervical intraepithelial neoplasia grade 1 (CIN1), cervical intraepithelial neoplasia grade 2 (CIN2), and cervical intraepithelial neoplasia grade 3 (CIN3), which takes about 10 years (1). Many studies have shown that the main cause of cervical cancer is

the persistent infection of HR-HPV (2, 3). Chen et al. (4) pointed out that a higher HPV infection rate and a lower incidence of cervical cancer showed that an HPV infection is not the only factor in the malignant transformation of cells. Molecular pathology shows that the normal operating of the body lies in the regulation of the cell cycle (5). The proteins p16 and p27 are important negative regulatory factors in the Cyclin-CDK-CKI cell cycle regulation system (6), both play a role in the G1/S stage and are responsible

*Key words: cervical cancer; HR-HPV; regulation disorder; p16 and p27 protein**Corresponding Author:*

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for checking whether the DNA is damaged or not. Furthermore, the expression of p16 and p27 protein in cervical exfoliated cells was detected and the relationship between HR-HPV and cervical lesions is discussed.

MATERIALS AND METHODS

From January 2016 to November 2017 at the Second Affiliated Hospital of Mu Danjiang Medical University, 120 cases of cervical cytology specimens were selected from cervical cancer screenings (combined detection of TCT and Cervista™ HR-HPV). According to the double screening, the results were divided into: double negative group (24 cases), single positive group (36 cases), and double positive group (60 cases) [including: TCT = LSIL and HPV positive group (36 cases); TCT = HSIL and HPV positive group (24 cases)]. Among them, the single positive group was HR-HPV positive after at least one year of follow-up, and the double positive group was routinely examined by cervical colposcopy and biopsy. The HR-HPV positive group (single positive group) was also divided into A9, A5/A6, A7 and mixed-infection groups. Flow cytometry was used to detect p16 and p27 protein expression, the average fluorescence intensity of p16, and double screen p27 protein negative group specimens of the threshold. The expressions of p16 and p27 proteins remaining in each group were compared for differences in statistical analysis. Colposcopic cervical biopsy was performed on double positive patients (concurrently, the double positive group was divided into three subgroups: inflammatory and CIN1 group, CIN2 group, and CIN3 group). The pathological results are the gold standard of analyzing the differentially expressed proteins in different groups.

Samples

This study was carried out in the gynecologic clinic of The Second Affiliated Hospital of Mudanjiang Medical University. None of the groups were in the period of menstruation or pregnancy, and the cervical cytology had not been examined or treated in the previous 6 months. No vaginal operation had been performed during the 48 h prior to taking the material. At the time of sampling, the cervical sampling brush (disposable cervical cell sampler, Zhengzhou Zhongpu Medical Instrument Co., Ltd., China) was used to rotate 5 circles at the outer part of

the cervix under satisfactory exposure of the cervix, and the cells from the cervical surface and cervical canal were adequately brushed. After taking the materials, they were immediately placed in the cell preservation solution kit, sealed, and stored. All patients (or their families) signed an informed consent and this study was approved by the ethics committee of The Second Affiliated Hospital of Mudanjiang Medical University.

TCT cytology production

The specimens were checked, numbered, and recorded, then pre-processing of specimens (specimens with less blood and mucus need no pretreatment) was carried out: 400C medical centrifuge (Beijing Baiyang Medical Instrument Co., Ltd., China) 2500rpm/min, centrifuge 5 min, removal of supernatant. Next, 15-20 ml cell digestive juice was added, and the oscillating rocking bed of the circumference was run at 1500rpm/min up to 10 min. With the centrifuge on low speed, the solution was run 1500rpm/min centrifugally for 3 min, and the supernatant was discarded. After adding 20 mL of cell preservation solution (Beijing Yingshuoli Technology Co., Ltd., China), it was set aside for 5-10 min. The solution was then placed in the ThinPrep2000 automatic treatment instrument (Beijing Yingshuoli Technology Co., Ltd., China) to create 20 nm thin layer cells, which were mounted on glass slides and fixed with 95% alcohol. The slides were processed in hematoxylin dye for nuclear staining, then the slides were removed from the orange G6 dye cylinder and EA50 staining solution (Shanghai Bogu Biological Co., Ltd., China) in the cytoplasm. The treated glass slides were put into the TO-type transparent agent solution (Guangzhou Zhongnan Chemical Instrument Co., Ltd., China) for 2 min, so that the chromaticity and overlap of the cells would not affect examination under the microscope. Lastly, a 24 x 32 mm coverslip and neutral balata were used in preparation for the microscopic examination.

TCT cytological test

Two to three highly trained cytopathologists followed the 2001TBS diagnostic criteria for cytological diagnosis. After that, data input (lesion report mapping) was carried out, and the report was printed.

Cervista HPV DNA Extraction

In this study, HPV was detected with the TCT results

unknown. The first step was to shake a specimen bottle for 5s, and transfer 2 ml samples. 2.2 ml samples were added to a 96-well plate, which were sealed by aluminum foil. The liquid was centrifuged at 3000rpm/min for 10 min, and the supernatant was discarded, followed by rinsing twice with a distilled water aspirator. Lysate and Proteinase K (Beijing Yingshuoli Technology Co., Ltd., China) were mixed into the 5 ml cone centrifuge tube and reversed and mixed repeatedly. Then, 400 µl of the mixture was added to each sample hole. The constant temperature mixing instrument(Beijing Yingshuoli Technology Co., Ltd., China) was adjusted to 37°C, 1000rpm/min, and incubated for 15 min. Multiple reversals were combined to fully mix the liquid (Beijing Yingshuoli Technology Co., Ltd., China) and 200 µl were add to each sample hole. The 96-hole plate was laid on the thermostatic mixer for 2 min at 1000rpm/min and then put on the magnet, remaining static for 4 min, and consequently laid on the constant temperature mixing instrument. 400 µl of wash buffer was added to each sample hole and mixed for 2 min at a speed of 1000rpm/min. The 96-hole plate was then put on the magnet, remaining static for 4 min. The supernatant was discarded with a distilled water aspirator. The plate was laid on the magnet and 400 µl of 70% ethanol was added to each sample hole and incubated for 30 s. The supernatant was discarded by aspirating. The previous steps were repeated. The supernatant was flushed with a large amount of distilled water twice, and the magnetic beads were dried for 10-15 min; 120µl Tris-HCL solution in 10mmol/L was added to the bottom of the plate. The constant temperature mixing instrument 1000rpm/min was mixed for 3 min, with a 2-min oscillation interval. The 96-hole plate was placed on the magnetic plate and remained static for 10 min, and the 110 µl DNA was transferred to the skirt plate to be sealed and preserved.

Cervista HPV HR Test

To 3 holes in the 96-hole plate, 10 µl of each control and sample DNA was added with a 20 µl mineral oil cover before being sealed with plastic film. The Cervista™ reaction solution (Beijing Yingshuoli Technology Co., Ltd., China) was prepared, to which was added the reaction solution 1. HPV Oligo mix1 and Cleavase enzyme solution were added to a 2 ml test tube, to which reaction solution 2 was added. Lastly, HPV Oligo mix2 and enzyme solution were added to the test tube, then

the reaction solution was added. The three mixtures were mixed evenly around the eight-link tube, at a temperature of 95°C after incubation of 5 min using a thermal cycler (Hangzhou Bori Technology Co., Ltd., China). The temperature of the thermal cycler was reduced to 63°C, and 10 µl reaction liquid was added to the specimen; as quality control, the plastic sealing membrane and gasket were covered, and it was incubated for 4 h.

FCM tests of p16 and p27 protein

Direct immunofluorescence antibody labeling was used to treat the residual liquid-based cell preservation solution of TCT and HPV double sieves. The expressions of p16 and p27 were detected by flow cytometry. The coefficient of variation (CV) was adjusted to less than 5% during the detection period, which was adjusted by standard cell samples. Then, 10,000 cervical epithelial cells were analyzed using cellQuest™ software through the forward scattering angle/lateral scattering angle (FSC/SSC). The average fluorescence intensity of the positive cell protein = the sum of the fluorescence intensity of each positive cell/the number of positive cells.

When the results were analyzed, a negative threshold was set first. The mean fluorescence intensity of p16 and p27 protein in each specimen of the double screening negative group was the threshold value. The expressions of p16 and p27 protein in the remaining groups were analyzed statistically.

Statistical analysis

The data of this study were analyzed using SPSS 19 Statistical software. The mean fluorescence intensity of positive cell proteins in each group was expressed by mean value±standard deviation. The difference of protein expression in different groups was analyzed using an independent sample *t*-test, and the difference was statistically significant if $P < 0.05$.

RESULTS

FCM

The analysis showed that the expression of p16 in the TCT ≥ HSIL group was higher than that of the other groups ($P < 0.05$), and the expression of p27 was lower than the other groups ($P < 0.05$). The expression of p16 and p27 between TCT ≤ LSIL

group and the double negative group was statistically significantly different ($P<0.05$), however, it was not a significantly different from the expression of p16 and p27 in the single positive group ($P>0.05$). There was no significant difference in the expression of p16 and p27 between a single positive and double negative group ($P>0.05$). The specific data are shown in Table I and Fig. 1.

FCM and colposcopy biopsy

Sixty cases of double positive specimens of cervical biopsy under colposcopy were examined in accordance with the guidelines, including inflammation of 26 cases in the inflammatory and CIN1 group. The mean fluorescence intensity of p16 was 58.92 ± 2.67 , and the mean fluorescence

intensity of p27 was 280.43 ± 5.47 ; for 12 cases in group CIN2, the mean fluorescence intensity of p16 was 62.73 ± 3.62 , and the mean fluorescence intensity of p27 was 276.15 ± 5.18 ; for 22 cases in the CIN3 group, the mean fluorescence intensity of p16 was 71.36 ± 5.16 , and the mean fluorescence intensity of p27 was 265.48 ± 6.08 . The analysis showed that the expression of p16 was significantly higher in the CIN2 group than in the inflammatory and CIN1 group ($P<0.05$). Group CIN3 was significantly higher than group CIN2 ($P<0.01$). With the increase of the degree of cervical lesions in CIN, the trend of p16 up regulation was obvious ($P<0.05$). The expression of p27 in group CIN2 and the inflammatory and CIN1 group was significantly different ($P<0.05$), and there was a significant difference between the CIN3 group

Table I. FCM results and double screen results of p16 and p27 proteins in each group.

	Cases	P16	P27
Double negative	24	$57.88\pm2.32^{\&}$	$284.24\pm9.96^{\&}$
Single positive	36	60.35 ± 3.16	281.04 ± 9.14
Double positive TCT $\leq$ LSIL	36	$62.07\pm3.53^*$	$276.43\pm10.08^*$
Double positive TCT $\geq$ HSIL	24	$67.97\pm6.72^{*}\#\#\&$	$271.16\pm10.83^{*}\#\#\&$

Note: Comparison between the double negative group and others $^*P<0.05$, $^{**}P<0.01$; comparison between the TCT negative and HPV positive group and others $^{\#}P<0.01$; comparison between the TCT $\leq$ LSIL HPV positive group and others $^{\&}P<0.05$.

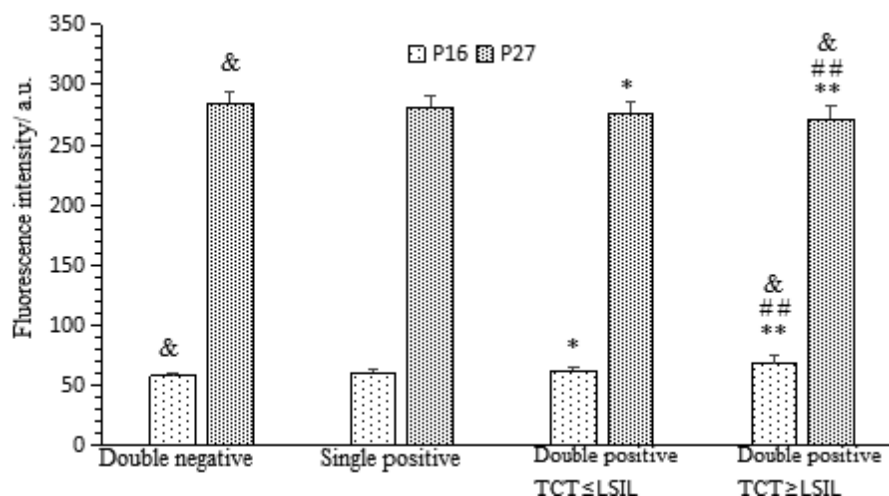


Fig. 1. The p16 and p27 double sieve results statistics.

and the inflammatory and CIN1 group ($P<0.01$). There was a significant difference between group CIN3 and group CIN2 ($P<0.01$). The specific data are shown in Fig. 2.

Of the single positive samples, the A9 group (61.53 ± 1.53) and the other group (60.24 ± 1.36) had a mean fluorescence intensity of p16 compared to $P<0.05$, and the average fluorescence intensity of p27

was 276.84 ± 6.32 , 282.2 ± 5.53 . There was significant difference between the two groups ($P<0.05$); the inflammation and CIN1 group, A9 group (60.56 ± 1.41), and the other group (58.75 ± 1.68) had a mean fluorescence intensity of p16 compared to $P>0.05$, and the average fluorescence intensity of p27 was 280.13 ± 3.49 , 284.65 ± 4.27 ($P>0.05$); for the CIN3 group, A9 group (71.04 ± 3.71), and the other

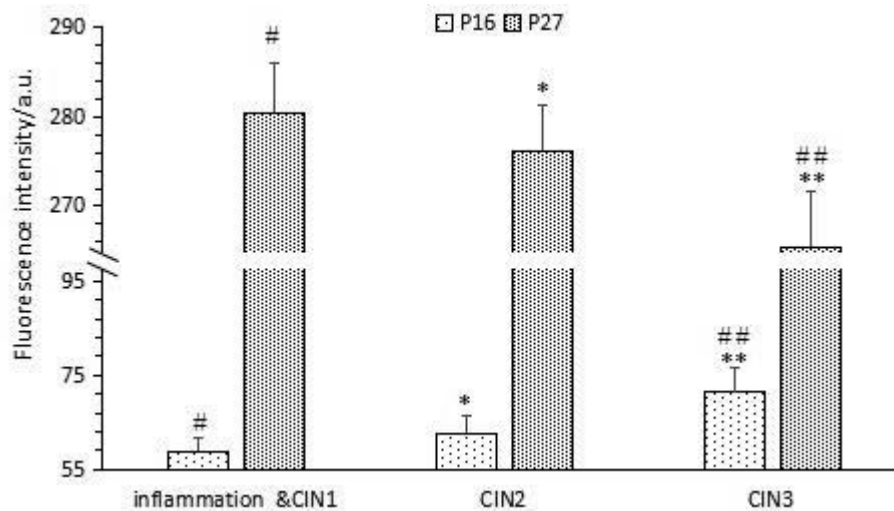


Fig. 2. The p16 and p27 fluorescence intensity statistics under colposcopy. Comparison between CIN1 and inflammation group and others * $P<0.05$, ** $P<0.01$; comparison between CIN2 group and others * $P<0.05$, ** $P<0.01$

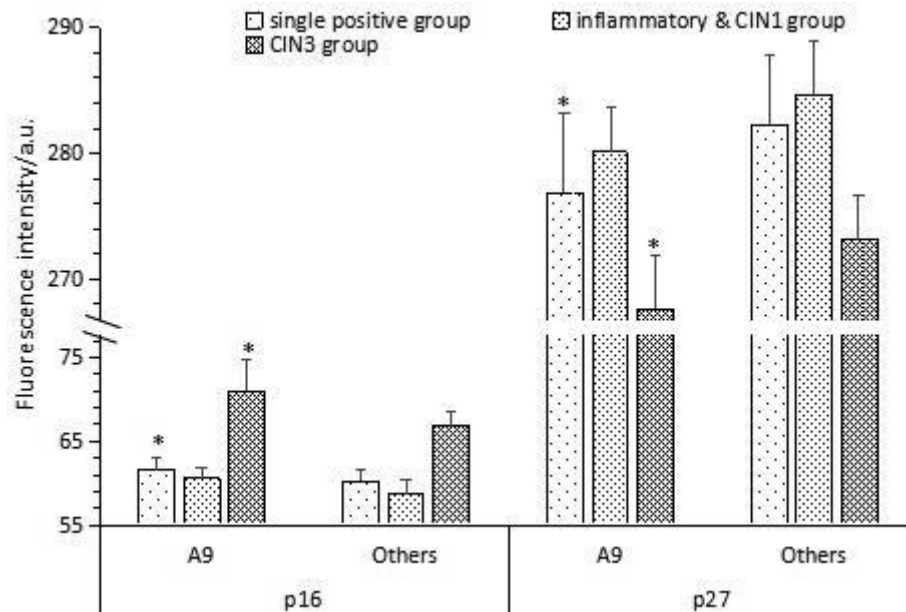


Fig. 3. A statistical graph of the expression of p16 and p27 in group A9 and other groups of viruses. When A9 is compared to others, * $P<0.05$.

group (66.83 ± 1.68), the p16 compared to the average fluorescence intensity $P < 0.05$, and the average fluorescence intensity of p27 was 267.65 ± 4.23 , 273.12 ± 3.61 ($P < 0.05$) (Fig. 3).

Infection statistics

In 60 cases of double positive specimens, CIN3 accounted for 22 cases, of which the infection rate of the A9 group was 16/22 (72.72%), and the inflammatory and CIN1 group accounted for 26 cases. The infection rate of the A9 group was 6/26 (23.08%) ($P < 0.05$), indicating that the high-risk group HPV infection rate in the A9 group was the highest.

In 36 single positive specimens, the A9 group, A5/6 group, A7 group, and mixed infection group accounted for 16/36 (44.44%), 9/36 (25%), 6/36 (16.67%), and 5/36 (13.89%), respectively. Therefore, the infection rate of the A9 group was significantly higher than that of other groups.

DISCUSSION

According to statistics, among the Chinese population, the HPV subtype of infection was dominated by type 16 in group A9 (7). In this study, in 36 cases of single positive specimens, the infection rate of group A9 was significantly higher than that of other groups. It is speculated that the incidence of cervical cancer is closely related to the HPV infection in group A9, indicating that the group A9 is closely related to high-grade lesions, which is a high-risk factor (8).

In the experiment, p27 was the highest in the normal and low grade lesions, but the lowest in the most severe group ($P < 0.01$), which is consistent with the conclusion of many scholars (9, 10). The imbalance of p27 expression is related to the infection of HR-HPV, and is closely related to the A9 subtype. This may be integrated with HPV DNA to interfere with cell cycle p53 and pRb pathways in human cell DNA, causing uncontrolled expression of tumor suppressor genes (11).

The results of this study showed that the expression of p16 in normal cervical epithelium was significantly lower than that of high level cervical

lesions ($P < 0.01$). However, there is a significant difference in the expression of p16 between the LSIL and the following lesions and normal cervix epithelium ($P < 0.01$), indicating that p16 can be used for differentiation of early lesions. Samir et al. (12) indicates that the strong positive expression of p16 in cervical tissue is often powerful evidence for the integration of HPV in the host cells. In the single positive group, the mean fluorescence intensity of p16 in group A9 was higher than that in the other groups ($P < 0.05$), and the expression of p16 in group CIN3 gave a similar outcome. The conclusion is that the expression of p16 is associated with HR-HPV infection.

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LETTER TO THE EDITOR

MOLECULAR REGULATION MECHANISM OF FARNESYL X RECEPTOR IN BILE ACID AND CHOLESTEROL METABOLISM IN HYPERLIPIDEMIC RATS

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The study was aimed to elucidate the molecular mechanism of Farnesyl X receptor (FXR), Bile salt export pump (BSEP) and sodium-taurocholate cotransporting polypeptide (NTCP) in the metabolism of bile acid and cholesterol in hyperlipidemia rats. Fifty male Wistar rats weighing 140.6±9.5g were randomly divided into two groups: the control group was given normal diet while the experimental group was given high-fat diet. Body weight was measured on a regular basis and liver weight was measured after sacrifice. Plasma glucose and lipids (total cholesterol, triglycerides, high density lipoprotein, low density lipoprotein) were determined by commercially available kits. Hepatic FXR, BSEP and NTCP gene expression and protein distribution were determined by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and immunohistochemistry. The results showed that the expression of FXR and BSEP mRNA in the experimental group was higher than that in the control group ($P < 0.05$). The expression of NTCP mRNA in the control group was higher than that in the experimental group ($P < 0.05$). Immuno-histological results showed that the positive rates of FXR, BSEP and NTCP expression in the experimental group were significantly different from those in the control group ($P < 0.05$). In conclusion, FXR and Bsep and Ntcp gene changes played an important protective role in lipid metabolism, especially for the rats with high-fat diet.

To the Editor,

Hyperlipidemia is a disease with one or more lipids higher than the normal range (1). In recent years, the incidence of hyperlipidemia has had a significant upward trend, which is frequently accompanied by disorders of cholesterol metabolism (2). Bile acid synthesis is an important metabolic pathway of cholesterol. Bile acids are secreted into the small intestine and are reabsorbed by the ileum and then transported back to the liver through the portal vein and re-secreted into the bile for ongoing enterohepatic circulation (3, 4).

The uptake of bile salts into hepatocytes occurs

mainly in a sodium-dependent manner which is mediated by sodium-taurocholate cotransporting polypeptide (NTCP). Canalicular export of bile salts is mediated by Bile salt export pump (BSEP), an ATP-binding cassette transporter. BSEP constitutes the rate limiting step of hepatocellular bile salt transport, and mutation of the BSEP gene causes severe progressive familial intrahepatic cholestasis. However, the role of FXR, BSEP and NTCP in the excretion and absorption of cholesterol into bile acids is still unclear (5). Studies have shown that decreased secretion of bile by hepatocytes results in cholestasis of bile acids and bilirubin (6, 7). It is

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believed that the conversion of cholesterol to bile acids can reduce the level of serum cholesterol, and the disorder of bile acid metabolism can lead to cholesterol metabolism disorders. As a result, our study was focused on the impact of hyperlipidemia on hepatic FXR, BSEP and NTCP gene and protein expression and effect of FXR, BSEP and NTCP in the pathogenesis of hypercholesterolemia.

MATERIALS AND METHODS

Animals

Fifty Wistar specific-pathogen free (SPF) rats (male, 140.6 ± 9.5 g) (Nanjing Junke Bioengineering Co., Ltd., China), 6 weeks of age, were selected. All animal experimental protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals. Rats were housed at $24 \pm 1^\circ\text{C}$, humidity $50 \pm 5\%$, on 12-hour light/dark cycle with ad libitum access to food and water for one week before the experiment. The protocol was approved by the ethics committee of Mudanjiang Medical University, and the experiment also followed the regulations of the State Science and Technology Commission on the management of laboratory animals.

Preparation of hyperlipidemia rat model

Pig lard was heated and melted in a water bath at 40°C . The basic feed (crude protein 18.28%, crude fat 7.4%, and dry matter 15.85 kJ/g) was added with 0.04 cholesterol, 0.1 lard, 0.05 sucrose, 0.005 sodium cholate and 0.002 propylthiouracil. The lard was mixed with water and stirred thoroughly to make a fist size pellet, dried in an oven and refrigerated for use.

Fifty Wistar rats were randomly divided into two groups: the control group (given basic feed) and the experimental group (given high-fat diet). After feeding for 90 days, the hyperlipidemia model was established. Blood was collected regularly to detect the cholesterol content by an automatic biochemical analyzer. Plasma cholesterol level was measured between 8-12 weeks to monitor the extent of hyperlipidemia in the rats.

Determination of physiological parameters

Body weight was measured monthly and pictures taken, and liver weight was detected. Blood glucose (Glu) was measured by a blood glucose meter (Zhejiang

Mingzhong Medical Devices Co., Ltd., China). Serum total cholesterol (TC), triglyceride (TG), high density lipoprotein cholesterol (HDL-C), and low density lipoprotein cholesterol (LDL-C) were detected by a blood lipid tester (Redumit Medical Equipment (Shanghai) Co., Ltd., China).

Specimen collection

All the experimental animals fasted for 24 hours prior to surgery and were not given water for 12 hours prior to the operation. Anesthesia was induced by intraperitoneal injection of 0.25% sodium pentobarbital (1mL/kg). Blood (2 mL) was collected from the abdominal aorta for biochemical testing. The first hepatic hilum was exposed and ligated, liver tissues were sliced into $2 \times 1.5 \times 1.5 \text{ cm}^3$ size pieces. Liver tissues were further washed with distilled water containing Taq (Thermus Aquaticus) enzyme and dried with sterile gauze. Part of the liver tissues were placed in aseptic cryopreservation tube and frozen at -70°C ; the remaining liver tissue was fixed in 4% freshly made formaldehyde for 48 h.

Reverse transcription - polymerase chain reaction

Total RNA of liver tissue was extracted by TRI Reagents (Shanghai Huashun Biotechnology Co., Ltd., China) according to the manufacturer's instruction. The concentration of the RNA was determined by micro-nucleic acid quantitative analyzer (Nano Drop, USA) and the concentration of RNA was determined. The samples were qualified when A260/280 reached 1.8-2.0. The RNA template, primers, dNTPMix, 5 x RTBuffer and RNase free water were dissolved and placed on ice. The reaction system was configured and the total volume was 12 μL . The reaction system contained 1 μL dNTP (10 mM), 1 μL Oligo-d T primers (100 μM) or Random primers (100 μM), 4 μL RNA Template, and 6 μL RNase-Free water.

The samples were incubated at 65°C for 5 min, followed by ice bath for 10 min and centrifugation (Cryogenic Centrifuge, Beckman, USA). The following reagents were added to the above reaction solution: 5 x 4 μL RT Buffer, 2 μL RNasin (20U/ μL), and 2 μL DTT (0.1 M 10 mM).

The samples were lightly beaten and mixed, followed by incubation at 37° for 2 min (GNP-9082 water-proof electro-thermal incubator, Shanghai Yuejin Medical Optical Device Factory, China). 1 μL HiFi-MMLV (200U/ μL) was added, gently stirred and mixed, and kept at 37°C for 50 min. After

the reaction, temporary centrifugation was performed and the reverse transcription products were stored at -20°C. The primer sequences were as follows:

Upstream primer of FXR was 5'-CCTCATTGTCTCCCCGACTTATCCT-3' and downstream primer of FXR was 5'-GCCTCTAGAAAGCAGTGTTCACTTT-3'; upstream primer of BSEP was 5'-CCCTCAACTGATGGGGCTCCAGT-3' and downstream primer of BSEP was 5'-CCCATGTCTGACTCAGTGATTCTT-3'; upstream primer of NTCP was 5'-GATGGAGGTGCACAACGTAT-3' and downstream primer of NTCP was 5'-CTGTCTCAGTTCATGGCTCC-3'; upstream primer of β -actin was used as internal control, upstream primer of β -actin was 5'-GATGGTGGGTATGGGTCAGAA-3' and downstream primer of β -actin was 5'-CTAGGAGCCAGGGCAGTAATC-3'.

The PCR reaction system included 25 μ L Taq Master Mix (2 $\times$), 2 μ L forward Primers (10 μ M), reverse primers (10 μ M) 2 μ L, 10 μ L C-solution (5 $\times$), 1 μ L template, and 10 μ L RNase-Free water, a total of 50 μ L.

The reaction conditions: pre-denatured at 95°C for 2 min, denatured at 94°C for 30 s, annealed at 50°C for 30 s, and finally extended at 72°C for 5 min.

Immunohistochemistry

Paraformaldehyde fixed liver tissues were embedded with paraffin and tissue blocks were sliced into 4- μ m thick sections (LEICA CM1850). The sections were treated at 68°C for 20 min, followed by conventional xylene dewaxing and gradient alcohol dehydration. Afterwards, the sections were incubated with 3% H₂O₂ in methanol for 10 min at 37°C to inactivate the endogenous peroxidase. Antigen retrieval (0.01 M citric acid buffer, PH6.0, 95°C, 15 - 20 min) was performed on these slides. The sections were further incubated with blocking buffer (5% normal sheep serum) at 37°C for 10min, followed by primary antibodies. They were kept in a refrigerator at 4°C overnight. Primary anti-body (Sheep anti rat FXR, 1:1000, AB100000, Britain ABCAM) was added and incubated overnight in 4°C. They were washed 3 times with PBS (Phosphate buffer saline) (Yanyu (Shanghai) Chemical Reagent Co., Ltd., China), 5 min each time (PBS buffer was used as negative control). The secondary antibody (Shanghai Huashun Biotechnology Co., Ltd., China)

was labeled with biotin (Hubei Hengjingrui Chemical Co., Ltd., China) and incubated at 37°C for 30 min, followed by PBS washing for 3 times, 5 min each time. Horseradish peroxidase labeled streptomycin ovalbumin solution (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., China) was dripped. The sections were dyed by DAB (Diaminobenzidine)/H₂O₂ and hematoxylin, and routinely dehydrated. They were then transparent, dried, sealed, and observed under a high-power microscope (Type A1130186, Shanghai Ai-ce Electronic Technology Co., Ltd., China).

Statistical analysis

The data obtained were analyzed by SPSS22.0 software (IBM Company, USA). The results of measurement data were expressed by $\bar{x} \pm s$; the two groups were compared by *t*-test; *P* < 0.05 was considered statistically significant.

RESULTS

Changes in basic indicators of rats

All experimental rats survived the 90 days feeding. The body weight change in the experimental group (204.45 $\pm$ 5.83g) within three months was compared with that of the control group (203.77 $\pm$ 6.57g). There was significant difference in the weight (174.02 $\pm$ 10.55g vs 166.32 $\pm$ 7.65g) within the first month between the experimental group and the control group. From the second month, the average body weight of the experimental group was lower than that of the control group (194.27 $\pm$ 7.76g vs 194.89 $\pm$ 9.11g). The liver weight of the experimental group was higher than that of the control group (21.09 $\pm$ 0.83g vs 18.12 $\pm$ 0.78g), with significant difference (*P* < 0.05). Serum total cholesterol (TC) levels increased, and low density lipoprotein (LDL-C) and triglyceride (TG) levels also increased, but high density lipoprotein (HDL-C) levels decreased in the experimental group compared with the control group, with significant difference (*P* < 0.05), as shown in Table I.

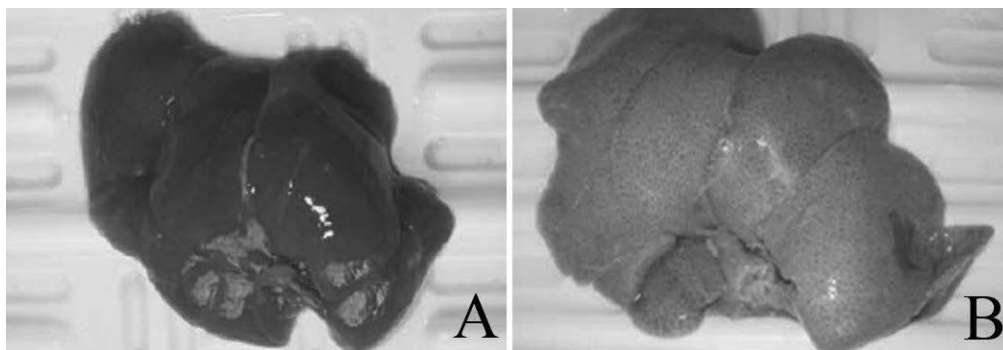
Morphological changes of rat liver

Fig. 1B shows that the liver of the experimental group showed diffuse enlargement, blunt, thick edges, smooth surface and granular changes. Depression

Table I. *Changes of serum indexes in rats of each group*

Indexes	Control group	Experimental group
C(Glu)/(mmol/L)	5.08±0.87	5.11±0.86
C(TC)/(mmol/L)	1.47±0.22	3.41±0.38*
C(TG)/(mmol/L)	0.58±0.16	2.79±0.14*
C(HDL-C)/(mmol/L)	0.79±0.14	0.48±0.13*
C(LDL-C)/(mmol/L)	0.21±0.09	0.53±0.11*

Note: compared with the control group: * $P < 0.05$.

**Fig. 1.** *Liver morphology. A) Control group; B) Experimental group).*

occurred when pressed, the surface color was pale or gray yellow, and the section was yellow-red or light yellow, greasy feeling. These morphological changes further confirmed the successful construction of rat hyperlipidemia.

Electrophoretic and semi-quantitative results of gene expression

The results of electrophoresis showed amplification bands of β -actin gene at 425bp. The amplification bands of FXR, BSEP and NTCP genes were shown at 283 bp, 289bp and 325bp, respectively. As shown in Fig. 2, more robust FXR and BSEP gene amplification bands were observed in the experimental group compared to the controls, reflected by brighter bands on agarose gel. In contrast, the brightness of NTCP gene amplification band in the experimental group was much weaker than that in the control group (Fig. 2C).

As shown in Table II, the semi-quantitative analysis indicated that the expression of FXR mRNA in the control group was low (0.3802 ± 0.0311), and

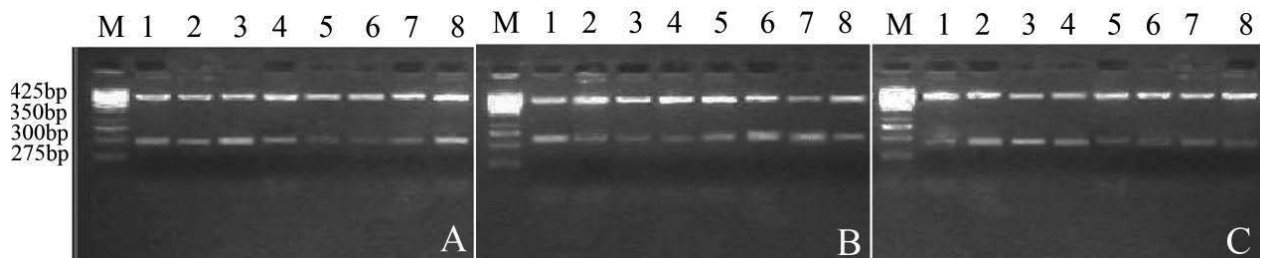
was high in the experimental group (0.8563 ± 0.0268); the expression of BSEP mRNA in the control group was low (0.3893 ± 0.0377), and was high in the experimental group (0.8656 ± 0.0289), the expression of NTCP mRNA in the control group was high (0.8661 ± 0.0231), and in the control group was relatively low (0.3451 ± 0.0409). The expression of FXR, BSEP and NTCP genes in the control group and the experimental group were significantly different ($P < 0.05$).

Immuno-histochemical results

The expression and distribution of FXR, BSEP and NTCP proteins in the liver of all rats were determined by immuno-histochemical analysis (arrow), as shown in Fig. 3. The positive rate of FXR expression in the experimental group was 74.8%, and in the control group, was 18.5% ($P < 0.05$). The positive rate of BSEP protein expression in the experimental group was 76.2%, and in the control group was 18.9% ($P < 0.05$). The positive rate of

Table II. RT-PCR semi-quantitative results of *FXR*, *BSEP*, *NTCP* gene mRNA expression.

No.	FXR/ β -actin		BSEP/ β -actin		NTCP/ β -actin	
	Experimental group	Control group	Experimental group	Control group	Experimental group	Control group
1	0.895	0.405	0.876	0.405	0.854	0.302
2	0.820	0.379	0.872	0.383	0.871	0.376
3	0.861	0.388	0.861	0.383	0.857	0.388
4	0.875	0.316	0.865	0.401	0.835	0.301
5	0.854	0.388	0.864	0.403	0.851	0.386
6	0.796	0.332	0.877	0.312	0.865	0.311
7	0.819	0.353	0.835	0.388	0.896	0.303
8	0.821	0.396	0.834	0.396	0.901	0.298
9	0.858	0.385	0.858	0.381	0.857	0.321
10	0.902	0.401	0.863	0.392	0.863	0.278
11	0.811	0.387	0.821	0.394	0.886	0.387
12	0.863	0.397	0.863	0.367	0.843	0.389
13	0.853	0.374	0.859	0.297	0.847	0.379
14	0.837	0.379	0.845	0.317	0.843	0.320
15	0.862	0.401	0.879	0.401	0.885	0.296
16	0.864	0.378	0.884	0.495	0.853	0.295
17	0.832	0.408	0.844	0.385	0.879	0.396
18	0.831	0.331	0.836	0.403	0.889	0.297
19	0.836	0.378	0.908	0.393	0.838	0.292
20	0.835	0.321	0.833	0.367	0.831	0.376
21	0.865	0.377	0.873	0.386	0.901	0.331
22	0.886	0.402	0.851	0.311	0.862	0.375
23	0.837	0.372	0.902	0.407	0.903	0.387
24	0.857	0.395	0.867	0.398	0.865	0.312
25	0.858	0.393	0.857	0.392	0.868	0.301
T	63.291		61.482		62.831	
P	<0.05		<0.05		<0.05	

**Fig. 2.** Electrophoresis results. Experimental group: swimming lanes 1, 5, 6, 7, and 8; control group: swimming lanes 2, 3, and 4). **A)** 283bp: *FXR* gene, 425 bp: β -actin gene; **B)** 289bp: *BSEP* gene, 425 bp: β -actin gene; **C)** 325 bp: *NTCP* gene, 425 bp: β -actin gene.

NTCP protein expression in the experimental group was 25.2%, and in the control group was 70.5% ($P < 0.05$).

DISCUSSION

The discovery that FXR regulated the expression of BSEP and NTCP indicates that FXR plays an important role in the metabolism of bile acid and cholesterol in hyperlipidemia, thus providing a new direction for the prevention and treatment of hyperlipidemia (8, 9). In this study, rats fed on high fat high cholesterol diet showed obvious disorders of fat metabolism. The increase of liver weight indicates that the disorder of liver fat metabolism, which may lead to fatty liver and even liver cirrhosis over a prolonged period. It has been proved that FXR gene expression increases, BSEP gene expression increases, and NTCP gene expression decreases in hyperlipidemia rats. Therefore, in hyperlipidemia, the liver bile acid excretion rate increases, the uptake rate decreases, the burden on the liver is lightened, and the liver function is maintained.

Previous studies (10, 11) have shown that partial

hepatectomy results in a decrease in bile acid absorption. The discovery of phospho-lipid transfer protein (PLTP), the target gene of FXR, also suggests that FXR may play an important role in lipoprotein metabolism. The increase of FXR, BSEP and NTCP expression directly and effectively affects the absorption of bile acids *in vivo*, thus affecting the serum cholesterol level. It is hypothesized that FXR and its regulation of BSEP and NTCP expression, especially in the case of high-fat feeding conditions, can effectively regulate lipid metabolism. Therefore, enhancing the expression of BSEP or inhibiting the expression of NTCP might be potential therapeutic approaches for treating human hypercholesterolemia, by further affecting the secretion and absorption of bile acids in the liver. Therefore, BSEP and NTCP might be important therapeutic targets in the prevention and treatment of hyperlipidemia and atherosclerosis.

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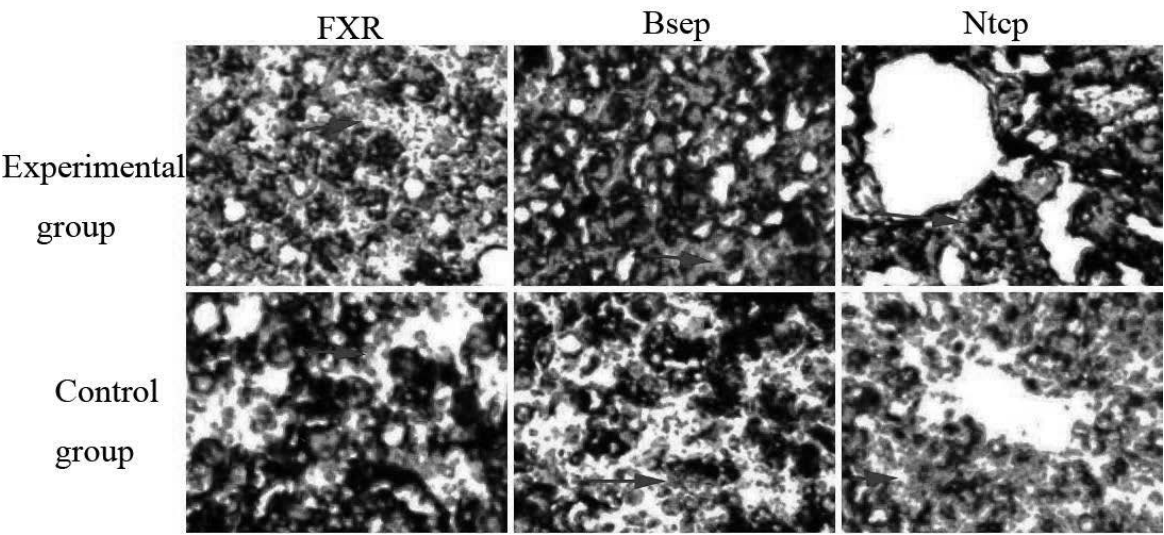


Fig. 3. The positive expression of various proteins under optical microscope (arrows refer to the corresponding positive cells) (* 400).

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LETTER TO THE EDITOR

EFFECT OF CHOLECALCIFEROL ON CD3⁺CD25⁺ T CELLS IN PATIENTS WITH SEVERE SEPSIS

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Sepsis is a common complication in surgical patients and is the first cause of death in Intensive Care Unit (ICU) patients. The aim of this study was to identify the effect of cholecalciferol on the expression level of T helper 17 cells (Th17) and regulatory T cells (Tregs) in patients with severe sepsis, and to explore their role in immune function in severe sepsis. Forty patients with severe sepsis from Intensive Care Unit (ICU) were selected as the experimental group. Blood samples were collected from all patients on day 1 after their admission to ICU. CD3⁺CD25⁺ cells were isolated from blood specimens and used to measure the differentiation of CD3⁺CD25⁺ to Th17 cells and Treg cells under different concentrations of cholecalciferol: 0 nM, 1 nM, 5 nM and 10 nM. Apache II scores, Sofa Scores, blood pressure and platelet count decreased in the group with low cholecalciferol levels compared to the group with normal cholecalciferol level. The percentage of CD4⁺IL-17⁺ and CD8⁺IL-17⁺ cells decreased significantly in the 4 groups under cholecalciferol treatment in a dose-dependent manner. The percentages of CD4⁺CD25⁺Foxp3⁺ and CD8⁺CD25⁺Foxp3⁺ cells increased significantly in the 4 subgroups under cholecalciferol treatment in a dose-dependent manner. Cholecalciferol modulates the differentiation of CD3⁺CD25⁺ T cells into Th17 cells and Treg cells by decreasing the Th17 cells levels and increasing the Treg cell levels in a dose-dependent manner, suggesting that cholecalciferol level can affect the progression of severe sepsis patients.

To the Editor,

Sepsis is a common complication in surgical patients and is the first cause of death in Intensive Care Unit (ICU) patients (1). Severe sepsis refers to sepsis patients with organ failure. Even though antimicrobial therapy and organ function support technology have progressed greatly in recent years, the mortality rate is still very high, 30-70% (1, 2).

Therefore, research on the pathogenesis and effective treatment and prognosis of severe sepsis has become the primary task of intensive medical research.

Previous studies have shown that CD4⁺CD25⁺ T cell subsets play a key role in combatting pathogens, infection prevention, and maintaining the stability of organism immunity (3). It was shown that the imbalance between regulatory T cells and Th17

Key words: severe sepsis; T helper 17 cell; Regulatory T cells; sequential organ failure assessment score

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cells was correlated with the severity of disease and prognosis of patients with severe sepsis (4). Regulatory T cells express Foxp3 and play an immunosuppressive role, such as CD3⁺CD25⁺. In contrast, Th17 cells secrete pro-inflammatory cytokines that prevent the installation of infections. Therefore, we hypothesized that regulating the balance of regulatory T cells and Th17 cells may be beneficial in severe sepsis treatment.

Cholecalciferol regulates the metabolism of calcium phosphate. In clinical practice it is commonly used in osteoporosis and secondary parathyroid dysfunction treatment. Studies suggest that the onset of severe sepsis is associated with low cholecalciferol levels, and is closely related to the prognosis of the disease (5, 6). Cholecalciferol has been shown to inhibit the differentiation of Th17 cells whereas it promotes the expansion of regulatory T cells and modulates the function of immune regulation (5). The aim of this study was to observe the levels of cholecalciferol in patients with severe sepsis, to analyze the correlation with the disease and to demonstrate *in vitro* its implication in the differentiation of regulatory T cells and Th17 cells.

MATERIALS AND METHODS

Patients

Forty patients admitted to the emergency department and then transferred to the ICU department of Hang Zhou Red Cross Hospital due to severe sepsis, for the period from October 2015 to September 2017, were included in this study.

Patients were divided into two groups. The first group included 20 severe sepsis patients who presented normal level cholecalciferol whereas the other group included 20 severe sepsis patients that presented low cholecalciferol levels. Patient information was obtained from the medical records of Hang Zhou Red Cross Hospital. Patients included in the study received standard care according to the ACCP (The American College of Clinical Pharmacology) and SCCM (The Society of Critical Care Medicine): Surviving Sepsis Campaign 2001 guidelines. Antibiotics were administered for identified infections.

Exclusion criteria: Patients with poor disease

prognosis that could lead to death such as: craniocerebral injury, postoperative cardiopulmonary resuscitation (CPR) and those with late malignant tumors; patients with autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus (SLE), asthma and other diseases for which they had used corticosteroids or immunosuppressants within 3 months prior to admission.

Blood pressure and platelet count were recorded for all the patients included in the study. APACHE II (Acute Physiology and Chronic Health Evaluation) and SOFA (Sequential Organ Failure Assessment) scores were performed. Patients were enrolled within 24 hours after meeting the inclusion criteria. Written informed consent was obtained from either the patient or, in the event that the patient lacked capacity, a personal or professional legal representative, before enrollment in the trial. Retrospective written informative consent was signed by the patient once capacity was regained. The study was carried out with the approval of the Ethics Committee of Hang Zhou Red Cross Hospital, Hangzhou, China. On day 1 the percentages of peripheral blood, lymphocyte subsets, CD3⁺CD25⁺T cells were measured, and the ratio of the latter two was calculated.

Determination of cholecalciferol level in the blood of sepsis patients

Cholecalciferol ELISA kit (K4806 BioVision, Inc.) was used to determine the cholecalciferol level in the blood. The test was performed according to the instructions of the kit. A level of cholecalciferol <0.002 g/L was considered deficient and a level of cholecalciferol >0.003g/L was considered normal (7).

Cell sorting and T-cell suppression

Isolation of peripheral blood mononuclear cells (PBMCs) was performed by density gradient centrifugation using human CD3⁺T cells (Miltenyi Biotec selected with magnetic beads, and enriched according to the manufacturer's instructions (Miltenyi Biotec). The obtained CD3⁺T cells were incubated using anti-CD25Apc antibody (eBioscience), and the anti-CD25⁺T cells were removed using anti-apc magnetic beads, and CD3⁺CD25⁺T cells were obtained and counted.

The purity of CD3⁺CD25⁺T cells obtained by the magnetic bead separation and enrichment was determined.

5×10^7 cells were added to 80 μ L PBS, 10 μ L CD3-PE antibody (eBioscience) and 10 μ L CD25-Apc antibody (eBioscience). After mixing, the mixture was incubated for 30 min. After centrifugation for 10 min at 300 g, the supernatant was discarded. 1 mL PBS was added, the mixture was again centrifuged for 10 min and then the supernatant was discarded; then 400 μ L of PBS with 2% FBS was added to the cells and mixed; the ratio of CD3+25- cells was detected by flow cytometry following standard procedure.

The proportion of CD4+CD25- T cells and CD8+CD25+ T cells in CD3+CD25+T cells obtained from the above methods was obtained. 5×10^7 cells were added to 80 μ L PBS, 10 μ L CD4-FTC and 10 μ L CD25-apc antibody (eBioscience). After mixing, the mixture was incubated for 30 min. After centrifugation for 10 min, the supernatant was discarded. 1 mL PBS was added, centrifugation was performed for 10 min at 300 g and again the supernatant was discarded; 400 μ L were added to PBS with 2% FBS and cells were mixed; the ratio of CD4+CD25+ T cells and CD8+CD25+ T cells was detected by flow cytometry with standard procedure.

Cholecalciferol effect on CD3+ CD25+T cells differentiation

The obtained cells were divided into two groups; one used for determination of cholecalciferol effect on differentiation of CD3+ CD25+T cells into Th17 cells and the other for the determination of cholecalciferol effect on differentiation of CD3+ CD25+T cells into Treg cells. After cell culture, each group was subdivided into 4 subgroups according to cholecalciferol level (0 nM, 1 nM, 5 nM and 10 nM).

Cell culture for differentiation of CD3+CD25+T cells into CD4+IL-17+ and CD8+IL-17+ cells

The CD3+CD25+T cells were separated by supernatant by centrifugation (600 g, 5 min), divided in 4 groups and cultured in RPM1640 culture media with cholecalciferol in doses of 0 nM, 1 nM, 5 nM and 10 nM, respectively. On the first day of culture anti-CD3 antibodies (5 μ g/mL) (eBioscience, China), anti-CD28 antibodies (5 μ g/mL) (eBioscience, China), anti-IL-4 antibodies (5 μ g/mL) (eBioscience, China) and anti-IFN- γ -antibodies (5 μ g/mL) (eBioscience, China) were added to each group, together with the cytokine TGF- β (2 ng/mL) (eBioscience, China). IL-23 (10 ng/mL) (eBioscience, China) was added

to culture at 72 h. After the culture 50ng/mL PMA5 (eBioscience, China), 750ng/mL ionomycin (eBioscience, China) and 10 g/mL brefeldin A (Bioscience, China) were added to each group to stimulate the differentiation and after 4 h the CD4+ IL-17+ cells and CD8+ IL-17+ cells were detected by flow cytometry (BECKMAN).

Cell culture for differentiation of CD3+CD25+ T cells into CD4+CD25+Foxp3+ cells and CD8+CD25+Foxp3+ cells

The CD3+CD25+T cells after separation were resuspended in the cell culture medium with anti-CD3 antibodies (eBioscience, China) and different concentrations of cholecalciferol (0 nM, 1 nM, 5 nM and 10 nM). The next step was to add anti-CD28 antibodies (2 μ g/m) (eBioscience, China), TGF- β 1 (5 ng/mL) (Peprotech (100-21)), and IL-2 (50 U/mL) (Peprotech) and culture for 5 days. After culture, the concentration of CD4+CD25+Foxp3+ cells and CD8+CD25+Foxp3+ cells in each group was detected by flow cytometry.

Statistical analysis

All results were analyzed using SPSS 13.0 software, and all quantitative data was presented as mean $\pm$ standard deviation (SD). For other analysis, the Student-Newman-Keuls or Student-Newman-Keuls test were used for comparison of means between two groups. Pearson momentum-correlation coefficient was used to measure the correlation between measured parameters and cholecalciferol levels. $P < 0.05$ was considered statistically significant.

RESULTS

Correlation between blood cholecalciferol level and admission parameters in severe sepsis patients

Apache II scores, Sofa Scores, blood pressure and platelet count decreased in severe sepsis group with low cholecalciferol levels compared to severe sepsis patients with normal cholecalciferol level ($P < 0.05$). No difference was observed between the groups regarding age and gender (Table I).

Cholecalciferol effect on the CD3+ CD25+T cell differentiation

After CD3+CD25+T cells differentiated into CD4+IL-17+ and CD8+IL-17+ cells, the percentages

of CD4⁺IL-17⁺ and CD8⁺IL-17⁺ cells in the control group were 6.15±0.07% and 5.67±0.15%, respectively. Exposure to different concentrations of cholecalciferol (1 nM, 5 nM and 10 nM) determines the inhibition of CD4⁺IL-17⁺ and CD8⁺IL-17⁺ cells, that is dose-dependent. The maximum effect was observed after the treatment with 10 nM cholecalciferol when the proportion of CD4⁺IL-17⁺

cells and CD8⁺IL-17⁺ cells was respectively 3.37±0.18% and 2.72±0.09% (Fig.1).

After differentiation, the percentages of CD4⁺CD25⁺Foxp3⁺ and CD8⁺CD25⁺Foxp3⁺ cells in the control group were 6.16±0.12% and 6.36±0.30%, respectively. After the exposure to different concentrations of cholecalciferol 1 nM, 5 nM and 10 nM, the percentages of CD4⁺CD25⁺Foxp3⁺

Table I. Correlation between blood cholecalciferol level and admission parameters in severe sepsis patients.

	Age	Gender Male/Female	APACHE II (scores)	SOFA (scores)	Blood pressure (mmHg)	Platelet count(X10 ⁹ L ⁻¹)
Low Cholecalciferol level severe sepsis patients	40.6±1.5	20/20	2.25±2.13	0.05±0.54	89.66±7.42	89.4±5.62
Normal level Cholecalciferol severe sepsis patients	38.5±1.2	20/20	6.12±2.11	1.56±0.49	110.36±5.12	101.21±1.23
R	-0.029	0.024	0.482	0.561	0.324	0.625
P	0.551	0.741	0.000	0.000	0.000	0.000

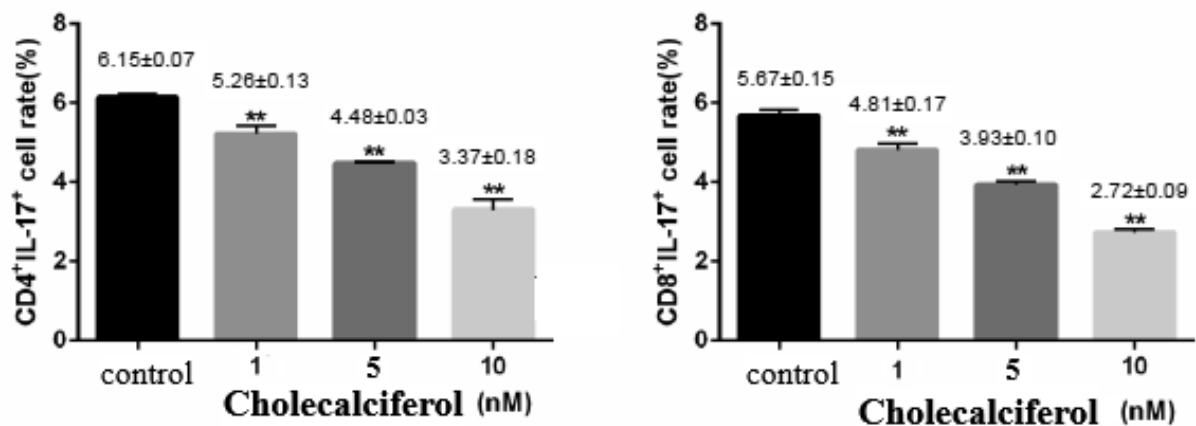


Fig. 1. Effect of different concentrations of cholecalciferol on CD4⁺ IL-17⁺ and CD8⁺ IL-17⁺ differentiation (% , mean ± SD). **P<0.01 vs 0 nM cholecalciferol (control).

and $CD8^+CD25^+Foxp3^+$ cells increased in a dose-dependent manner. After treatment with 10 nM Cholecalciferol, the percentage of $CD4^+CD25^+Foxp3^+$ cells and $CD8^+CD25^+Foxp3^+$ cells was $20.76 \pm 0.56\%$ and $20.91 \pm 0.78\%$, respectively (Fig.2).

DISCUSSION

Th17 cells which include many CD cells, such as $CD4^+IL-17$ and $CD8^+IL-17$, play a role of barrier in the prevention of infection. The increase of Th17 cells percentage results in an increase of IL-17 production by the human body (8). By activating a series of signaling pathways, the immune response to sepsis is further mediated, and the inflammatory process is accelerated, resulting in organ damage (8). IL-17 promotes mature neutrophil expansion, stimulates the synthesis of proinflammatory cytokines, mediating the interaction of lymphocytes (9) and induces sepsis in the process of inflammation. In our experiment, blood pressure, platelet count, SOFA and APACHE II scores decreased with the decrease of cholecalciferol levels.

Cholecalciferol has recently been studied for its immunomodulation function (10). Alvarez-Rodriguez et al. (11) found that the innate immune function decreased in patients with low cholecalciferol levels, and Mora et al. (12) also found that active

cholecalciferol could reduce the differentiation and activity of lymphocytes. This is the first study to show that in patients with severe sepsis cholecalciferol modulates the dynamic of Treg, which include $CD4^+CD25^+Foxp3^+$, $CD8^+CD25^+Foxp3^+$ and Th17 cells, and to confirm this effect *in vitro*. Braun's study (13) showed that ICU patients with low cholecalciferol levels had a higher mortality rate.

In conclusion, this study shows that cholecalciferol can inhibit the differentiation of $CD4^+CD25^+$ cells and $CD8^+CD25^+$ cells into $CD4^+IL-17^+$ cells and $CD8^+IL-17^+$ cells and stimulate the differentiation of $CD4^+CD25^+$ cells and $CD8^+CD25^+$ to $CD4^+CD25^+Foxp3^+$ cells and $CD8^+CD25^+Foxp3^+$ cells, respectively in a dose-dependent manner. These findings suggest that the administration of cholecalciferol in severe sepsis patients can be beneficial for their recovery.

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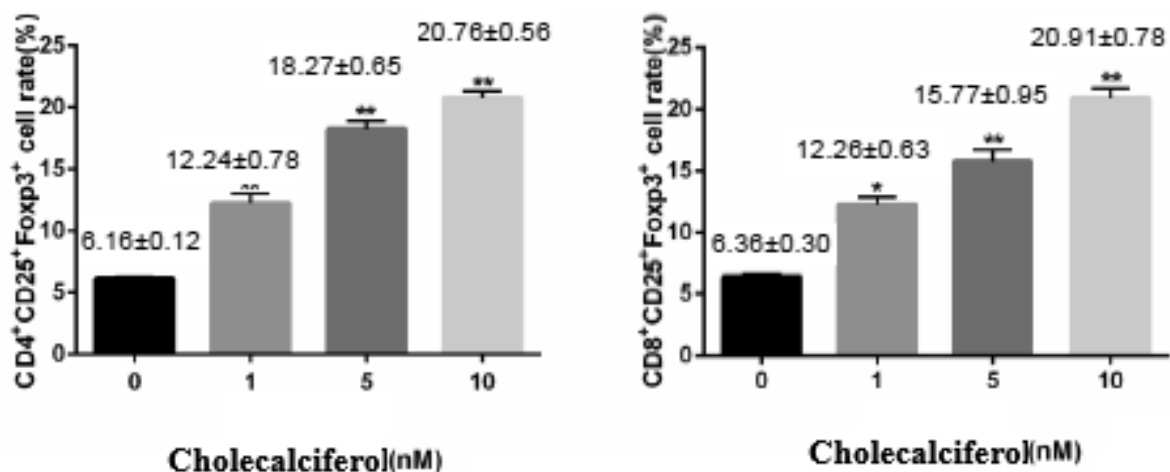


Fig. 2. The effect of different concentrations of cholecalciferol on $CD4^+CD25^+Foxp3^+$ and $CD8^+CD25^+Foxp3^+$ cell differentiation (% , mean $\pm$ SD). $^{**}P < 0.01$ vs 0 U/mL cholecalciferol (control).

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LETTER TO THE EDITOR

ANALYSIS OF CHANGES IN HEPATIC GENE EXPRESSION AND TISSUE ARCHITECTURE OF HEART AND KIDNEY IN ASSOCIATION WITH DIET-INDUCED OBESITY IN *RATTUS NORVEGICUS*

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Obesity is an epidemic issue associated with low-grade inflammation and in most of the cases with poor iron condition that influences nearly every community. The present research experiment was designed to inspect the consequences of obesity on the micro architecture of heart and kidney tissues as well as on the expression of mRNA of some hepatic genes like Hcpidin, Transferrin and TNF- α in association with their role in iron regulation and proinflammatory responses in the body. Two tentative groups of *Rattus norvegicus* (each supplied with different proportions of fat in their food) and one control group were analysed. After a period of sixteen weeks, the rats were dissected and their organs, liver, heart and kidneys, were excised and preserved accordingly. Histological analysis of heart tissues of obese rats showed hemorrhagic condition along with interstitium widening and interstitial inflammatory condition (myocarditis) and also necrosis of some myocardial fibers. Microanalysis of kidney tissues of obese rats showed glomerular distortion, glomerular basement membrane thickening and enlargement of Bowman's capsule along with mesangial expansion. mRNA expressional studies of some hepatic genes of the obese rats showed significant elevation in the expression of hepcidin (P-value < 0.001) and TNF- α (P-value < 0.001) and at the same time significant decline in the expression of transferrin (P-value < 0.001) was also observed as compared to the control group. Taken together, from these findings it can be concluded that fatty diet induces many changes in the cellular architecture of heart and kidney as well as in the expression of hepatic genes involved in iron regulation (Hepcidin and transferrin) and proinflammatory responses (TNF- α).

To the Editor,

Obesity is an epidemic problem linked with low-grade inflammation and in many cases with poor iron condition, affecting every region of the world (1). High fat diet contributes towards obesity due to increased lipoprotein lipase activity at adipose tissue and by failure in oxidation of extra fats (2). According to one estimate, 500 million adult people are obese throughout the world and every year approximately

30,000 deaths occur in the UK because of obesity (this value is ten times greater in the United States) (3). Among adults 'overweight' is attributed to those who have body mass index (BMI) from 25 kg/m² to 29.9 kg/m², and 'obesity' is attributed to those who have BMI $\geq$ 30kg/m² (4).

As carbohydrates are known to cause obesity, minimizing only the quantity of fat ingestion from the diet does not help to prevent obesity; carbohydrates

*Key words: histopathology, inflammation, obesity, hepcidin, TNF- α , transferrin**Corresponding Author:*

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must also be in balanced quantities in our daily food intake. When a person takes 200 g of sucrose per day then a prominent increase in serum triacylglycerols level can be observed (5).

Obesity involves both hyperplasia (increase in adipocyte numbers) and hypertrophy (increase in size of adipocytes) (2). In general, adipocytes regulate body fat mass and nutrient homeostasis. However, adipose tissues of obese subjects have macrophage infiltration and storage of different kinds of inflammation enhancing cytokines, such as tumor necrosis factor- α (TNF- α), interleukin (IL)-6, and IL-1 (6). Accumulation of fats in the adipose tissues also leads to the development of secondary complications such as type 2 diabetes mellitus (T2DM), hypercholesterolemia, atherosclerosis, Alzheimer's disease and arthritis. Hyperinsulinemia and dyslipidemia are also associated with fatness, which may further contribute to renal diseases. Hyperinsulinemia is linked with glomerular hypertrophy (7).

High fat deposition is also linked with the inhibition of iron uptake from the duodenum and hence leads to iron deficiency in the body. It also contributes towards hyperglycemic and hypertriglyceridemic conditions which further lead to chronic hepatic diseases known as Nonalcoholic fatty liver diseases (NAFLD) (8).

The aims of the present study were to investigate the changes in cell structures of heart and kidney by using histological techniques as well as investigation of mRNA expression of iron regulatory genes (Hepcidin & transferrin) and proinflammatory cytokine gene (TNF- α) in hepatic tissues of diet-induced obese animals in comparison to normal ones.

MATERIALS AND METHODS

Animals

In this study 15 male Wistar rats (*Rattus norvegicus*) were used. The rats were reared in the animal house of the department of Zoology of the University of the Punjab Lahore, Pakistan. The room where the animals were kept was well ventilated with temperature maintained at 20-25°C. All animals were

maintained according to the guidelines prescribed by the institute, and this study was approved by the Ethics Committee of the Department of Zoology the of University of the Punjab, Lahore.

Diet design and administration

Two experimental groups were established against one control group (n=5 in each group). Group 1 was given a diet containing a mixture of rat chow (33%), commercially available powdered milk (33%) and sucrose (33%) in crushed form with water *ad libitum*, and Group 2 was provided with a diet comprised of commercially available dry milk and rat chow separately but in equal amounts with water *ad libitum*. While only rat chow (20% crude protein) and water *ad libitum* was given to the control group. These different food compositions were given to each group daily for a continuous period of 16 weeks.

Tissue sampling and processing

Animals were sacrificed after 16 weeks, under aseptic conditions after being anesthetized with intra-peritoneal injection of ketamine (50mg/ml of ketamine) with distilled water in 1:1. Heart, kidneys and liver of all animals were excised and cut into 1×1cm pieces. Heart and kidney tissues were preserved in 10% formalin in glass vials while liver tissues were stored at -80°C in falcon tubes after proper cleaning in 0.9% saline solution.

Evaluation of histopathological changes

Cardiac and kidney tissues that were fixed in 10% formalin solution were further dehydrated by using different grades of alcohol in ascending order (30-100%). After clearing in xylene solution and embedding in paraffin wax, tissues were cut into 5- μ m thick sections by using microtome (Nippon Optical Works CO LTD Japan). These tissue sections were then stained with hematoxylin and eosin and were examined under the microscope (Olympus VANOX) at 100X and 400 X resolutions, in order to carry out the histopathological analysis.

Total RNA extraction

For total RNA extraction from stored liver tissues, TRIzol reagent was used for homogenization. After

homogenization the tissue sample was treated with chloroform which segregated the sample RNA in upper clear layer. Then RNA was precipitated with the help of isopropanol. The precipitated RNA thus separated was then washed to remove the impurities. Lastly RNA was quantified by using nanodrop UV/VIS Spectrophotometer (Model # Optizen NANO Q) and resuspended for further downstream applications. The ratio of O.D at 260 nm and 280 nm gave the measurement of the purity of the RNA.

cDNA synthesis

cDNA was prepared in small PCR tubes from total RNA by using RevertAid first strand cDNA synthesis kit (containing 5X reaction buffer, Ribolock RNase inhibitor, dnTPs mix and RevertAid) in a thermo cycler (Labnet, Model: MultiGene OptiMax) by applying different reaction conditions at different steps. cDNA thus formed was stored at -20°C.

Polymerase chain reaction

Already reported primers of β -actin, TNF- α , Hepcidin and Transferrin were used. β -actin was used as housekeeping gene (Table I).

For each targeted gene a thermo cycler was run by using cDNA sample along with primers (forward and reverse), dnTPs mixture, $MgCl_2$ and TAQ Polymerase under the conditions shown in Table II.

Agarose gel electrophoresis

Two percent agarose gel was prepared by using agarose, 1X TAE (Tris acetate EDTA) buffer and EtBr (ethidium bromide). Gel was run for each PCR product (specific for TNF- α , Hepcidin, and transferrin) along with Thermo Scientific GeneRuler™ 100bp Plus DNA Ladder ranging from 100-3000 bp. The gel was further analyzed to find the expression of mRNA of different genes by using imageJ software and GraphPad Prism 5 software.

RESULTS

H&E Staining

The heart and kidney sections of the animals from the control group were clear of any kind of histopathological abnormality. In both experimental groups the heart cells were deformed, showing cytoplasmic fragmentation and hemorrhagic

Table I. List of primers of different genes used for PCR reaction.

Primers	Forward 5' → 3'	Reverse 3' ← 5'
β -actin	TGT CAC CAA CTG GGA CGA TA	AAC ACA GCC TGG ATG GCT AC
TNF- α	ACA AGG CTG CCC CGA CTA T	CTC CTG GTA TGA AGT GGC AAA TC
Hepcidin	GAA GGC AAG ATG GCA CTA AGC A	TCT CGT CTG TTG CCG GAG ATA G
Transferrin	GGC ATC AGA CTC CAG CAT CA	GCA GGC CCA TAG GGA TGT T

(G, C, A, T stands for different nucleotides in the primer sequence)

Table II. Different temperature conditions for different stages of PCR.

Sr. No.	Steps	Temperature	Time
Stage 1	Initial Denaturation	94°C	5 minutes
Stage 2	Denaturation	94°C	45 seconds
	Annealing	60°C	45 seconds
	Extension	72°C	45 seconds
Stage 3	Final extension	72°C	10 minutes

condition. A prominent macrophage infiltration in heart tissue was also observed that may lead to inflammatory state (myocarditis). In some areas in group 2, widening of the intersitium was also observed (Fig. 1).

The kidney tissues of group 1 were abnormal, manifested by glomerular distortion and collapse of renal tubules in some areas. Enlargement of Bowman's capsule was also observed. The kidney tissue of group 2 showed extensive hemorrhagic infiltration of cells. Other structural changes observed were mesangial expansion, glomerular basement membrane thickening, and more glomerular distortion along with enlargement of bowmans capsule as compared to group 1 (Fig. 2).

PCR analysis

Gene expression of TNF- α (an acute phase proinflammatory cytokine gene) in liver tissue was significantly upregulated in both group 1 (1.83-fold) and group 2 (3.88-fold) in comparison to the control group.

Similarly, a significant increase in hepcidin expression was observed in both group 1 (4.66-fold) and group 2 (9.31-fold) in comparison to controls.

PCR analysis of transferrin gene expression revealed that there was a significant decrease in the expression of transferrin in group 2 (8.39-fold) in comparison with controls. Similarly, in group 1 decrease (1.42-fold) in expression was also observed (Fig. 3).

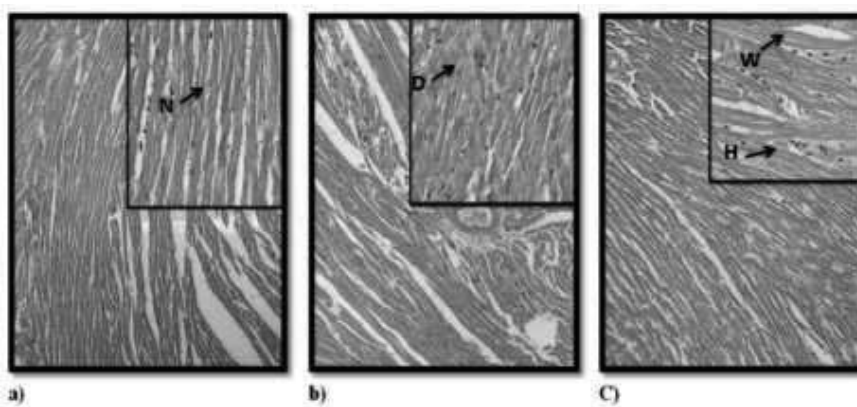


Fig. 1. H&E staining on cardiac tissue of (a) Control group, (b) Group 1 and (c) Group 2. Microphotographs were taken at 100X & 400X magnifications. N: normal cardiac fibers cell, D: deformed cardiac fibers; W: interstitium widening; H: hemorrhagic condition.

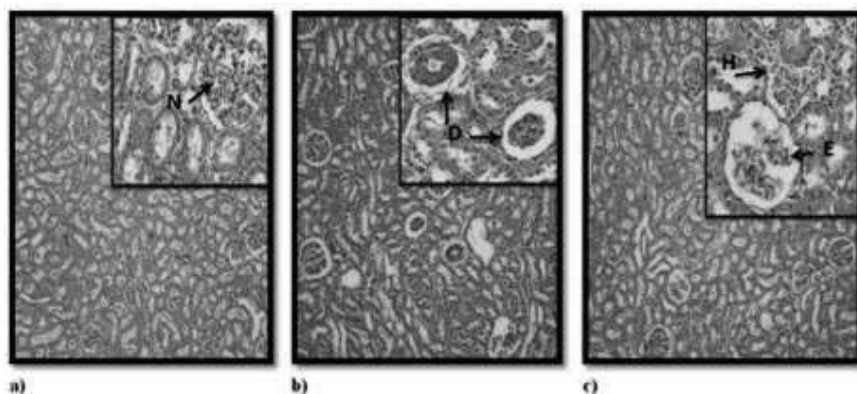


Fig. 2. H&E staining on kidney tissue of (a) Control group, (b) Group 1, and (c) Group 2. Microphotographs were taken at 100X and 400X magnifications. N: Normal Bowman's capsule; D: deformed Bowman's capsule; H: hemorrhagic condition; E: enlargement/widening of cell.

DISCUSSION

In their study, Neels and his colleagues found that obese subjects have inflammatory state which may be due to increased infiltration of macrophages in adipose tissues which contributes to developing chronic inflammation and state of insulin insensitivity which is concurrent with the present study findings that high fat storage in the body leads to a state of inflammation (9).

Results of the current study involving histological changes in heart muscles of obese rats are in accordance with the previous studies where it was suggested that intake of high fat diet (obesity) leads to increase in width of interstitium of the heart muscles

along with the interstitial fibrosis of atria. It also leads to lipodosis and inflammation of myocardial tissue. It was also described that obese animals show alteration in cardiac fibers, and hemorrhagic symptoms along with monocyte infiltration that cause inflammation (10).

Results of the microanatomy of kidney tissue in the present study were similar to other studies in which basement membrane thickening, mesangial expansion with glomeruloseclerosis were observed in obese subjects. Interstitial fibrosis, interstitial inflammation, renal tubule dilation, tubular atrophy, and arteriolar wall thickening in obese subjects were also described in previous studies. In obese rats, expansion of Bowman's area due to infiltration of fluid in the space

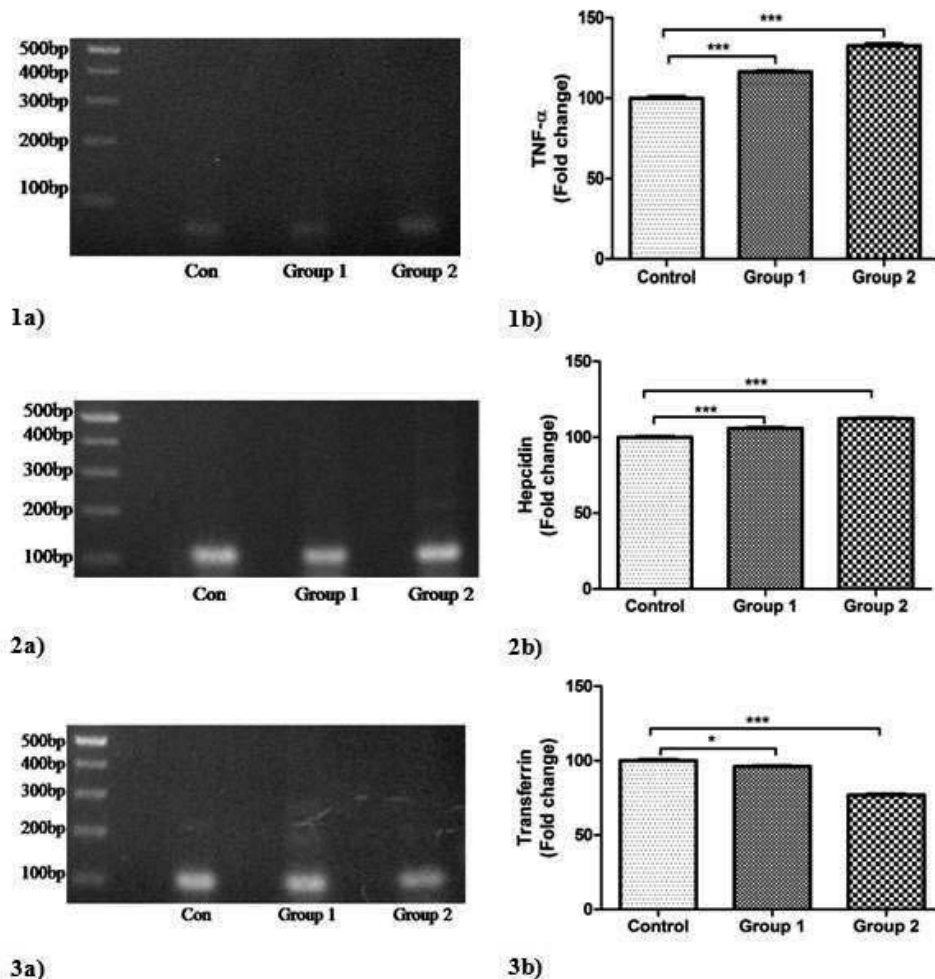


Fig. 3. Expression of mRNA on 2% agarose gel; **1a)** TNF- α , **2a)** Hepcidin, **3a)** transferrin and statistical analysis showing fold change in the expression of mRNA **1b)** TNF- α , **2b)** Hepcidin and **3b)** transferrin gene in the liver tissue of *Rattus norvegicus*. Statistical significant changes are marked with asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; Mean $\pm$ S.E.M.; $n = 3$).

of Bowman's capsule was also observed (11).

In this study it was found that expression of TNF- α was upregulated in hepatic tissue of obese individual, which was in accordance with the study of Feldstein et al., in which they found that TNF- α is over expressed in the hepatic tissue of obese mice (12). From the current study, the results of mRNA expression of hepcidin were similar to those of Chung J et al. who observed that rats fed on HFD showed increased expression of hepcidin in liver tissue even in the absence of iron accumulation in liver (13). Bekri et al. also observed in their study that the hepatic level of transferrin was low in obese patients which could further contribute to an anemic condition (14) which agrees with our results.

From all of these findings, it can be concluded that diet-induced obesity leads to many of the structural changes in the micro architecture of heart and kidney tissues. It was also found that expression of iron regulatory genes as well as proinflammatory cytokines in obese subjects were dysregulated, which contributes towards many other health problems. Thus, there is a need to develop the awareness of the public in order to avoid the many risks caused by a HFD, and more research is needed in this area to overcome the problems of diet-induced obesity.

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LETTER TO THE EDITOR

PRECISE HEPATECTOMY IN THE TREATMENT OF PATIENTS WITH PRIMARY LIVER CANCER

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Primary liver cancer is a commonly seen tumor, and early surgical resection can achieve a favourable effect, however traditional laparotomy has the shortcomings of great trauma and multiple postoperative complications, which are not beneficial to the recovery of patients. To explore the characteristics and effectiveness of precise hepatectomy, 116 patients with primary liver cancer who were admitted to Chenzhou No. 1 People's Hospital, Hunan, China, between August 2015 and December 2016 were selected. They were randomly divided into a conventional hepatectomy group and a precise hepatectomy group, 58 in each group. The surgical condition, changes of liver function indexes, prognosis and incidence of postoperative complications were observed and compared between the two groups. The results suggested that patients who underwent precise hepatectomy had less average intraoperative bleeding and shorter hospital stay compared to those who underwent conventional hepatectomy, but the duration of the surgery was longer ($p < 0.05$). Compared to pre-surgery, the content of total bilirubin in serum (TBIL), alanine transaminase (ALT) and aspartate transaminase (AST) of the two groups decreased, and the content of albumin (ALB) increased after treatment. The changes of the indexes of the precise hepatectomy group were more obvious, and the liver function of was superior to that of the conventional hepatectomy group; the comparison within group and between groups had statistical significance ($P < 0.05$). The recurrence rate of tumor of the precise hepatectomy group was significantly lower than that of the conventional hepatectomy group ($P < 0.05$). The lesion removal rate, one-year survival rate, and two-year survival rate of the former were obviously higher than that of the latter ($P < 0.05$). The difference of the incidence of complications between the two groups had statistical significance ($P < 0.05$). Precise hepatectomy has a favourable effect in the treatment of primary liver cancer as it can effectively control cancer recurrence and improve prognosis; hence it is worth promotion.

To the Editor,

Primary liver cancer refers to cancer caused by the deterioration of liver cells or intrahepatic biliary epithelial cells. It is one of the most common malignant cancers, featured by insidious onset, rapid progress, poor prognosis and high death rate (1, 2). Hepatectomy is always considered as the most

effective surgery for primary cancer. One study (3) suggested that the peri-operative mortality of hepatectomy was below 5%, but the 5-year survival rate was between 26% and 50%. Therefore how to further enhance the resection rate of primary liver cancer has been a serious challenge in the liver surgical field. Traditional conventional surgery

*Key words: primary liver cancer; precise hepatectomy; prognosis**Corresponding Author:*

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used in the past has shortcomings of poor liver function recovery and high volume of bleeding, and the postoperative recurrence rate and incidence of complications are high (4, 5). With the improvement of medical technical level in recent years, the surgery of primary liver carcinoma has transformed from conventional to precise. Precise hepatectomy is a new concept and technical system for the treatment of liver cancer, which aims for minimum invasion, highest protection for the liver and best rehabilitation effect (6, 7). However, few studies regard the effectiveness of precise hepatectomy in the treatment of primary liver cancer, therefore to verify its clinical value, patients treated by precise hepatectomy or conventional hepatectomy were compared.

MATERIALS AND METHODS

General data

One hundred and sixteen patients with primary liver cancer who were admitted to Chenzou No. 1 People's Hospital, Hunan, China, between August 2015 and December 2016 were selected, and all of them had surgical indications and satisfied the diagnostic criteria. The subjects were divided into a conventional hepatectomy group and a precise hepatectomy group, 58 in each group. The conventional hepatectomy group included 32 males and 26 females aged 26–65 years (average 45.8 ± 5.7 years). There were 45 cases of positive alpha fetoprotein (AFP), 39 cases of positive hepatitis B surface antigen (HBs Ag) and 4 cases of positive antibody to hepatitis C virus (anti-HCV); as to the Child-Pugh grade, there were 45 cases of grade A and 13 cases of grade B; the diameter of cancer was between 5 cm and 13.2 cm (average 9.4 ± 0.9 cm). The control group included 36 males and 22 females aged 23–66 years (average (45.7 ± 5.6) years); there were 39 cases of AFP, 41 cases of HBs Ag and 2 cases of anti-HCV; as to Child-Pugh grade, there were 39 cases of grade A and 19 cases of grade B; the diameter of cancer was between 4.5 cm and 11.8 cm (average (8.7 ± 1.1) cm). The differences of gender, age, liver function, Child-Pugh grade and size of diameter between the two groups had no statistical significance ($P > 0.05$); therefore the results were comparable. This study was reviewed and approved by the ethics committee of our hospital.

Inclusive and exclusive criteria

Patients who satisfied the standards of primary liver cancer described in the Rules of Diagnosis and Treatment of Primary Liver Cancer (2011) (8), were confirmed by preoperative conventional imaging and postoperative pathological examination, and signed informed consents were given. Those who had serious heart and lung diseases, had Child-Pugh grade C, had ascitic fluid, jaundice, dyscrasia or distant metastasis of lesions, or whose lesion occupied 4/5 of the whole liver were excluded.

Preoperative evaluation

Three-dimensional reconstruction was performed for patients in the precise hepatectomy group based on Child-Pugh grade, indocyanine green retention rate at 15 min (ICGR15) and enhanced computed tomography (CT) examination results. The boundary of cancer was precisely calculated. The total estimated liver volume, cancer volume, resection volume and residual liver volume were calculated. The standard liver volume could be calculated using the formula standard liver volume = $11.508 \times \text{weight} + 334.024$. Before surgery, the residual liver volume of normal liver/standard liver volume should be higher than 30%, and the residual volume of liver with liver cirrhosis/standard liver volume should be higher than 40%.

Vascular reconstruction was performed for patients in the conventional hepatectomy group based on Child-Pugh grade, ICGR15 and enhanced CT examination results. The anatomic relationships between size, location and number of cancer and important vessels and adjacent organs were evaluated. Postoperative liver function evaluation and liver resection range of patients in the two groups referred to the relevant content in Consensus on Evaluation of Hepatic Functional Reserve before hepatectomy (9). Treatments such as liver protection, virus resistance and improvement of coagulation function were carried out before surgery.

Intraoperative operations

Patients in the conventional hepatectomy group were given general anesthesia. Patients underwent laparotomy in a supine position. Hemihepatic occlusion was performed by occluding the porta hepatis with traditional Pringle technology to reduce intraoperative bleeding. Regular or irregular segmental hepatectomy was selected

according to the actual situation.

Patients in the precise hepatectomy group were treated by tracheal intubation anesthesia in a supine position. The site which was 5~6 cm below the umbilicus was punctured and inserted with a 10-mm Trocar to establish pneumoperitoneum, and the pressure was controlled at 13 mm Hg. Two to four operation holes were made below the xiphoid process, midline costal margin of the right axilla, midline costal margin of the axilla and frontline costal margin and then inserted with a 12-mm Trocar to probe abdominal cavity and determine the location and size of lesions. The diaphragm was cut apart, and then the perihepatic ligament, left triangular ligament and ligamentum teres hepatis were dissociated along the edge of ligamentum hepatoduodenale to separate the hepatic artery. After, the hepatic artery was clipped using a haemostatic forceps. Liver parenchyma was dissociated using an ultrasound knife. For patients with calculus of bile duct, choledocholithotomy was performed using a choledochoscope. After the lesions were cleaned thoroughly, the wound surface was staunched using an electric coagulation knife. The existence of active bleeding and bile leakage was closely observed. Next the incision was sutured using suture 4. Then the abdominal cavity was cleaned, and a drainage tube was inserted.

Postoperative management

Patients in the conventional hepatectomy group were given treatment to protect the liver, resist virus, correct low protein and control peritoneal fluid. The stomach tube was removed after passage of gas by anus. Then the patients were given food and pain relief treatment when necessary.

The concept of enhanced recovery after surgery was followed in the precise hepatectomy to achieve excellent postoperative management and accelerate recovery. The patients were encouraged to take food after surgery. They took food on the first postoperative day: firstly, liquid food in a volume between 100 ml and 500 ml; then semi-liquid food and normal food were taken. Pain management was standardized after surgery. Moreover the patients were encouraged to do off-bed activity to promote the recovery of gastrointestinal function, reduce incidence of cardiopulmonary complication and enhance postoperative living quality. The urinary catheter and abdominal cavity drainage tube were removed as soon as possible according

to the condition of postoperative recovery. The stomach tube was removed from two patients on the second postoperative day because of abdominal distension, but removed from the others on the first postoperative day.

Observation index

The duration of surgery, intraoperative bleeding amount, length of hospital stay and postoperative complications were observed and recorded.

Fasting elbow venous blood was collected before surgery and one week after surgery. After anticoagulant treatment, the serum biochemical indexes of the patients including total bilirubin in serum (TBIL), alanine transaminase (ALT), aspartate transaminase (AST) and albumin (ALB) were detected using a fully automatic biochemical analyzer.

The patients were followed-up for two years after treatment. The recurrence rate, lesion clearance rate, one-year survival and two-year survival rates were statistically analyzed.

Statistical processing

Data were statistically analyzed using SPSS ver. 21.0. The comparison between data in the same group was performed using *Chi*-squared test. Measurement data were processed by *t*-test. Enumeration data were expressed as mean $\pm$ standard deviation (SD). Difference was considered as statistically significant if the value of *p* was smaller than 0.05.

RESULTS

Comparison of surgical condition between the two groups

In the precise hepatectomy group (*n*=58), 5 patients underwent hemihepatectomy, 16 patients underwent heplobectomy, 8 patients underwent segmental hepatectomy, 19 patients underwent partial hepatectomy, and 10 patients underwent regular hepatectomy. The average duration of surgery was (97.32 ± 9.65) min, the intraoperative bleeding amount was (221.21 ± 27.96) mL, and the length of hospital stay was (8.36 ± 0.78) days. In the conventional hepatectomy group (*n*=58), 11 patients underwent hemihepatectomy, 21 patients underwent heplobectomy, 16 patients underwent

partial hepatectomy, and 10 patients underwent segmental hepatectomy. The average duration of surgery was (78.17 ± 6.96) min. The intraoperative bleeding amount was (382.55 ± 22.67) mL. The length of hospital stay was (12.19 ± 1.92) days. The precise hepatectomy group had less average bleeding amount and shorter hospital stay compared to the conventional hepatectomy group, and the differences had statistical significance ($P < 0.05$), however, the duration of surgery of the precise hepatectomy group was longer (Table I).

Comparison of preoperative and postoperative liver function between the two groups

The ALT, TBIL, AST and ALB content of the two groups had no significant difference before surgery ($P > 0.05$). The ALT, TBIL, and AST content of the

two groups obviously decreased after surgery, but the content of ALB increased. The variation of different liver function-associated indexes in the precise hepatectomy group was more apparent than that of the conventional hepatectomy group, and the difference had statistical significance ($P < 0.05$, Table II).

Comparison of prognosis between the two groups

In the two-year follow up, the recurrence rate of the precise hepatectomy group was significantly lower than that of the conventional hepatectomy group ($P < 0.05$); the lesion clearance rate, one-year survival rate and two-year survival rate of the precise hepatectomy group was obviously higher than that of the conventional hepatectomy group ($P < 0.05$, Table III).

Table I. Comparison of basic surgical parameters between the two groups.

Group	Duration of surgery (min)	Intraoperative bleeding amount (mL)	Length of hospital stay (d)
Precise hepatectomy group	97.32 ± 9.65	221.21 ± 27.96	8.36 ± 0.78
Conventional hepatectomy group	78.17 ± 6.96	382.55 ± 22.67	12.19 ± 1.92
t	0.386	16.218	5.987
P	< 0.05	< 0.05	< 0.05

Table II. Comparison of liver function associated indexes before and after surgery.

Group	Time	ALT (U/L)	TBIL ($\mu\text{mol/L}$)	AST (U/L)	ALB (g/L)
Precise hepatectomy group	Before surgery	39.96 ± 5.47	19.74 ± 2.57	43.11 ± 2.73	38.11 ± 1.72
	One week after surgery	11.27 ± 2.17	6.39 ± 1.39	29.38 ± 1.65	55.30 ± 2.25
Conventional hepatectomy group	Before surgery	40.14 ± 5.48	19.86 ± 2.45	43.09 ± 2.67	38.18 ± 1.69
	One week after surgery	18.72 ± 3.26	8.89 ± 1.96	36.41 ± 1.86	47.43 ± 2.16

Table III. *Comparison of prognosis between the two groups.*

Group	Recurrence rate	Lesion clearance rate	One-year survival rate	Two-year survival rate
Precise hepatectomy group	4 (6.9)	58 (100)	49 (84.5)	41 (70.7)
Conventional hepatectomy group	15 (25.9)	43 (74.1)	36 (62.1)	26 (44.8)
χ^2	4.294	9.187	3.966	4.227
P	<0.05	<0.05	<0.05	<0.05

n(%)

Comparison of postoperative complications between the two groups

In the precise hepatectomy group, there was 1 case of peritoneal fluid, 3 cases of bile leakage and 1 case of intestinal adhesion; the incidence of complications was 8.6%. In the conventional hepatectomy group, there were 3 cases of pleural effusion, 4 cases of hepatic failure and 2 cases of portal vein thrombosis; the incidence of complications was 15.5%. The incidence of complications of the precise hepatectomy group was significantly lower than that of the conventional hepatectomy group, and the difference was statistically significant ($\chi^2=4.321$, $P<0.05$).

DISCUSSION

Precise hepatectomy has been the preferred treatment for liver cancer in recent years. Approximately 85% of patients with liver cancer have either liver cirrhosis or chronic hepatitis. Hypohepatia induced by insufficient residual hepatic volume is prone to occur in those patients after surgery. It is extremely important to accurately evaluate hepatic functional reserve before surgery and precisely resect the liver during surgery.

Relevant studies (10, 11) pointed out precise hepatectomy could effectively eliminate lesions, protect the liver to the highest extent, effectively reduce the death rate, and enhance the postoperative survival rate. The two-year follow up found that the recurrence rate of the patients who had undergone precise hepatectomy was apparently lower than the conventional hepatectomy two years after surgery, and the survival rate of the precise hepatectomy group was higher than that of the conventional hepatectomy group ($P<0.05$). The results demonstrated that precise hepatectomy had little influence on the residual liver of patients and could ensure sufficient blood supply. Moreover, it was found that the content of serum TBIL, ALT and AST of patients in both groups decreased, and the level of ALB increased; the liver function indexes of patients in the precise hepatectomy group were superior to those in the conventional hepatectomy group ($P<0.05$), which were similar to the results of Azoulay (12). The results revealed that hepatectomy could effectively eliminate target lesions and ensure stable haemodynamics and full compensation of function of residual liver. In conclusion, precise hepatectomy has advantages of less intraoperative bleeding, fast recovery of liver function, low

incidence of complications and favourable prognosis compared to traditional hepatectomy, therefore is worth promotion.

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LETTER TO THE EDITOR

CLINICAL EFFECT OF NURSING INTERVENTION FOR PATIENTS UNDERGOING LAPAROSCOPIC CHOLECYSTECTOMY

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Nursing intervention is a positive method for the recovery of patients' health. To study the clinical effect of intraoperative nursing intervention in patients with cholecystolithiasis who underwent laparoscopic cholecystectomy, 100 patients with cholecystolithiasis were randomly divided into two groups: an experimental group that was given nursing intervention, and a control group given conventional nursing care. The average length of stay, average hospitalization expenditure, postoperative recovery and nursing satisfaction of the two groups were compared. We found that there were significant differences in the average hospitalization time, average hospitalization expenses, postoperative recovery and nursing satisfaction between the two groups. The average hospitalization time of the experimental group was 4.4 ± 0.7 days, and the cost was 7985 ± 561 yuan, which were lower than those in the control group. Postoperative recovery and nursing satisfaction of the experimental group were better than those of the control group, indicating that nursing intervention achieved a favorable effect.

To the Editor,

Cholecystolithiasis is a common biliary tract disease that occurs frequently in middle age and older people, and women have a higher incidence than men (1). The main causes of gallstone formation are the secretion of biliary cholesterol and the stasis of bile (2). Cholecystolithiasis often has many complications, such as jaundice and pancreatitis, and the main treatment is cholecystectomy (3). Laparoscopic surgery allows physicians to perform rather complex intra-abdominal operations with a small incision (4). This is the preferred method for many intra-abdominal operations (5), and is also the best choice for cholecystectomy (6). Compared to other cholecystectomies, laparoscopy has the advantages of smaller, less painful incisions and

shorter recovery times (7). Nguyen et al. (8) found that laparoscopic surgery could significantly reduce the risks of infection at the surgical site compared to open surgery. Nursing intervention refers to various nursing measures to promote patient recovery, which has a major influence on clinical surgical efficacy. Shen et al. (9) found that patients undergoing nursing intervention felt more comfortable than patients having ordinary nursing care and had significantly lower risks of complications. Through the study of 98 cases of gastric ulcer, Dai (10) found that nursing intervention could improve nursing satisfaction and epidermal growth factor level and reduce the rate of recurrence of gallstones. For patients with gallstones undergoing laparoscopic surgery, nursing intervention can produce positive effects. In the present study,

Key words: nursing intervention; cholecystolithiasis; laparoscope; clinical effect

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100 patients with cholecystolithiasis were randomly selected and divided into an experimental group which received nursing intervention and a control group that received conventional nursing care. The average hospitalization time, expenses, postoperative recovery and nursing satisfaction were compared between the two groups. The clinical efficacy of the nursing intervention was analyzed based on the results obtained.

MATERIALS AND METHODS

Baseline data

One hundred patients with cholecystolithiasis were randomly grouped into an experimental group and a control group. Fifty patients belonged to the experimental group, 28 males and 22 females, aged 25-67 years (mean 48.72 ± 1.05 years). The control group consisted of 50 patients, 26 males and 24 females, aged 24-68 years (mean 47.38 ± 1.43 years). There was no significant difference in gender and age among the groups ($P > 0.05$). All patients were informed of the objective and procedures of the experiment and signed the information consent.

Nursing methods

Patients in the control group received conventional nursing care including conventional health education, instrumental examination, detection of vital indicators, guidance for coordination with physicians, and simple issues that needed attention; while the patients in the experimental group received high quality nursing intervention.

Preoperative preparation

The conventional clinical examination was carried out to exclude surgical contraindications and potential pathologies prior to surgery. Before a patient entered the operating room, the name, sex and other basic information of the patient was checked by a nurse. After entering the operating room, patient information was again checked by the anesthesiologist and the operating room nurse. If there were no problems, the vital signs of the patients were examined and the patient's position was adjusted. The head was raised from 15 to 20 degrees and tilted 15 degrees to the left. The patient's knees were fixed with appropriate straps. In addition, the shoulders were supported with a

support to promote the absorption of the anesthetic, to prevent the damage of the local compression to nerves and muscles of the patients in the process of general anesthesia and to keep the body away from metal objects. The operating room temperature was controlled between 22 and 25°C. Humidity was also controlled. In our study, intravenous inhalation was used in combination with general anesthesia: fentanyl, sufentanil and remifentanyl were used for anesthesia.

Patient care

Before the intervention, the patients were interviewed by the nurse and informed about the procedures and issues that needed attention to alleviate their tension and sense of insecurity. During the operation, the nurse told the patient to follow the instructions and reduce negative emotions. The nurse held the patients' hands to increase their confidence. Furthermore, changes in vital signs of patients such as body temperature, heart rate and blood pressure were tightly controlled. Any abnormality had to be communicated to the surgeon. Sufficient care allowed the patients to maintain good mental status, which was useful for postoperative recovery.

Surgery cooperation

Surgical progress was performed closely by the nurse prior to surgery. All the instruments for surgery were prepared and checked. In addition, the nurse assisted the surgeon to disinfect the instruments, listened to the instructions of the doctors, accurately transferred the necessary instruments to the surgeon, cleaned the used ones and repaired the clean laparoscopic lens promptly, to ensure a clear surgical field. Efficient surgical coordination ensured the smooth running of the surgery. 5 ~ 10 mg dezocin were injected intravenously 15 min before surgery to prevent postoperative pain.

Observational indexes

The average hospitalization time, expenses, postoperative recovery and nursing satisfaction were considered as observation indices. Nursing satisfaction was examined using a questionnaire: over 90 points (satisfactory), 80~90 points (substantially satisfactory) and 80 points (unsatisfactory). The patients filled out the questionnaire under the guidance of the nurse and the nurse gave the patients a

detailed explanation to obtain more accurate results.

Statistical analysis

SPSS 18.0 was used for statistical analysis. The count data was processed using the *chi*-squared test, while the measurement data was processed by the *t*-test. $P < 0.05$ indicated that the difference had a statistical significance, whereas $P > 0.05$ indicated an insignificant difference.

RESULTS

Average hospitalization time and expense

As seen in Table I, the average hospitalization time of the experimental group and control group were 4.4 ± 0.7 days and 9.8 ± 3.1 days respectively, and the difference had statistical significance ($P < 0.05$). The average hospitalization expense of the experimental group and control group was 7985 ± 561 yuan and 10863 ± 749 yuan respectively, and there was also a notable difference ($P < 0.05$).

Postoperative recovery

Time to food intake, defecate and other activities

are shown in Table II and Fig. 1. The postoperative recovery of the two groups presented a dramatic difference. The time to food intake for the first time in the experimental group was 22.39 ± 1.03 h, significantly different from that of the control group; the time to defecate, for the first time, and to perform activities (for the first time) were 9.4 ± 4.98 h and 8.23 ± 4.61 h, respectively, and were significantly different from those of the control group. It was seen that the recovery time of the experimental group was shorter than that of the control group, suggesting that the experimental group recovered better after surgery.

Satisfaction on nursing

The experimental group had more nursing satisfaction than the control group, suggesting a significant difference. Furthermore, each patient was satisfied with nursing care (one patient in the control group was dissatisfied) and 8 patients in the control group were substantially satisfied. This indicated that the patients in the experimental group had greater nursing satisfaction (Table III).

Table I. *The comparison of the average hospitalization time and expense between the two groups.*

	Average hospitalization time (days)	Average hospitalization expense (yuan)
Experimental group	4.4 ± 0.7	7985 ± 561
Control group	9.8 ± 3.1	10863 ± 749
P value	0.001	0.027

Table II. *The comparison of postoperative recovery between the two groups.*

	Experimental group	Control group
Time to take food for the first time (h)	22.39 ± 1.03	21.54 ± 10.45
Time to defecate for the first time (h)	9.42 ± 4.98	13.56 ± 9.72
Time to do activities for the first time (h)	8.23 ± 4.61	11.21 ± 8.35

DISCUSSION

It was found from the results that the postoperative recovery of patients who received nursing intervention was better than that of patients who received conventional nursing; the patients in the experimental group started activities about three hours earlier than that of the control group. Some patients in the control group did not eat until 30 hours after the operation, which was not found in the experimental group. Under the high-quality nursing intervention, the patients received meticulous care during the operation, both physically and

psychologically, so they showed good postoperative recovery status, not only because the wound was well treated, but also because of psychological ease and comfort. The attitude of patients was reflected on the satisfaction of nursing. The satisfaction rate of the experimental group was 98%, while that of the control group was only 82%. Only one patient in the experimental group was basically satisfied; eight in the control group were basically satisfied, and one patient was not satisfied; there was a significant difference between the two groups. In the course of treatment, the patients in the experimental group always maintained a relaxed and positive

Table III. The comparison of the satisfaction of the patients on nursing between the two groups.

	Experimental group	Control group
Satisfactory	49	41
Basically satisfactory	1	8
Unsatisfactory	0	1
Satisfaction rate	98%*	82%

Note: * indicated $P < 0.05$ comparing to the control group.

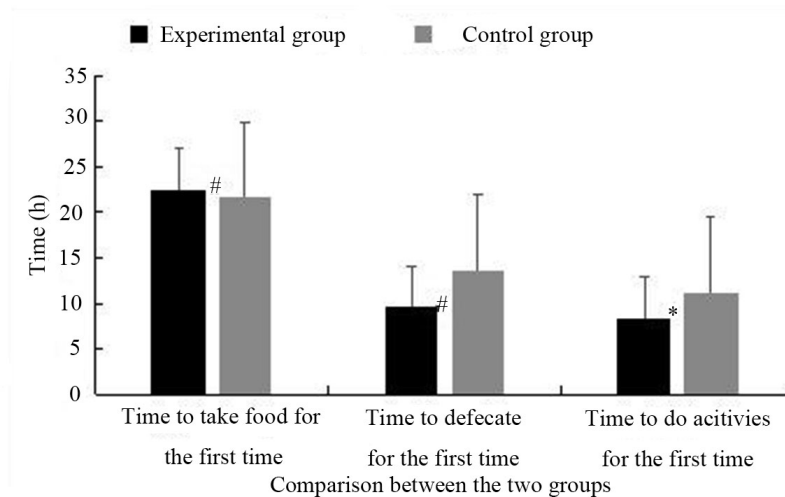


Fig. 1. The comparison of the postoperative condition between the two groups.

Note: # $P < 0.01$; * $P < 0.05$ comparing to the control group.

attitude and had a better understanding of their own conditions, so that they had a higher degree of satisfaction. However, there may be unavoidable omissions in conventional nursing, sometimes the emotion of some patients may not be taken care of, and conflicts between doctors and patients are possible, which can be seen from the degree of satisfaction of patients in the control group. It indicated that nursing intervention had significantly positive effect on the recovery of patients and the patients had high satisfaction degree in the process of treatment.

The average length of hospital stay in the experimental group was about five days shorter than that in the control group, and the average hospitalization cost was also much lower than that in the control group. High-quality nursing intervention can help nursing works proceed in an orderly manner, improve the efficiency of nursing, and accelerate recovery. Nursing intervention makes a scientific and reasonable arrangement for examination and nursing in the course of treatment, avoiding unnecessary examination and reducing the average hospitalization cost. It is not only valuable for patients; for hospitals, short hospitalization time is conducive to the circulation of patients and improvement of economic benefits of the hospital, and high satisfaction is of great help in establishing the image of a hospital.

In conclusion, laparoscopic cholecystectomy is the best therapy for gallstones and nursing intervention has a major impact on the clinical effect. By comparing 100 patients, it was found that intraoperative nursing intervention could provide patients with high quality and highly efficient medical services, help patients recover from surgery as soon as possible, reduce the duration of hospitalization, and reduce the cost of hospitalization. It not only improves the clinical effect, but also increases the benefits of the hospital. Therefore, nursing intervention can be widely promoted.

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LETTER TO THE EDITOR

ESTABLISHMENT OF MOUSE MODEL OF
QI-DEFICIENCY GASTROINTESTINAL FAILURELQ. HUANG¹, W. SHEN², T. QIU³, YH. ZHI¹, LC. WANG¹, SH. MAO¹, X. XING¹,
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This study aimed to prepare an animal model of qi-deficiency gastrointestinal failure, and verify feasibility of the model. One hundred and twenty healthy adult male ICR mice were randomly divided into a control group (24 mice), a placebo group (24 mice), and an experimental group (72 mice). Mice in the control group were given a normal diet for 10 days, those in the placebo group were additionally given normal saline solution at 4°C for 10 days, and mice in the experimental group were additionally given 4°C supersaturated sodium sulfate solution for 10 days. After 10 days, mice in the experimental group were randomly divided into three subgroups: a model group, a self-healing group and a Sijunzi decoction group, with 24 mice in each group. Mice in the model group were immediately sacrificed, those in the Sijunzi decoction group were fed with Sijunzi decoction for 7 days, and mice in the self-healing group were given the same amount of normal saline for 7 days. Mice in each group were randomly divided into three subgroups, namely, pathological group, immunohistochemistry group and toner propelling test group, with 8 mice in each subgroup. The general condition of mice in the toner propelling test group was observed. The changes of intestinal villi, intestinal gland and intestinal epithelial cells in the intestinal mucosa of mice in the pathological group were observed. In the immunohistochemical group, apoptosis and 5-HT of the intestinal epithelial cells were detected. All indicators were found to be significantly different from those of the normal control group. After 7 days of traditional Chinese medicine treatment, mice in the Sijunzi decoction group had gained weight, reduced stool, and increased gastrointestinal propulsion index, and the pathological damage scores of intestinal mucosa were decreased. In conclusion, the mouse model of qi-deficiency gastrointestinal failure was successfully established, which is a reliable, stable and simple method to investigate the disease.

To the Editor,

Gastrointestinal failure (1, 2) refers to severe stress responses of the gastrointestinal tract when the body is severely damaged by trauma, infection,

blood loss, etc. The clinical manifestations include abdominal distension, diarrhea, slowing or even cessation of gastrointestinal motility. It is a common complication of severely diseased patients, and the

Key words: qi-deficiency; gastrointestinal failure; animal model; Sijunzi decoction

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incidence is as high as 59% among ICU patients (3, 4). At present, there are few studies on animal models of gastrointestinal failure. In some countries, short-intestinal syndrome piglets have been established as gastrointestinal failure models (5), and cecal ligation and perforation models (CLP) are also available (6). However, there are few experimental studies involving animal models of gastrointestinal failure in China. In fact, many long-term bedridden patients who have neither undergone abdominal surgery nor suffered from sepsis infection develop gastrointestinal failure, and it is difficult to classify them into the three types of gastrointestinal failure above. Gastrointestinal failure can be divided into several common types in traditional Chinese medicine, such as heat toxic type, blood stasis type, qi-deficiency type and damp heat type (7). The model of traditional Chinese medicine can also be verified by the counter-evidence method. Many scholars (8, 9) have verified and treated the qi-deficiency model by Sijunzi decoction, and obtained the desired effect. Therefore, Sijunzi decoction was employed for counter-evidence. This study aimed to establish a stable and simple mice model of qi-deficiency gastrointestinal failure which is suitable for clinical research using Glauber's enema.

MATERIALS AND METHODS

Animals

One hundred and twenty healthy adult male ICR mice (18-22 g, 8 weeks old) were selected. They were purchased from the Animal Experimental Center of Zhejiang Academy of Medical Sciences, with the animal license number of SCXK (Zhejiang) 2008-0033. The mice were of clean grade, and adaptively fed for 3 days under a constant temperature of $(24 \pm 1)^{\circ}\text{C}$ and humidity of $(50 \pm 5)\%$ with 12h/12h light-dark cycle. During the experiment, the mice were allowed free access to water and solid food in the Experimental Animal Center of Zhejiang University of Traditional Chinese Medicine. The protocol was approved by the ethics committee of Zhejiang University of Traditional Chinese Medicine, and the experiment also followed the regulations of the State Science and Technology Commission on the management of laboratory animals.

Preparation of drugs

The Sijunzi decoction was made up of *Codonopsis pilosula* 9g, *Atractylodes* 9g, *Poria cocos* 9g, and *Radix Glycyrrhizae Preparata* 6g. These traditional Chinese medicines were soaked in water for 30 min, and were decocted by the Chinese Pharmacy of Zhejiang Provincial Hospital of Traditional Chinese Medicine to 500 mg of raw medicine per mL. According to the body surface coefficient exchange algorithm, the dose equivalent to the human clinical dose was 54.6mg/10g.d (the formula: mouse dosage g/kg.d = human daily dose/60kg x 1.0 / 0.026).

Grouping

One hundred and twenty healthy adult male ICR mice (weight, 18-22g) were selected. According to the random number table, the mice were randomly divided into three groups, namely, control group (24 mice), placebo group (24 mice), and experimental group (72 mice). After 10 days, mice in the experimental group were randomly divided into three subgroups: model group, self-healing group and Sijunzi decoction group, with 24 mice in each group. Then, the mice in each group (control group, placebo group, model group, self-healing group and Sijunzi decoction group) were further randomly divided into pathological group, immunohistochemistry group and toner propelling test group, with 8 mice in each group.

Operation steps

Mice in the control group were given a normal diet for 10 days, those in the placebo group were given normal saline (0.3 ml/10g) at 4°C for 10 days, and those in the experimental group were given supersaturated sodium sulfate solution (0.3 ml/10 g) at 4°C for 10 days. After 10 days, mice in the model group were anesthetized by intraperitoneal injection of ketamine (100mg/kg; Qiqiha'er Second Pharmaceutical Co., Ltd.) and then sacrificed. Mice in the Sijunzi decoction group were given Sijunzi decoction (0.1 ml/10 g) for 7 days. Mice in the self-healing group were given normal saline (0.1 ml/10 g) for 7 days. Mice in the control group and placebo group were normally fed, without special treatment. The body weight of mice was measured daily during the experiment, and the hair color, food intake, and feces were observed.

All mice in the pathology group were fasted but water was allowed. After 12 h, the mice were anesthetized by intraperitoneal injection of ketamine (100mg/kg) and then

sacrificed by cervical vertebrae dislocation. The gastric tissue specimens were taken by laparotomy, and the container (Chengdu Taimeng Software Co., Ltd., China) was used to measure the volume. Small intestinal tissue specimens were taken and 10% formaldehyde was used for fixation. The specimens were paraffin-embedded and HE staining was carried out. The changes of intestinal villi, glands and epithelial cells in the small intestinal mucosa were observed by light microscopy (Shanghai Caikang Optical Instrument Co., Ltd., China). The ultrastructure of intestinal mucosal epithelial cells was observed by transmission electron microscopy (Beijing Precise Instrument Co., Ltd., China).

The small intestine tissue section was 3-5 μm in thickness. After HE staining (Mouse 5-Hydroxytryptamine, 5HT, Shanghai Yaji Biotechnology Co., Ltd., China), a 100-fold microscope (Shanghai Caikang Optical Instrument Co., Ltd., China) was used to observe the tissue structure and give Chiu's intestinal mucosal lesion scores.

5-HT immunohistochemical staining. Frozen sections, 8 μm thick, were hydrated and fixed with 4% paraformaldehyde for 15 min, and were inactivated by 3% H_2O_2 solution for 10 min, and washed 3 times with 0.01 mol/L PBS for 5 min each time. The primary antibody was added with a dilution ratio of 1:100, and incubated at 37°C for 60 min. PBS was used for washing 3 times, 3 min each time. The secondary antibody was added and incubated for 30 min at 37°C, followed by PBS washing for 3 times, 5 min each time. DAB was used to develop color for 2 min, followed by hematoxylin counterstaining, conventional dehydration and transparent post-sealing.

TUNEL (TdT-mediated dUTP Nick-End Labeling) staining. The citrate microwave was used to repair the sections for 5 min. 3% hydrogen peroxide was inactivated for 15 min. Tris-HCL+BAS 3%+20% bovine serum was added at room temperature (15-25°C)

for 30 min. The sections were washed by PBS solution several times. The Tunel reaction solution was added and incubated at 37°C (thermotank, Shanghai Keken Experimental Equipment Co., Ltd. China) for 60 min, followed by PBS washing for 3 times, 3 min each time. The POD (Peroxidase) was added and placed under a constant temperature at 37°C for 20 min. PBS washing was carried out 3 times, 3 min each time. DAB was used to develop color for 2 min and then the sections were counterstained.

Ultrastructure of small intestinal mucosa epithelia. 1 mm³ of small intestine tissues fixed by glutaraldehyde were fixed with 1% citric acid for 1 h (4°C). The tissues were dehydrated twice by alcohol with a gradient of 50%, 70%, 80%, 90%, and 100%, followed by acetone dehydration twice. Acetone: the tissues were infiltrated by Epon812 embedding agent, and the infiltrated tissue blocks were placed in a capsule containing dipropylene phthalate and polymerized for 72 h (35°C, 45°C, 60°C for 24 h each). The embedded blocks were trimmed, and the ultrathin sections were stained with acetic acid pomelo-lead citrate (Suzhou Yuya Huagong Chemical Technology Co., Ltd., China) double electrons. The ultrastructure of intestinal mucosal epithelial cells was observed by transmission electron microscopy.

The mice in the toner propelling group were fasted for 12 h, and the toner (0.3 ml/10 g mice) was intragastrically administered. After 30 min, the mice were anesthetized by intraperitoneal injection of ketamine (100mg/kg) and then sacrificed by cervical vertebrae dislocation. The gastrointestinal tract was taken out by laparotomy. The gastrointestinal propulsion index under no tension was measured and calculated.

Observation index

Changes in the coat color, food intake and grade of

Table I. The standard of loose stool grading.

Grade	0	1	2	3	4	5	6	7
Diameter (cm)	0	0-0.9	1.0-1.9	2.0-2.9	3.0-3.9	4.0-4.9	5.0-5.9	6.0-6.9

Note: The average loose stool grade was calculated based on the following formula: $M = (X_1 + X_2 + X_3 + \dots + X_n) / (1 + 2 + 3 + \dots + n)$, where X_n represents the grade of a certain loose stool.

loose stools of the mice were noted. Diameter of the contaminated area in the filter paper by the loose stool was graded (Table I). Body weight of the mice was measured at 10 a.m. every day. Each mouse was measured three times, and the average was recorded.

Gastric volume and toner propelling index

The mice were sacrificed, the whole stomach was taken by laparotomy, and the gastric volume was measured with a container. Toner Advance Index is the distance from the toner tip to pyloric sphincter (cm)/distance from pyloric sphincter to the small intestine end (cm) $\times$ 100%. Chiu's rating scale for small intestinal mucosal damage - 0: Normal intestinal mucosa; 1: Gruenhagen space under the intestinal mucosal epithelia appears in the middle axis of the villi, which is often accompanied by capillary congestion; 2: Intestinal mucosal epithelial elevation from the lamina propria and expansion of the intestinal subepithelial space; 3: A large area of intestinal mucosa is elevated, the villi fall to the sides, and some of the villi are detached; 4: The villi and the lamina propria are detached, the naked capillaries are dilated, and the proportion of cells in the lamina propria is increased; 5: Degradation or digestion of lamina propria, with bleeding or ulcer formation.

Statistics analysis

Statistical analysis was performed using SPSS 17.0 software. The measurement data were expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$). The Dunnett *t*-test was used for the comparison between groups. $P < 0.05$ indicated significant difference.

RESULTS

Mice in the control group and the placebo group had bright coats, ruddy tails and normal appetite. The stool was spindle-shaped, with a moderate water content. There were no abnormalities or death of the mice. In the experimental group, body hair brightness decreased, the coat color was yellowish, the claw ending was pale, and the appetite was significantly reduced. The feces were soft and mushy, the odor of the excrement was heavy, activity was reduced, and the spirit had different degrees of wilting. After 7 days of treatment, the body hair of mice in the self-

healing group was still dull and hairy yellow, with pale tails. The food intake increased slightly, and the stool was soft and mushy. The mice were quiet and rarely moved. In the Sijunzi decoction group, brightness of the body hair of the mice was still not very good, but the food intake was significantly higher compared to the self-healing group, and the stool was shaped. Moreover, the activity became normal.

Changes in the detection index of mice in each group

The average grade of loose stools in the experimental group was higher than that of the control group and the placebo group, with significant difference ($P < 0.01$). After 7 days of treatment, the average grade of loose stools in the self-healing group and Sijunzi decoction group was significantly lower compared to the model group ($P < 0.05$). Moreover, the average grade of loose stools in the Sijunzi decoction group was significantly lower than that of the self-healing group ($P < 0.05$).

After 10 days, the body weight of mice in the experimental group was significantly lower than that of the control group and the placebo group ($P < 0.01$). On the seventeenth day, mice in the Sijunzi decoction group gained more weight than the model group and the self-healing group ($P < 0.05$).

Gastric volume of mice in the model group was significantly higher than that in the control group ($P < 0.01$). After 7 days of treatment, the gastric volume of the self-healing group and the Sijunzi decoction group decreased compared with the model group ($P < 0.01$). There was no significant difference between the Sijunzi decoction group and the self-healing group in the gastric volume ($P > 0.05$).

The gastrointestinal toner propelling index of mice in the experimental group was significantly lower than that in the control group ($P < 0.01$). The gastrointestinal toner propelling index of the self-healing group and Sijunzi decoction group was significantly higher than that of the model group ($P < 0.01$). There was no significant difference between the self-healing group and the Sijunzi decoction group in the gastrointestinal toner propelling index ($P > 0.05$).

The degree of intestinal mucosal damage in the model group was significantly higher than that in the control and placebo groups. The degree of intestinal

mucosal damage in the self-healing group and Sijunzi decoction group was significantly lower than that in the model group ($P<0.01$). The degree of intestinal mucosal damage in the Sijunzi decoction group was lower than that in the self-healing group ($P<0.05$). The specific data are shown in Table II.

The degree of intestinal mucosal damage in each group of mice was scored according to Chiu's grading scale. In the control group, the intestinal mucosa was structurally intact, and villi were clear, with the score of 0. In the placebo group, the intestinal mucosal epithelia from the lamina propria was elevated, and the inferior space of the intestinal epithelia was enlarged, with the score of 2. The intestinal mucosal damage of the model group was the most severe, and pathological changes of the lamina propria, hemorrhage and ulceration were observed, with the score of 5. After 7 days of treatment, the degree of damage in the self-healing group was reduced, and the large intestinal mucosa was elevated. Villi fell to the sides, and part of villi fell off the top. The score was 3. The degree of intestinal mucosal damage was significantly reduced in the Sijunzi decoction group. Only elevation of the intestinal mucosa from the lamina propria and expansion of the intestinal subepithelial space were observed, with the score of 2.

Immunohistochemical staining of 5-HT in small intestine

The morphology of small intestine mucosa in the

normal control group was normal. A large amount of 5-HT was expressed, which was brownish yellow. A large amount of 5-HT was expressed in the placebo group. Only a small amount of 5-HT was observed in the model control group. The expression of 5-HT in the Sijunzi decoction group increased. The expression of 5-HT was observed in the self-healing group, which was significantly increased compared to the model group, as shown in Fig. 1.

TUNEL staining results of small intestine

In the normal control group, the small intestinal mucosa showed positive staining of individual cells. There was a small amount of positive cells in the self-healing group. There was apoptosis of numerous cells in the model control group, which was indicated by brown-yellow. There was only apoptosis of few local cells in the Sijunzi decoction group. Local apoptosis of numerous cells was observed in the self-healing group, but there were less cells compared to the model control group.

Electron microscopy changes of intestinal mucosa

In the control group, villi were arranged neatly, and the tight junctions were clear. The endoplasmic reticulum and mitochondria were normal, and the organelles were not swollen and dissolved. In the placebo group, villi were broken, and the tight junction was twisted. The mitochondria were swollen and vacuolated. In the model group, villi

Table II. *Changes in the detection index of mice in each group.*

Category	Control group	Placebo group	Model group	Self-healing group	Sijunzi decoction group
10 days of loose stools	1.47±0.11	2.74±1.38	3.72±1.26 ^{*Δ}	3.69±0.36 ^{*Δ}	3.83±1.42 ^{*Δ}
17 days of loose stools	-	-	-	3.05±1.04 [#]	2.82±1.59 ^{#&}
10 days of weight change (g)	+3.47±0.13	+3.21±0.16	-1.84±0.15 [*]	-1.52±0.17 [*]	-1.85±0.15 [*]
17 days of weight change (g)	-	-	-	+1.04±0.14	+2.22±0.16 ^{&#}
Gastric volume (ml)	0.97±0.39	1.03±0.45	2.19±0.41 [*]	1.42±0.26 ^{*#}	1.27±0.20 ^{*#&}
Advancement index	73.95±5.49	70.25±7.10	48.10±3.36 [*]	62.45±10.09 ^{*#}	69.94±7.90 ^{*#}
Chiu's rating scores	1.00±0.63	2.00±0.05 [#]	3.83±0.75 [*]	2.33±0.52 [#]	2.00±1.04 ^{Δ&}

Note: ^{*} $P < 0.01$, compared with the control group; ^Δ $P < 0.01$, compared with the placebo group; [#] $P < 0.05$, compared with the model agent group; [&] $P < 0.05$, compared with the self-healing group.

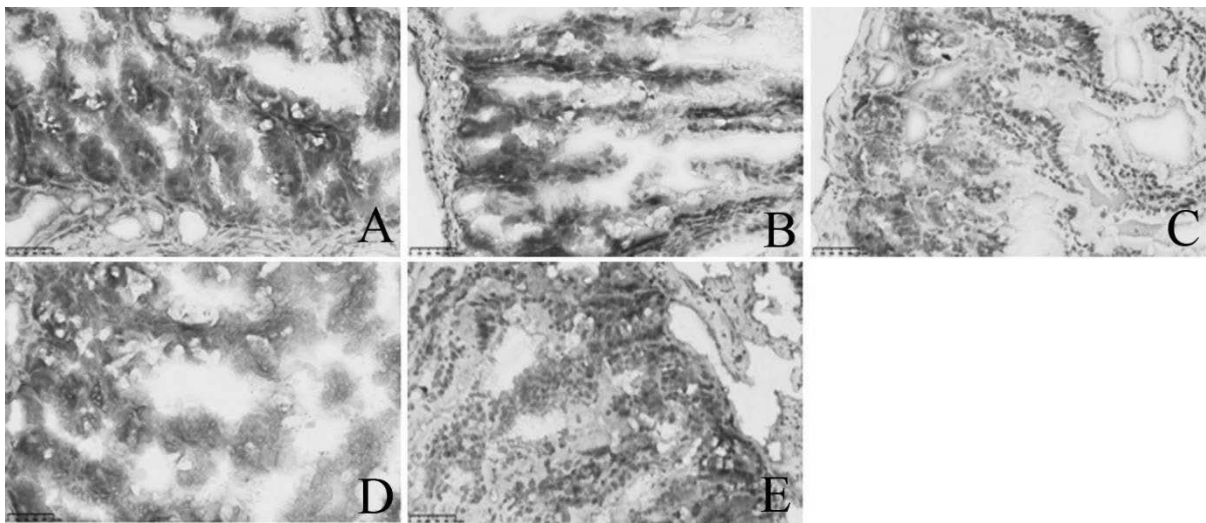


Fig. 1. Immunohistochemical staining results of 5-HT in small intestinal mucosa of mice in each group. **A)** control group: a large amount of 5-HT was observed, which was brownish yellow. **B)** Placebo group: Positive 5-HT expression was observed, which was brownish yellow. **C)** Model group: positive 5-HT expression was observed, which was indicated by brownish yellow. **D)** Self-healing group: positive 5-HT expression was shown, which was indicated by brownish yellow. **E)** Sijunzi decoction group: positive 5-HT expression was observed, which was indicated by brownish yellow. (5-HT, $\times 400$)

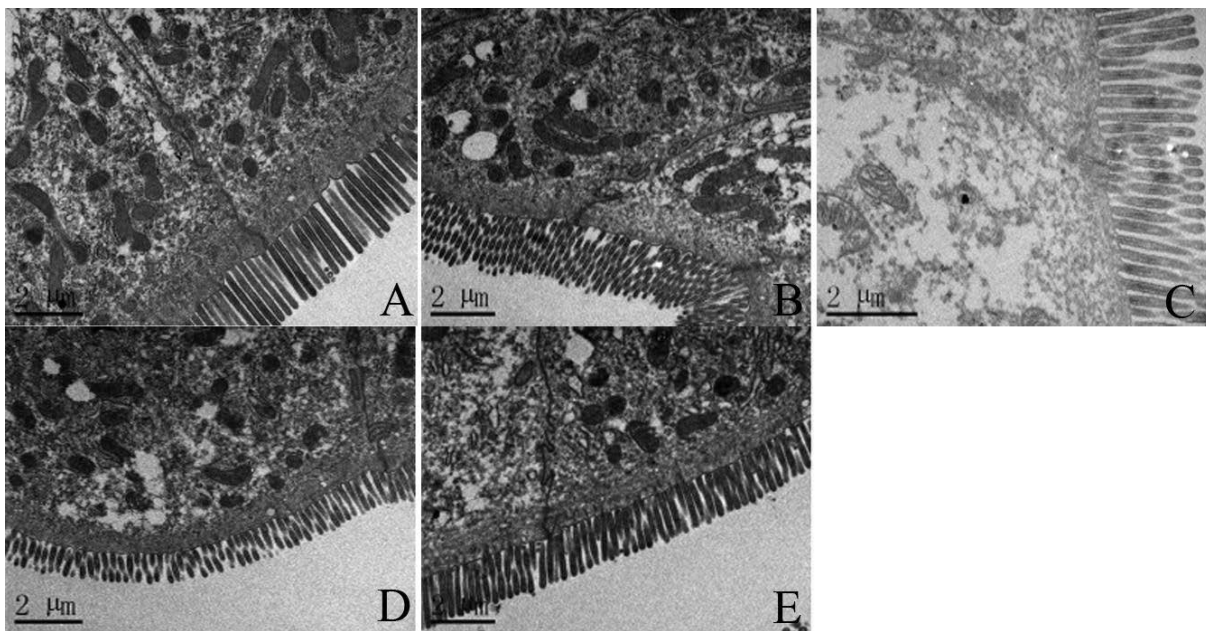


Fig. 2. Changes of intestinal mucosal epithelia under an electron microscope in each group. **A)** Control group: he villi were neatly arranged and the tight connections were clearly defined. Endoplasmic reticulum and mitochondria were normal. The organelles were not swollen and dissolved. **B)** Placebo group: The villi were broken and the tight connection was twisted. The mitochondria were swollen and vacuolated. **C)** Model group: the villi were detached, the tight junction was broken, the mitochondria were swollen and vacuolized, and a large number of organelles were autolyzed. **D)** Self-healing group: The villi were sparse and broken. Tight connections were widened and distorted. The mitochondria were vacuolated and disappeared. The organelles were autolyzed. **E)** Sijunzi decoction group: the villi were partially restored. The endoplasmic reticulum and mitochondria partially restored growth.. The organelles were reduced in autolysis. Tight connections were distorted, but the boundaries were clear.

were detached and the tight junction was broken. The mitochondria were swollen and vacuolated. A large number of organelles were autolyzed. The pathological damage of the Sijunzi decoction group was characterized by partial recovery of villi. The endoplasmic reticulum and mitochondria partially restored growth. The autolysis of the organelles was reduced, and the tight junctions were distorted but the boundaries were clear. In the self-healing group, villi were sparse and fractured. Tight connections were widened and distorted. Mitochondria became vacuoles and disappeared. The organelles were autolyzed. The results are shown in Fig. 2.

DISCUSSION

Sijunzi decoction is a representative prescription for Chinese medicine treatment of qi-deficiency syndrome. Many scholars have verified the reliability and therapeutic effects of the qi-deficiency model by Sijunzi decoction (10, 11). In the experiment, mice in the Sijunzi decoction group experienced weight gain and reduced stools after 7 days of Chinese medicine treatment. The gastrointestinal propelling index increased and the score of intestinal mucosal pathological damage decreased. This validates reliability of the qi-deficiency syndrome model. Sijunzi decoction has a good therapeutic effect on mice models with qi-deficiency gastrointestinal failure, and can be further investigated and applied to clinical practice. It provides a new approach for the treatment of qi-deficiency gastrointestinal failure. In this experiment, a mice model of qi-deficiency gastrointestinal failure was prepared by gavage of supersaturated sodium sulfate solution. The experimental results indicated that the mouse model of qi-deficiency gastrointestinal failure prepared by gavage of supersaturated sodium sulfate solution is in accordance with the diagnostic criteria of animal models of gastrointestinal failure in Western medicine.

In summary, the mouse model of qi-deficiency gastrointestinal failure prepared in this experiment is in line with the natural human diseases. The method is reliable, stable, and simple, which indicates that the model is established successfully. The advantages

of this model are as follows: the occurrence and development of qi-deficiency gastrointestinal failure are similar to the human disease; the success rate is high, mortality is low, and the method is simple and easy; the cost is low, the experimental period is short, and the experimental materials can meet the demand of determination of numerous indicators, which makes the experimental research more targeted and repetitive. In addition, the therapeutic effect of Sijunzi decoction on qi-deficiency gastrointestinal failure needs to be further studied.

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LETTER TO THE EDITOR

EFFECTS OF VITAMIN D ON PANCREATIC ISLET β CELL INJURY INDUCED BY LOW CONCENTRATION CADMIUM IN MICEW. DENG^{1*}, KY. CHEN^{2*}, WY. LIU², ZM. LV² and C. ZHANG²¹Endocrinology Department, Shanghai Songjiang Jiuting Hospital, Shanghai, China; Endocrinology Department, First Affiliated Hospital of Jiamusi University, Jiamusi, Heilongjiang, China

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The aim of this study was to investigate the effect of Vitamin D on pancreatic islet β cell injury induced by low concentration cadmium in mice. The islet β cell line NIT-1 cells were divided into normal control group, CdCl₂ group and CdCl₂+vitamin D₃ (VD) group. Cell viability was detected by CCK-8 assay. Insulin content in the supernatant was detected by enzyme-linked immunoassay (ELISA). Reactive oxygen species (ROS) content was detected by flow cytometry, and changes of insulin-related genes were detected by real-time fluorescence quantitative PCR. Changes of insulin receptor substrate-1 (IRS-1) and insulin receptor substrate-2 (IRS-2) were detected by Western blotting. The results showed that the concentration of CdCl₂ (0.05 μ M) which had no significant effect on the viability of NIT-1 cells was screened by CCK-8 method, where the concentration of active vitamin D (1 nM) for the cytotoxicity induced by a low concentration of cadmium could be reduced. The insulin secretion ability of NIT-1 cells was detected. The results showed that under 4-day exposure to a low concentration of cadmium, the insulin secretion of NIT-1 cells increased at first ($P<0.05$), and then decreased with the prolongation of cadmium exposure ($P<0.05$). The intervention of active vitamin D eventually increased the insulin secretion of the cadmium-exposed cells ($P<0.05$). At the gene expression level, long-term exposure of a low concentration of cadmium did not significantly change the expression of insulin gene, but significantly increased the ROS content in NIT-1 cells ($P<0.05$), while the ROS content in NIT-1 cells was decreased by the intervention of active vitamin D ($P<0.05$). In conclusion, the insulin secretion of islet β cells increased at the early stage of long-term low concentration cadmium exposure, and then decreased with the prolongation of cadmium exposure. This effect was affected by active vitamin D intervention.

To the Editor,

With the aging of China's population and the improvement of living standards, the number of people suffering from diabetes mellitus has been increasing. Type 2 diabetes mellitus (T2DM) is a chronic, systemic metabolic disease caused by long-term interaction of genetic and environmental

factors. Its main characteristics are insulin resistance and decline of insulin secretion function of islet β cells, so the exact cause of islet β cell dysfunction is worthy of investigation (1). Wallia et al. reported that urinary cadmium levels were significantly associated with the risk of pre-diabetes mellitus by abating the islet β cell function (2). Despite the obvious

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hazards of cadmium, the effects of long-term low dose cadmium on human health remain to be further studied because the half-life of cadmium is up to several decades and cadmium can be accumulated in the body for a long time.

Vitamin D is necessary for maintaining the life of animals. The presence of vitamin D receptors in islet β cells suggests that vitamin D may be involved in the regulation of islet β cell function. It has been shown that vitamin D can reduce the risk of diabetes and improve β cell function (3, 4). The long-term exposure of low concentration cadmium in the environment was simulated, so as to find the concentration of cadmium that did not affect the activity of islet β cells and to investigate the effects of cadmium on the function of islet β cells. Furthermore, the effects of long-term vitamin D intervention on the cytotoxicity of islet β cells induced by cadmium were also investigated to provide targets for treatment of T2DM.

MATERIALS AND METHODS

Experimental materials

The mouse islet β cell line NIT-1 (American ATCC cell bank) was used. Before the experiment, NIT-1 cells were cultured in RPMI-1640 complete medium containing 10% fetal bovine serum and placed in an incubator under 5% CO_2 and 37°C. The medium was changed every two days. NIT-1 cells were randomly divided into normal control group, CdCl_2 group and CdCl_2 +VD group. The CdCl_2 and CdCl_2 +VD groups were subdivided according to the concentration of CdCl_2 and VD. $1\alpha,25(\text{OH})_2\text{D}_3$ is the main active form of vitamin D. The following experiments used $1\alpha,25(\text{OH})_2\text{D}_3$ as research materials.

Detection of cell viability by CCK-8 assay

NIT-1 cells were obtained at their logarithmic growth phase and seeded on 96-well plates. Different concentrations of cadmium and vitamin D were added, and 100 μL serum-containing medium was added to the control group. There were 3 parallel accessory holes for each group. After cell attachment, the cells were treated with different concentrations of CdCl_2 , $1\alpha,25(\text{OH})_2\text{D}_3$ and CdCl_2 were added simultaneously, then 10 μL CCK-8 was added to each hole after incubation at 37°C for 1 h. The

absorbance value A was determined by enzyme-labeling instrument at a wavelength of 450 nm. Cell viability was calculated according to the following equation: viable cell rate (%) = (value A of experimental group/value A of control group) $\times$ 100%.

Insulin content test

Cell culture supernatant was centrifuged at 3000rpm/min for 10 min to remove particles and polymers. According to the instruction of ELISA kit, the OD value of each well was determined using the enzyme-labeling instrument at a wavelength of 450 nm. Excel was used to draw the standard regression equation, where the sample concentration was calculated according to the curve equation. The total number of cells was measured to calibrate the insulin secretion of each cell. Trypan blue staining and automatic cell counting were also conducted.

Detection of cell apoptosis rates and ROS release by flow cytometry

The apoptosis rate was measured by Annexin V-FITC/PI double labeling method; DCFDA probe with a final concentration of 10 $\mu\text{mol/L}$ was prepared by serum-free RPMI-1640 medium. The as-prepared probe was added to the collected cells, incubated at 37°C for 30 min, and washed three times by PBS. Then, the fluorescence intensity was measured by a cell analyzer and the concentration of ROS was finally determined.

The changes of insulin receptor substrate-1 (IRS-1) and insulin receptor substrate-2 (IRS-2) were detected by Western blotting: The total protein was extracted by taking cells in logarithmic growth phase and adding equal volume of cell lysate containing PMSF. The supernatant was collected, *i.e.*, the total protein of cells. The operation was performed according to the quantitative BCA kit manual. Electrophoresis was conducted in SDS polyacrylamide gel using the extracted protein (40-50 μg), and the target protein position was determined by reference to protein molecular weight marker. On the 330 mA and 90 min electro-migration instrument, the target protein in the gel was transferred onto the PVDF film, and after addition of the corresponding diluted I-antibodies, was maintained overnight at 4°C and washed 3 times in a shaker. After the incubation of corresponding diluted II-antibodies, it was incubated in a slow shaker for 1.5 h, washed in a shaker for 3 times, and developed with a developer.

Chemiluminescence imaging system was used for color development, photographing and data processing.

Statistical analysis

The experimental data were expressed in the form of $\bar{x} \pm SD$. SPSS 19.0 software was used for statistical analysis and variance analysis test was conducted. SNK-q test was used for comparison between two groups. $P < 0.05$ indicated significant difference.

RESULTS

Effects of cadmium on apoptosis rates

NIT-1 cells were exposed to different concentrations of $CdCl_2$ to investigate the effect of high concentration cadmium on apoptosis rates. $CdCl_2$ with concentrations of 5, 10, 20 and $40 \mu\text{mol/L}$ was applied. After 24-hour cadmium exposure, the total apoptosis rate of NIT-1 cells increased significantly, and with the increased $CdCl_2$ concentration, the apoptosis rate was also sharply increased, showing a positive concentration-effect correlation. In addition, the cell death rate (late apoptosis) was significantly increased, thus cadmium had a significant effect on cell death, as shown in Table I.

Effects of cadmium on cell viability

The results of cell viability test showed that a high concentration of $CdCl_2$ significantly decreased

the cell viability of NIT-1 compared with the control group, and there was significant difference between the two groups at all time points ($P < 0.05$); a low concentration of $CdCl_2$ had no effect on cell viability compared with the control group ($P < 0.05$). Using 1 nM and 10 nM $1\alpha,25(OH)_2D_3$ to interfere with 0.1 μM $CdCl_2$ exposed NIT-1 cells, it was found that 1 nM $1\alpha,25(OH)_2D_3$ improved the damage of cadmium to cell viability at 72 h (Fig.1).

Effects of $1\alpha,25(OH)_2D_3$ and low concentration cadmium on insulin secretion in NIT-1 cells

NIT-1 cells were intervened with 0.05 M $CdCl_2$ and 1 nM $1\alpha,25(OH)_2D_3$. It was firstly demonstrated that long-term low concentration cadmium and active vitamin D intervention did not change cell viability, as shown in Fig. 2A. Then, the ability of individual NIT-1 cells to secrete insulin was detected. The results showed that the insulin secretion of NIT-1 cells increased after exposure to a low concentration of cadmium for 4 days, and then decreased with prolonged exposure of cadmium. Compared with the control group, the difference was statistically significant ($P < 0.05$). However, $1\alpha,25(OH)_2D_3$ intervention eventually increased the insulin secretion of cadmium-exposed cells. On the 16th day, the difference was statistically significant ($P < 0.05$) compared with the $CdCl_2$ group (Fig. 2B). In addition, it was found that long-term

Table I. Apoptosis induced by cadmium of different concentrations.

$CdCl_2$ concentration ($\mu\text{mol/L}$)	Early apoptosis (Mean $\pm$ SD)	Late apoptosis (Mean $\pm$ SD)
0	0.03 $\pm$ 0.01%	0.14 $\pm$ 0.12%
5	65.79 $\pm$ 4.71%*	6.28 $\pm$ 2.14%*
10	87.22 $\pm$ 1.19%*	7.05 $\pm$ 0.27%*
20	55.01 $\pm$ 5.95%*	31.85 $\pm$ 1.52%*
40	25.48 $\pm$ 2.10%*	70.68 $\pm$ 3.05%*

Note: * Compared with the control group (the $CdCl_2$ concentration was $0 \mu\text{mol/L}$), $P < 0.05$.

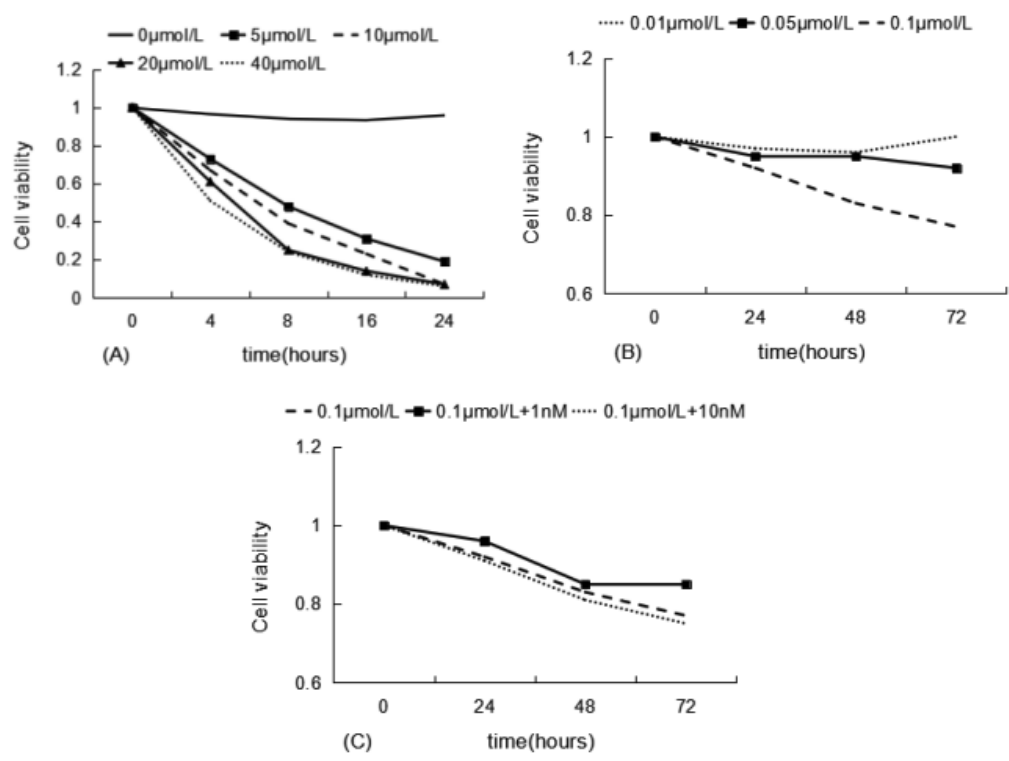


Fig. 1. Effects of cadmium of different concentrations and vitamin D on cell viability. **A)** Cell viability induced by a high concentration of CdCl₂. **B)** Cell viability induced by a low concentration of CdCl₂. **C)** Effects of vitamin D on CdCl₂-induced cell viability.

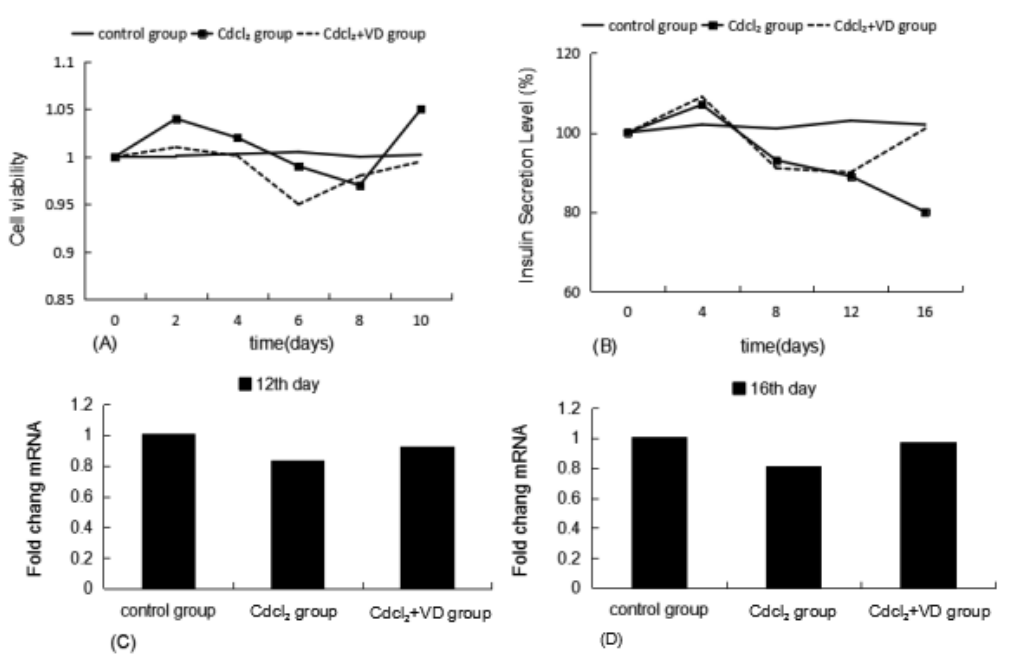


Fig. 2. Insulin content test results. CdCl₂; 0.05 μM; VD: 1 nM. **A)** Effects of CdCl₂ and vitamin D on cell viability. **B)** Effects of vitamin D and CdCl₂ on the ability of NIT-1 cells to secrete insulin. **C)** Expression of insulin gene in NIT-1 cells on the 12th day. **D)** Expression of insulin gene in NIT-1 cells on the 16th day.

exposure to low concentrations of cadmium resulted in a decrease in insulin gene expression, but no significant difference was found with the control group; the expression of insulin genes increased in the active vitamin D-intervened $CdCl_2$ group, but there was no significant difference, (Fig. 2C).

Effects of $1\alpha,25(OH)_2D_3$ and low concentration cadmium on ROS content in NIT-1 cells

NIT-1 cells were intervened with 0.05 M $CdCl_2$ and 1nM $1\alpha,25(OH)_2D_3$. The results showed that long-term exposure of low concentration cadmium could significantly increase the content of ROS in cells compared with the control group. The difference was statistically significant at each time point compared with the control group ($P<0.05$). The ROS level of the $CdCl_2$ +VD group was significantly decreased compared with the $CdCl_2$ group, and there was significant difference between $CdCl_2$ +VD group and $CdCl_2$ group on the 8th, 12th and 16th day ($P<0.05$). Therefore, active vitamin D reduced the reactive oxygen content in cadmium-treated cells.

Effects of $1\alpha,25(OH)_2D_3$ and low concentration cadmium on expressions of IRS-1 and IRS-2 in NIT-1 cells

Both the $CdCl_2$ group and the $CdCl_2$ +VD group showed decreased expressions of IRS-1 in NIT-1 cells, and the difference was statistically significant compared with the control group ($P<0.05$). Compared with the $CdCl_2$ group, the expression level of IRS-1 in the $CdCl_2$ +VD group did not change significantly. Compared with the control group, the expression of IRS-2 in NIT-1 cells was not affected in the $CdCl_2$

group and the active vitamin D exposure group, as shown in Fig. 3.

DISCUSSION

Cadmium concentrations which did not cause cell death were investigated, and it was found that at a low concentration, cadmium still caused islet β cell damage. The main manifestation is that the insulin secretion of pancreatic islet β cells firstly increased after exposure to a low concentration of cadmium, and then decreased with the prolongation of exposure time. This process is similar to the formation of T2DM (5). Although the mechanism of islet β cell dysfunction caused by cadmium is not clear, it has been confirmed that long-term exposure to low concentrations of cadmium can increase the level of reactive oxygen species in islet β cells.

ROS is an important cause of islet β cell dysfunction (6, 7). It was also found that active vitamin D decreased ROS levels in cadmium-exposed cells, and long-term active vitamin D intervention eventually increased insulin secretion in cadmium-exposed cells. In other studies (8-10), vitamin D also reduced the content of ROS in other types of cells, such as macrophages and retinal photoreceptors. Therefore, vitamin D may improve the function of islet β cells by lowering the ROS level of the cells exposed to cadmium.

To sum up, damage of β cell activity and insulin secretion is induced by long-term low concentration cadmium. This effect is changed by active vitamin D intervention. The study provides some reference for the treatment of related diseases.

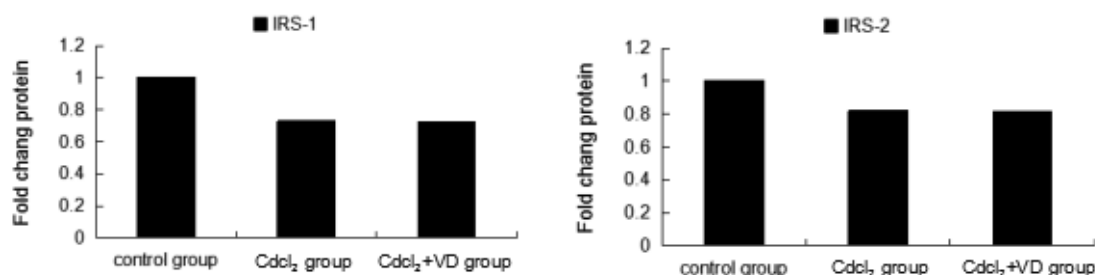


Fig. 3. Effects of vitamin D and $CdCl_2$ on expressions of IRS-1 and IRS-2 in NIT-1 cells ($CdCl_2$: 0.05 μ M; VD: 1 nM.).

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LETTER TO THE EDITOR

EFFECT OF THE APP17 PEPTIDE ON DIABETIC ENCEPHALOPATHY

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This study aimed to investigate the effect of the APP17 peptide on diabetic encephalopathy. Streptozotocin (STZ)-induced type 2 diabetes mellitus rats were used to obtain a model of diabetic encephalopathy with cognitive impairment. After treatment with APP17 peptide, the changes in blood glucose levels, body weight, and memory ability in the model rats were observed. Glucose metabolism was detected by Positron Emission Tomography/Computerized Tomography (PET/CT) in brain of rats from each group. After 12 weeks of STZ-induced diabetes, the escape latency of the diabetic encephalopathy (DE) group was significantly prolonged compared with the normal control (NC) group ($p < 0.05$) and the uptake of glucose in the cerebral cortex and hippocampus was decreased compared with the NC group. The glucose uptake was significantly increased in the group treated with APP17 (APP17+DE), compared with the DE groups and the level of glucose metabolism in brain tissue was greatly improved ($p < 0.05$). In conclusion, it was confirmed that APP17 peptide could improve the cognitive function and glucose metabolism in diabetic encephalopathy rats.

To the Editor,

Diabetic encephalopathy is defined as the damage of the brain caused by diabetes that leads to symptoms that range from mild memory impairment to severe memory impairment, including dementia, seizures and coma (1). Diabetic encephalopathy has great effect on learning, memory process, analysis, problem-solving ability, thinking, intelligence and reaction speed. Diabetic encephalopathy may occur in patients with either type 1 or type 2 diabetes (2, 3). The pathogenesis of diabetic encephalopathy is very complex, being a multifactorial disease (4-6). B-amyloid precursor protein (APP) is widely found in vertebrates and mammals in various

tissues and cells. Almost all cells can synthesize APP, but the highest expression is found in neurons (7). The APP is hydrolyzed to a soluble fragment, N-terminal fragment of the alpha amyloid precursor protein (sAPP α), by α -secretase (8). APP17 can play a protective role in nerve cells, and prevent and control the ultrastructure of the nervous system and neurodegeneration by influencing the nerve cell protein metabolism, improving learning and memory in different brain disorders (9). This study aimed to assess the effect of the APP17 peptide on diabetic encephalopathy and the mechanism that led to this effect in a diabetic encephalopathy rat model. The *in vitro* experiment could help us to better evaluate

Key words: PET/CT imaging technology; diabetes; insulin signaling

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the brain function under the influence of diabetic encephalopathy.

MATERIALS AND METHODS

Animals

Twelve-week-old clean grade male Wistar rats (weight 200–250 g) were used for the study. The animals were purchased from Jilin University Experimental Animal Center and kept in standard conditions with a temperature of $22\pm 3^{\circ}\text{C}$, relative humidity of 40~70% and a 12 h light/dark cycle with free access to standard rat diet and drinking water. Before the experiment, they were acclimatized for one week to the new standard conditions. The animal experiment had the approval of the Ethics committee of Nanfang Hospital, Southern Medical University, Guangzhou and all the procedures were according to laboratory animal guidelines.

Cell lines

XL1-Blue, 293T, and PC12 cells were purchased from the Chinese Academy of Medical Sciences Union cell bank. The ΔIGF1R-PC12 cell line was made in our laboratory.

Preparation of major reagents

Solution A, 0.5% STZ (Streptozotocin), was prepared by dissolving 2.101 g of citric acid (Sigma, USA) into 100 mL double distilled water and solution B was prepared by dissolving 2.941 g of citric acid (Sigma, USA) into 100 mL double distilled water. Solution C (pH 4.6) was prepared by accurately measuring the amount of A liquid 51 mL and B solution 49 mL, mixing well, and filtering after which it was stored at 4°C until use. The C solution was used to configure 0.5% of the STZ solution (Sigma-Aldrich, USA). The preparation process was carried out on ice in the dark. After the preparation was completed, had to be injected within 30 min.

PBS (Phosphate-buffered saline): 0.24g NaH_2PO_3 (Alfa Aesar, UK), 0.2gKCl, 3.63g $\text{Na}_2\text{HPO}_3 \cdot 12\text{H}_2\text{O}$ (Alfa Aesar, UK) and 8 g of NaCl (Alfa Aesar, UK), dissolved in 1000 mL double distilled water. The pH of the solution was then adjusted to 7.4, sterilized at 121°C for 30 min, and stored at 4°C .

DMEM (Dulbecco's Modified Eagle's medium): 3.0 g NaHCO_3 (Alfa Aesar, UK) and 14.7 g DMEM powder

(Hubei Guangao Biotechnology Co., Ltd., China) were added to 800 mL of ultra-pure water. After stirring until complete dissolution, streptomycin and 100 U/mL of penicillin (Shanghai Jizhi Biochemical Technology Co., Ltd., China) at a final concentration of 100 U/mL were added and mixed to 1000 mL. 0.22 μm filter (Tianjin Dongkang Technology Co., Ltd., China) was used for sterilization, and then stored 4°C .

Trypsin solution (0.25%): 2.5 g trypsin (Shanghai Yuanye Biotechnology Co., Ltd., China) was dissolved in 800 mL PBS (Wuhan Ke Enke Technology Development Co., Ltd., China). NaOH (Alfa Aesar, UK) (0.1mol/L) solution was used to adjust the pH to between 7.4 and 7.8, and then it was set to 1000 mL. A 0.22 μm filter was used for sterilization. After packing, and stored at 4°C .

0.5 mM and 1.5 mM Tris-HCl solution: 15.1 g, 45.4 g of Tris (TAKARA BIO INC., JAPAN) were dissolved in 200 mL of ultrapure water, and the pH was adjusted to 6.8 and 8.8 with 2 mol/L HCl (Alfa Aesar, UK). Then, it was set to 250 mL.

N, N'-methyleneacrylamide/acrylamide solution (30%): 58g of acrylamide (Acr) (Thermo Fisher Scientific, USA) was dissolved in 100 mL of ultrapure water. 2 g methylene bis-propylamide (Bic) (Thermo Fisher Scientific, USA) was dissolved in 20 mL of ultrapure water. The volume was mixed to 200 mL, filtered, and stored at 4°C .

TTBS (Tris-buffered saline) (10 x TBS at pH 7.6): 100 g of NaCl and 24.2 g of Tris were dissolved in 800 mL of ultrapure water. 2mol/L HCl was used to set the pH to 7.6, constant volume to 1000mL. Working solution was prepared by diluting the stock 10 times and adding 0.1% Tween-20 (Beijing Baiao Laibo Technology Co., Ltd., China).

Protein loading buffer (5×): 3.75 mL of 30% glycerol (Shanghai Jizhi Biochemical Technology Co., Ltd., China), 0.5 mL of β -mercaptoethanol (Sigma, USA), 4 mL of 5% SDS (Sigma, USA), 0.8 mL of Tris-HCl (Sigma, USA) with a pH of 6.8 and 10 mg of bromophenol blue (Amresco, USA), ultra-pure water volume was add to 10 mL, mixed, 1 mL sub-installed, and stored at 4°C .

Streptozotocin (STZ)-induced diabetic rat model

From 100 male Wistar rats, 10 were randomly selected as a normal control group (NC), and the rest were used the experimental group. After 1 week of acclimatization

the rats were fasted for 12 h and then injected with 0.5% STZ solution intraperitoneally, in doses of 65 mg/kg body weight. On the third day after injection, blood was collected from the tail vein of the rats and fasting blood glucose was measured. Ninety rats with a blood sugar level above 16.7mmol/L were included in the diabetes model group. The NC group received intraperitoneal 0.1mmol/L citrate buffer (pH4.6). For 12 weeks after diabetes induction, the rats from the control and model groups were allowed to have free access to standard diet and water. After 12 weeks the rats were examined if they developed diabetic encephalopathy using the Morris water maze test.

Morris water maze test screening for diabetic encephalopathy rat model

The conditions for Morris water maze experiment were: water depth – 30 cm and water temperature 22–25°C. For the Morris water maze (Technology Institute, Chinese Academy of Sciences) test the rats had 4 days of training, 8 times per day, 4 times in the morning and 4 times in the afternoon, 2 minutes each training. During training, the rats were randomly placed from the four water points into the water, recording the time from entry into the water, to find the time to escape. If the experimental rats did not find the platform within 2 min, the escape latency is 2 min. Then, the rats were drawn to the platform by the operator and stayed 30 s. The rats were put back in their cages and rested for half an hour, the second test began. The fifth day was the test period, recording escape latency.

APP17 peptide effect on memory ability of diabetic encephalopathy rats

The rats with diabetic encephalopathy were randomly divided into 4 groups, 10 rats per group.

The groups included in the experiment were:

Normal control group (NC) – 10 normal rats received same volume of saline intraperitoneally once per day.

Diabetic encephalopathy group (DE) – 10 rats with diabetic encephalopathy received the same volume of saline as the test groups intraperitoneally once per day.

Low dose APP17 peptide group (LAPP17 + DE) – 10 rats with diabetic encephalopathy received 13 µg/kg body weight APP17 peptide (Shanghai Jill Biochemical, China) intraperitoneally once per day.

High dose APP17 peptide group (HAPP17 + DE) - 10

rats with diabetic encephalopathy received 26 µg/kg body weight APP17 peptide intraperitoneal injection once per day. The experiment lasted four weeks. At the end of the administration period, the effect of the APP17 peptide on the behavior of diabetic encephalopathy was examined by Morris water maze test.

Effects of the APP17 peptide on glucose metabolism in the brain of diabetic encephalopathy rats

Regarding the *in vivo* experiments, the PET/CT scan was used to evaluate the effects of APP17 on animals from NC, DE, LAPP17+DE and HAPP17+DE groups. Thirty minutes before the test, 0.75 mCi/kg contrast agent [¹⁸F-FDG (Sigma, USA)] was injected intravenously and then visualized in a PET/CT instrument. Before imaging, rats were anesthetized using chloral hydrate (TRC, Canada) (3mL/kg body weight) injected intraperitoneally. The CT images were first collected followed by PET images. CT and PET and their fusion images were obtained using a Xeleris workstation (GE Healthcare, USA). The brain glucose metabolism was determined by collecting the image information. CT acquisition conditions: voltage 80KV, current 30 mA, scanning layer thickness 5 mm, reconstruction layer thickness 4.25 cm, tube rotation time 0.8 s. The acquisition time of PET was 15 min, and the number of overlaps between the acquisition horizons was 1. The collected images were processed by attenuation correction. PET/CT tomographic images were analyzed using the Xeleris image fusion station to obtain cross-sectional data. The glucose metabolism of the brain was analyzed by using the standardized uptake values (SUV) of the region of interest (ROI) on the PET image. SUV value is the commonly used quantitative parameters. It can accurately reflect the distance between the intake of radioactive drugs and the average body intake. The formula used for calculation is:

$$SUV = \frac{\text{The average radioactivity per gram of tissue}}{\text{The radionuclide activity of the injected nuclide} \times \text{body weight}}$$

Immunohistochemical staining

At the end of the *in vivo* experiment, the rats were sacrificed with barbiturate overdose. Blood was taken from the abdominal aorta, and the brain was removed by rinsing with precooled saline to 0°C and separated on ice. The brain was dried from excess water, and one side of

the tissue was fixed overnight in 4% paraformaldehyde for immunohistochemical staining. Immunohistochemical staining was performed using the streptomyacin avidin-peroxidase (S-P) method. The other side of the brain was washed with saline solution at 0°C and used for tissue homogenate. A 10% tissue homogenate (3500 r/min, 10 s/time, interval 30s, performed 4 consecutive times) was prepared by using an internal trimming homogenizer, on ice bath. Then, it was centrifuged (3000r/min, 10min) for supernatant removal and stored at -80°C.

The apoptotic effect of WST-1 (Water Soluble Tetrazolium Salts-1) on PC12 cells

The APP17 peptide was digested with Iscove's Modified Dulbecco's Media (IMDM) medium prepared into 8 concentration gradients. From high to low, they were 20, 10, 5, 1, 0.5, 0.25, 0.12 and 0.06µg/mL. In the 96-well culture plate, 0.1 mL of PC12 cells and 0.1 mL of APP17 peptide were tested. 0.1 mL of PC12 cells and 0.1 mL of the culture medium were added to the negative control group. The positive control group was arsenic

trioxide. Each group had 6 wells. They were placed in a 37°C, 5% CO₂ incubator for 48 h; 4 h before the cell culture was terminated, 10 µL of WST-1 (5mg/mL) was added. After the cell culture was terminated, 100 µL of 0.1 mol/L isopropyl alcohol hydrochloride was added. The OD value was measured at 450 nm on an enzyme-linked immunosorbent assay.

Statistical analysis

SPSS16.0 software was used for single factor analysis of variance (ANOVA) post hoc test. Student's *t*-test was used to compare the experimental groups two by two. The results were expressed as a mean ± standard error. *P* < 0.05 was considered statistically significant.

RESULTS

The establishment of a rat model of diabetic encephalopathy

The rats from DM and DE showed obvious polydipsia, polyuria, excessive feeding, and had

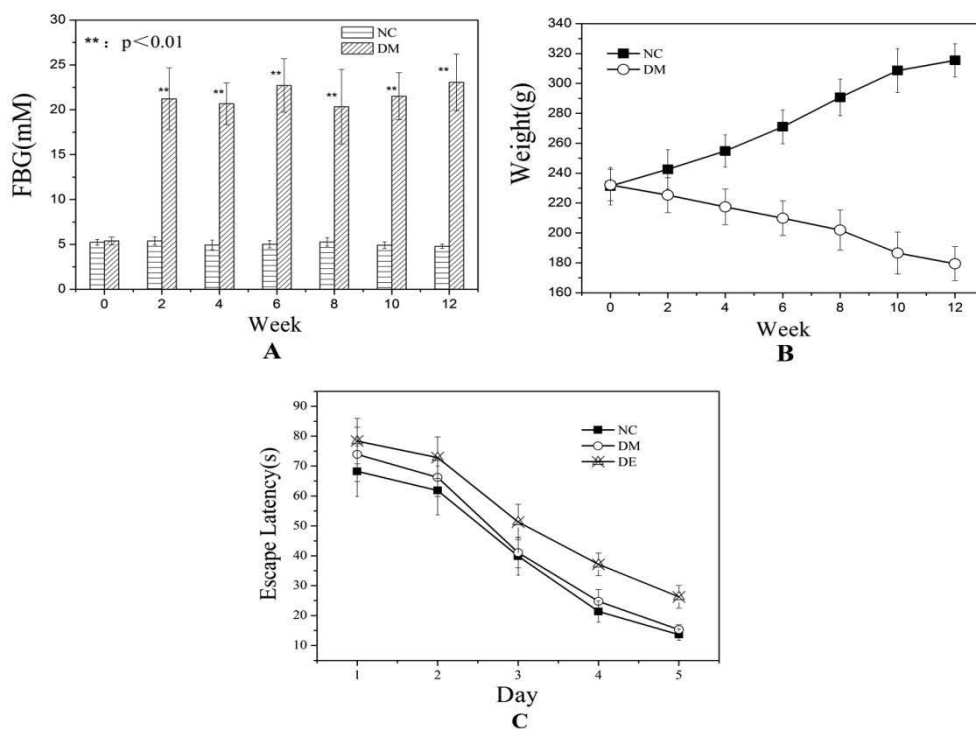


Fig. 1. A) Changes in blood glucose levels in an STZ-induced rat model; B) Changes in body weight in an STZ-induced diabetic rat model; C) Learning and memory in rats from STZ-induced diabetic group and diabetic encephalopathy group. FBG: fasting blood glucose

gray hair color, were apathetic, and actions were slow. Compared with the normal control group, the body weight of the diabetes mellitus group decreased gradually from the time of onset. However, the blood glucose level was maintained at a high level. The changes in blood glucose levels, body weight gain and memory ability of the rats in the NC and DM and DE rats are presented in Fig. 1A and Fig. 1B. Between NC and DM groups, there was no difference in escape latency of animals in the Morris water maze.

In the DE group the escape latency was significantly increased ($p < 0.05$) (Fig. 1C).

Effect of the APP17 peptide on the cognitive ability of diabetic encephalopathy rats

Before and after treatment, the level of blood glucose in diabetic encephalopathy group was significantly higher than that in the normal control group ($p < 0.01$). Compared with the diabetic encephalopathy group, the blood glucose of APP17

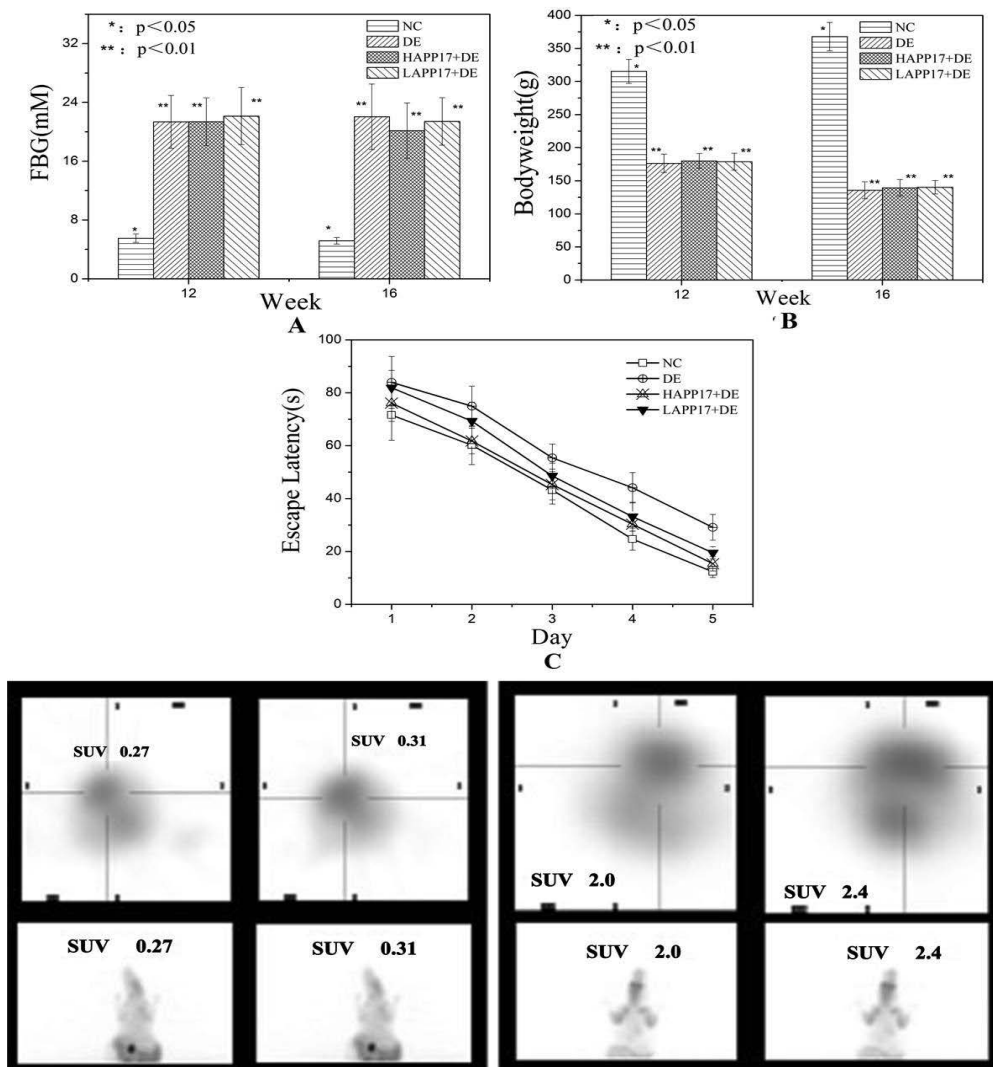


Fig. 2. Effect of the APP17 peptide in the treatment of diabetic encephalopathy. **A)** Changes in blood glucose levels in the 4 groups before and after APP17 peptide treatment; **B)** Changes in body weight in the 4 groups before and after APP17 peptide treatment; **C)** Learning and memory in rats in the 4 groups in the Morris water maze. FBG: fasting blood glucose; **D)** The PET/CT imaging and SUV values of the diabetic encephalopathy in DE group; **E)** The PET/CT imaging and SUV values of the diabetic encephalopathy in APP17+DE group.

peptide high dose group and APP17 peptide low dose group was decreased, but there was no statistical significance ($p > 0.05$) (Fig. 2A). Changes in body weight are similar to changes in blood sugar. The weight of the diabetic encephalopathy group was lower than that of the normal control group ($p < 0.01$) before and after treatment. Compared with the diabetic encephalopathy group, the weight of HAPP17+DE group and LAPP17+DE group was increased, but there was no statistical significance ($p > 0.05$) (Fig. 2B). These results showed that APP17 peptide could not improve the blood glucose and body weight of diabetic encephalopathy rats. The cognitive ability of diabetic rats treated with APP17 peptide was significantly improved compared with the diabetic encephalopathy group, the escape latency being significantly shorter ($p < 0.01$ or $p < 0.05$) (Fig. 2C).

The glucose metabolism in the brain of diabetic encephalopathy rats

In the DE and APP17+DE groups, 18F-FDG uptake had a significant positive correlation between the cerebral cortex and the hippocampus. Rat glucose metabolism in the brain showed that, compared with the NC group, the glucose uptake in the cerebral cortex and hippocampus was decreased in the DE

group. Compared with DE group, the glucose uptake in the APP17+DE group was significantly increased, and the level of glucose metabolism in brain tissue was greatly improved (Fig. 2D and Fig. 2E).

Effects of the APP17 peptide on insulin-related signaling pathway

Table I shows that the absorbance of the APP17 peptide in the OD 450 nm drug group was compared with the untreated negative control group. When the dose reached 5 $\mu\text{g/mL}$, the APP17 peptide produced a significant cytotoxic effect on the cells. When the dose was 1 $\mu\text{g/mL}$ or below the cytotoxicity of the APP17 peptide was not significant.

DISCUSSION

This study showed that the treatment with the APP17 peptide could not improve the blood glucose level, and body weight index of diabetic encephalopathy rats but can improve the memory function. PET/CT imaging was used to study the level of glucose metabolism in the rat brain. Compared with the normal control group, the glucose uptake of the cerebral cortex and hippocampus was reduced in the DE group. Compared with DE group, the glucose uptake of APP17 peptide group was increased significantly, and the level of glucose metabolism in brain tissue was improved. Also, it was found that the APP17 peptide could significantly increase the expression of Glut4, which had no significant effect on the expression of IGF1R and IR in the hippocampus of diabetic encephalopathy rats, suggesting that APP17 peptide may influence an insulin-related pathway.

Defects in PC12 expression in IGF1R are caused by lentiviral knockdown techniques (10). At the same time, the cell surface insulin receptor IR is preserved. By using APP17 peptides and IGF1, PC12 cells were stimulated (11). When IGF1R and IR are present, IGF1 stimulates a large number of phosphorylation of IRS in PC12 cells. The APP17 peptide can also stimulate the phosphorylation of IRS to a certain extent, but the stimulating effect is not strong. When IGF1R expression defects and the IR expression is normal, IGF1 can still induce a certain degree of IRS phosphorylation (12). From this, it is speculated

Table I. The apoptotic effect of the APP17 peptide on PC12 cells in vitro.

Group		OD value
NC group		1.46 $\pm$ 0.22
DE group		0.53 $\pm$ 0.06
APP17 peptide group ($\mu\text{g/mL}$)	0.06	1.35 $\pm$ 0.14
	0.12	1.24 $\pm$ 0.15
	0.25	1.30 $\pm$ 0.13
	0.5	1.19 $\pm$ 0.49
	1	1.08 $\pm$ 0.20
	5	0.80 $\pm$ 0.09*
	10	0.32 $\pm$ 0.03*
	20	0.27 $\pm$ 0.02*

($x \pm s$, $n = 6$); * $p < 0.05$, compared with the negative control group.

that APP17 peptide may seriously affect the signal transduction of IGF1 to IGF1R, which leads to the abnormal start of the pathway involved in IGF1R.

The results of this study can be a starting point for developing new drugs to be used in the treatment of diabetic encephalopathy.

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LETTER TO THE EDITOR

NASOPHARYNGEAL DRAINAGE WITH A NEW DEVICE: PRELIMINARY RESULTS OF NASAL SPIROMETRY

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The nasopharyngeal cavity is the crucial point of the upper airway; its obstruction is associated with allergic rhinitis, upper airway respiratory infections and is the basis of middle ear diseases. The purpose of this preliminary study was to evaluate the ability of a new device to empty the nasopharyngeal cavity from mucus and restore free nasal breathing. The present study evaluated the effects of nasopharyngeal drainage using a new device (Atomix wave®) on nasal expiratory flow rates in 20 volunteers (12 males and 8 females, mean age 44 ± 18 years) with nasal obstruction. Nasal spirometry was performed before and after nasopharyngeal cavity treatment with the new device. The nasal maximum mid-expiratory flow rate (n-MEF 25/75) was significantly increased after treatment (baseline value 0.92 ± 0.43 and 0.67 ± 0.33 ; post-treatment 1.31 ± 0.56 and 1.25 ± 0.44 ; p less than 0.05). The new device proved effective in clearing the nasopharyngeal cavity.

To the Editor,

The nasopharyngeal cavity represents a vital crossroad connecting the nose, the ears, and the pharynx. Located posteriorly to the nasal cavity and superior to the oropharynx, the nasopharynx physiologically receives the drainage of mucus from the paranasal sinuses and is directly connected to the middle ear by the Eustachian tubes. The interactions between the nasopharyngeal microbiome (bacteria, viruses and fungi) and the lymphatic system are responsible for the complex dynamics of the local immune response. The latter, conversely, mediates the interactions between resident (symbiotic) and pathogenic bacterial strains (1). Nasopharyngeal bacterial colonization is a frequent event from early childhood, eventually leading to the development of lower respiratory tract infections, asthma (2) and

otitis media with effusion (3).

Morphological and functional alterations of the nasopharynx also affect the function of adjacent anatomical regions such as the nasal cavity and paranasal sinuses, the Eustachian tube and the middle ear. The nasopharyngeal functions are mainly altered by the presence of phlegm, often gelatinous, induced by intermittent inflammatory states of the mucosa and, to a lesser extent, by the mechanical obstruction caused by hypertrophic adenoids (4). The common consolidation of the mucous secretions can form a plug that blocks the choanae and becomes a substrate and a receptacle for pathogens.

Nasal spirometry has been used as an objective and reliable tool to assess the degree of nasal obstruction before and after treatments (5). A portable spirometer connected to a nosepiece is able

Key words: nasal flow; nasal spirometry; nasopharyngeal cavity; nasal rinsing

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to measure the volume of air expired from each nasal passage during a slow vital capacity.

The aim of our study was to evaluate the ability of a new device (Atomix wave®) to clear the mucous plug from the nasopharyngeal cavity and restore free nasal breathing, by means of nasal spirometry.

MATERIALS AND METHODS

The study was conducted on twenty adult volunteers: 12 males and 8 females (mean age 44 ± 18 years) with nasal obstruction.

The selection criteria included patients with obstruction of the nasopharynx by dense mucous secretions observed at nasal fibro-endoscopy, and excluded patients with nasal septum deviations and nasal polyps. All selected patients suffered from chronic rhinosinusitis with post-nasal drip or post-nasal mucous rhinorrhoea as the main symptom.

The nature of the study was explained and informed consent was obtained from each subject prior to the initiation of the study. All subjects were treated and tested after at least 24 hours of persistence of the obstructive symptoms ("stuffy nose" and "thick mucus behind the nose"). After blowing their noses as needed, they underwent nasal spirometry before and immediately after the nasopharyngeal cavity treatment with a new device (Atomix wave®, Tred Medical, Milano, Italy).

The subjects were asked to bend their heads forwards over a wash basin and plug one nostril with the nosepiece before pressing the plastic reservoir of the device. The characteristics of the fluid flowing out of the opposite nostril were observed and recorded by the examining physician. A single administration of Atomix wave was allowed for each subject. Fibro-endoscopy of the

nasopharynx was performed before and after treatment and the subjects were asked to subjectively evaluate the benefit of nasopharyngeal drainage on nasal breathing by a visuo-analogic scale from 0 to 10.

Nasal spirometry

The Spirobank G spirometer (MIR; Rome, Italy) is a turbine with an infrared interruption; its parameters meet or exceed the latest American Thoracic Society (ATS) standards (6). Nasal spirometry was performed with the subjects in standing position; the nosepiece was connected to the device and inserted in each nostril, similarly to the study by Goyal et al. (7). The nasal spirometer measured the airflow from each nostril separately. The nasal maximum mid-expiratory flow rate (n-MEF 25/75) was recorded for analysis. The average of three repeated measures was retained.

The nasopharyngeal draining device

Treatment of the nasopharyngeal cavity was achieved with a new device for nasopharynx hygiene constituted by: i) pump dispenser that generates a washing flow; ii) a custom-made nasal ending adaptable to all nostrils; iii) 2 bottles of 250 ml of hypertonic saline solution buffered at pH 6. The pump container is filled with hypertonic saline solution buffered at pH6 and closed with the nasal nozzle. The method is for the patient to seal one nostril, and then hold a steady and firm pressure on the pump. The jet of solution activates the rhino-palatal reflex and forces the mucous exudate out of the nasopharyngeal cavity into the contralateral nasal cavity, finally flowing out of the free nostril.

All procedures contributing to this work comply with the ethical standards of the relevant national and institutional guidelines on human experimentation and

Table I. Nasal maximum mid-expiratory flow rate (n-MEF 25/75) before and after treatment.

Right nostril		Left nostril	
BEFORE	AFTER	BEFORE	AFTER
0.92±0.43	1.31±0.56	0.67±0.33	1.25±0.44
p < 0.004		p < 0.001	

with the Helsinki Declaration of 1964, as revised in 2008. The volunteers were not paid for the study and all gave signed written consent for publication.

Statistical analysis

Data were analysed by means of the statistical package SPSS (IBM Corp. Released 2016. IBM SPSS Statistics for MAC, Version 24.0. Armonk, NY: IBM Corp.). Non-parametrical tests were chosen for the verification of the hypotheses due to the low number of subjects analyzed. Differences between nasal spirometric parameters were calculated by Wilcoxon's test. The significance level was set at $p=0.05$.

RESULTS

All subjects performed clinical evaluation with fibro-endoscopy, nasal spirometry and treatment with Atomix wave[□] in a single session. Explanation was straightforward, and the simple testing and treatment procedures were easy and fast to administer.

The procedure was well tolerated: none of the patients reported any kind of discomfort during the nasopharyngeal washout, nor complained of fluid reaching the oropharynx or aural fullness. All studied subjects reported improved nasal breathing after the treatment. The subjective and spirometric clinical benefit was evident in both nasal cavities.

As shown in Table I, the nasal spirometry results demonstrated a significant improvement in the n-MEF 25/75 in both nostrils after treatment.

DISCUSSION

In this study, all subjects reported an improved nasal breathing after the treatment. However, since a discrepancy between nasal obstruction symptoms and clinical findings has been reported in the literature, objective measurements of nasal obstruction are as important as objective measurements of lung function (8). Rhinomanometry objectively measures the nasal obstruction but it is not an easy and quick method, conversely nasal spirometric parameters are commonly used to assess the degree of airway nasal obstruction (9). For this reason, nasal spirometry was preferred in this study.

Some papers report that forced expiratory flow (FEF) at 25-75% of forced vital capacity might be considered as a possible marker of early bronchial impairment in patients with allergic rhinitis (10), and this parameter has been considered as the most reliable and stable index for the management of asthma (11). Since 1994, n-MEF 25/75 was used to evaluate nasal obstruction (12), because it does not consider the initial and final part of the expiratory flow, thus resulting in the most stable nasal spirometric parameter (7), as well as in the lung (11).

Saline nasal irrigations have been reported to reduce nasal congestion and mucopurulent secretion, and have also been proved to decrease nasal resistance, induce restoration of muco-ciliary clearance and attenuate upper airway inflammation. Therefore, maintaining the rhino-pharyngeal cavity empty, in addition to cleaning with saline solutions, is certainly a necessity to prevent and/or improve the treatment of infections of the upper airways.

These preliminary results showed that the new device was able to clear the nasopharyngeal cavity; in order to verify this interesting effect, further studies are needed comparing different devices and different solutions.

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LETTER TO THE EDITOR

COMBINED SEA SALT-BASED ORAL RINSE WITH XYLITOL IN ORTHODONTIC PATIENTS: CLINICAL AND MICROBIOLOGICAL STUDY

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Orthodontic appliances protect the plaque from the actions of brushing, mastication, and salivary flow. Plaque control is a very difficult task for patients with fixed orthodontic appliances. It has been demonstrated that chemical agents are useful additions in plaque control for these patients. To improve mechanical plaque removal, studies in the field suggest to add a chemo–therapeutic agent, such as an antibacterial mouth rinse, to the oral hygiene regimen. Noteworthy evidence from clinical trials has shown that oral hygiene status is significantly improved when antibacterial mouth rinses are added to daily oral hygiene measures (tooth brushing and flossing) compared with tooth brushing and flossing alone. The purpose of this study was to evaluate the effect of a combined rinsing solution (sea salt, xylitol and lysozyme) on plaque and gingival index in orthodontic patients with fixed appliances.

To the Editor,

Dental plaque, which is typically accumulated during orthodontic treatment, is the major etiologic factor in the development of dental caries and gingivitis (1). Orthodontic metallic attachments may cause alterations in the oral microflora due to variation of pH and bacteria affinity to the metallic surfaces facilitated by electrochemical reactions, that lead to the development of new plaque retentive areas, which, in turn, result in increased microbe carriage (2).

Generally, orthodontic patients are unable to maintain adequate oral hygiene by mechanical

means alone due to the failure of plaque removal from difficult-to-access areas that are hindered by orthodontic attachments (3). A common strategy is to add a chemotherapeutic agent, such as an antimicrobial mouth rinse, to their mechanical oral hygiene routine (4).

Recent caries research has been mostly focused on xylitol, which is a caloric sugar substitute (5, 6). Most plaque bacteria lack the ability to ferment xylitol into cariogenic end products. Instead, xylitol accumulates intracellularly in *S. mutans* as a non-metabolizable metabolite, thus inhibiting bacterial growth, reducing bacterial population, and reducing

Key words: Streptococcus mutans; randomised controlled trial; fixed orthodontic treatment

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the amount of plaque. Moreover, the presence of sea salt in the mouth increases the pH level inside the mouth.

The goal of the present study was to investigate the impact of a unique mouth rinse formula (H2Ocean Sea Salt Mouthwash) on gingival health in orthodontic patients based on the reported health benefits from sea salt and xylitol in the oral cavity.

MATERIALS AND METHODS

Subjects and study schedule

Twenty orthodontic patients were enrolled in the study. During the study period the subjects underwent fixed orthodontic treatment in a private practice. The study was conducted as a double-blind parallel-group clinical trial. Written informed consent was obtained from patients in accordance with the Declaration of Helsinki.

The participants were asked to rinse their mouth twice a day for 4 weeks after brushing in the morning and at night before sleeping, and once per day for the following

4 weeks (before sleeping). The patients were instructed to rinse with 15 mL of the solution for 1 min followed by expectoration of the residual mouth rinse and avoid drinking and eating for 30 min after rinsing. To avoid the introduction of new variables, participants were asked to continue their usual daily brushing method during the study period.

Written instructions were given to the participants, explaining how to use the mouthwash. Rinsing was performed at home without supervision. To check for compliance, the participants were asked to note the times of day when they rinsed.

Sample size and blinding

All selected participants had mild gingivitis at the start of the study. All participants were randomly assigned to one of two treatment groups ($n=10$ in each group) by using two series of random numbers, one for the male participants and one for the female participants. The groups and assigned mouthrinses were as follows:

A: A combined mouth rinse containing sea salt,

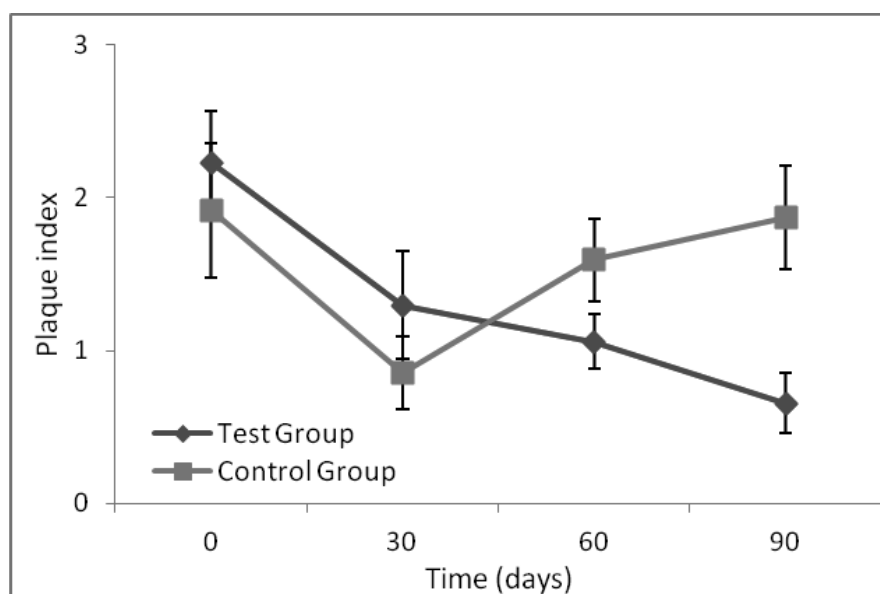


Fig. 1. Effect of oral solution on the plaque index at baseline (day zero) and 30, 60, 90 days after tooth scaling and correct oral hygiene instructions. Control group received placebo. The index for each patient was obtained by summing the indices for all the examined teeth and dividing by the total number of the examined teeth. Results are presented as the mean $\pm$ SD and significant differences between the test and control groups are indicated by * ($p<0.05$) or *** ($p<0.001$).

lysozyme and xylitol (H2Ocean Sea Salt Mouthrinse, a new mouthwash formula that contains sea salt, xylitol and lysozyme with no harsh chemicals, alcohol or fluoride).

B: A placebo mouth rinse (physiologic saline solution - no mouthwash dilution added).

All of the mouth rinse bottles had a similar appearance.

Inclusion criteria

Patients aged between 18 and 30 years, and suffering from mild gingivitis, under orthodontic treatment, with good-to-fair oral hygiene were selected for the study.

Exclusion criteria

Patients on antibiotic therapy or those who had been on antibiotic therapy in the previous month or during the trial period, suffering from pre-existing oral irritations or oral infections (rampant caries, severe/aggressive gingivitis and periodontitis, oral thrush, diagnosed halitosis, or diagnosed xerostomia), using any mouthwash within the previous month, smoking or consuming tobacco in any form, suffering from any systemic diseases, and patients

who were deemed to be uncooperative.

Clinical parameters

For the clinical parameters, scores including Bleeding Index (BI) and Plaque Index (PI), (7-9) were blindly taken from all participants by the same trained examiner at baseline and after 30, 60, and 90 days (investigation of supplementary 30 days from the end of oral rinsing to assess the long-term efficacy of the product). The measurements were recorded from central incisors, canines and second premolars of four quadrants.

A mean value of each parameter for both groups, was calculated in each pre- and post-rinse measurement.

Microbiologic examination

For microbial evaluation, saliva samples were collected by chewing on a piece of sterile paraffin wax. We conducted paired cultivation on 20 samples in order to test whether there were variations in the bacterial population caused by the laboratory methods. The samples were transferred, immediately upon collection,

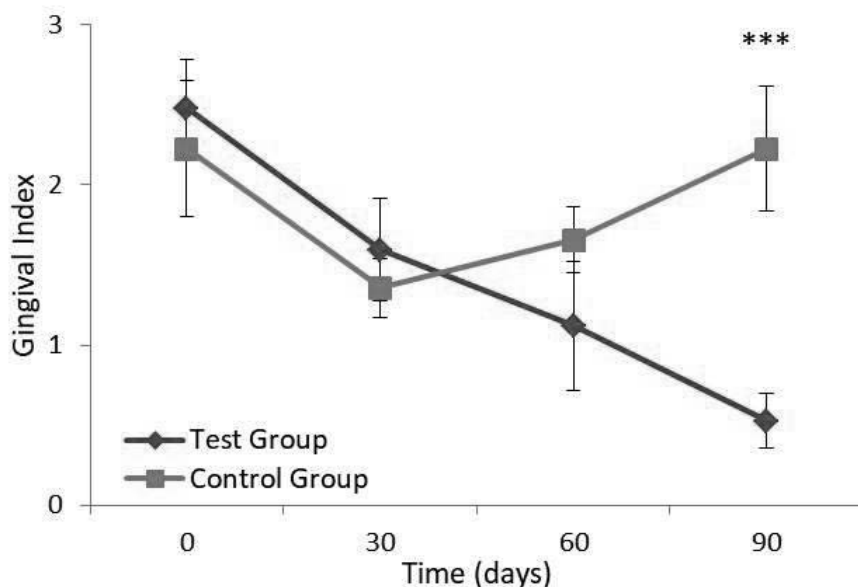


Fig. 2. Effect of oral solution on the gingival index at baseline (day zero) and 30, 60, 90 days after tooth scaling and correct oral hygiene instructions. Control group received placebo. The index for each patient was obtained by summing the indices for all the examined teeth and dividing by the total number of the examined teeth. Results are presented as the mean±SD and significant differences between test and control group are indicated by * ($p<0.05$) or *** ($p<0.001$).

to the Microbiology Laboratory for further processing. Then, 50 μ L of the specimen were cultured in Blood Agar and Eosin Methylene Blue (EMB) mediums. The colony counts were enumerated in 48-h incubation at 37°C. The total bacterial count was determined by visual counting and was multiplied by 20 to express the count in terms of colony forming units (CFU)/mL (4).

Statistical analysis

Statistical analysis was carried out by utilizing Two-way ANOVA and Tukey's multiple comparison tests to assess the statistical differences. For this purpose, GraphPad Prism software (version 7.0; San Diego, California, USA) was used. The probability (P) of <0.05 was considered to indicate statistical significance.

RESULTS

Clinical measurements

We obtained encouraging clinical results in our study. The participants provided positive feedback on the use of our suggested hygienic protocol.

Plaque index

Fig. 1 shows the effect of the oral solutions on the plaque index at baseline (day zero) and 30, 60, 90

days after tooth scaling and oral hygiene home care instructions. The control group received placebo. The index for each participant was obtained by summing up the indices for all the examined teeth and dividing by the total number of the examined teeth for that participant. The results are presented as the mean $\pm$ SD of ten participants per group and significant differences between the test and the control groups are indicated by * ($p < 0.05$) or *** ($p < 0.001$).

Gingival index

Fig. 2 shows the effect of the oral solution on the gingival index at baseline (day zero) and 30, 60, 90 days after tooth scaling and oral hygiene home care instructions. The control group received placebo. The index for each participant was obtained by summing up the indices for all the examined teeth and dividing by the total number of the examined teeth for that participant. The results are presented as the mean $\pm$ SD of ten participants per group and significant differences between the test and the control groups are indicated by * ($p < 0.05$) or *** ($p < 0.001$).

Microbiologic examination.

Large variations in inter- and intra-individual measurements were observed. Both examinations

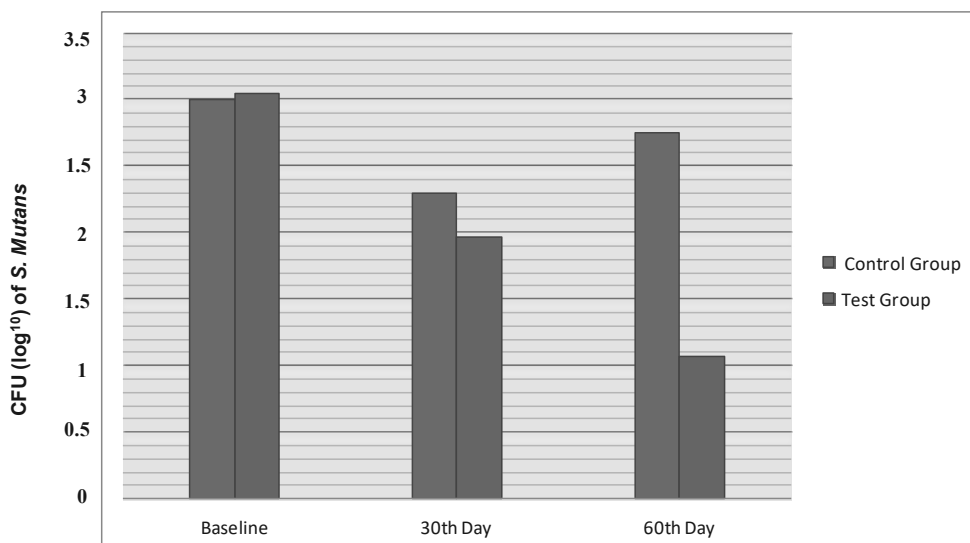


Fig. 3. Intergroup comparisons of mouth rinses based on microbiological parameters.

showed a significant decrease from the baseline.

Compliance.

Compliance with the course of the use of the tested product was documented. All participants completed the course of mouthwash protocol as indicated and returned the medication bottles.

DISCUSSION

The results of our study show a significant improvement of oral hygiene status when the natural antimicrobial mouth rinses were added to the daily oral hygiene regimen. The importance of such agent was even greater for the participants with oral appliances (7-11).

The present study was therefore designed to evaluate clinical and microbiological effects of H2Ocean Sea Salt Mouthwash when added to the oral hygiene regimen of orthodontic patients. We conducted a double-blind procedure in order to obtain unbiased results. The study groups were comprised of teenager and young adult orthodontic patients. Taking into consideration that such patients had difficulty in mechanical plaque control, it seems that they were an interesting category for this kind of study in terms of compliance. In order to better observe the efficacy of mouth rinses, all selected participants had mild gingivitis at the start of study.

One may try to attribute the observed findings to the fact that the participants knew about the goal of the study or to the mechanical effect of rinsing alone. Given that there was no improvement in the scores of the placebo group, the active mouth rinse effects could not be attributed to the Hawthorne effect or to the mechanical effect of rinsing alone (12). Subsequently, the limited number of published studies on the subject makes the comparison of our results with other groups rather limited. However, this research was a relatively short clinical study, and a longer and more detailed study may show a different outcome.

Finally, the clinical and *in vitro* data obtained in this study revealed that H2Ocean Sea Salt Mouthwash led to good inhibition of *S. mutans* and improved clinical conditions compared to those at baseline. We believe that a toothpaste or a mouthwash containing sea salt extract and xylitol, in combination with lysozyme,

might be useful for the prevention of dental-related diseases.

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LETTER TO THE EDITOR

DENTINOGENIC GHOST CELL TUMOUR IN A 20-YEAR-OLD MALE WITH PREVIOUS SQUAMOUS CELL CARCINOMA OF THE TONGUE

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Oral squamous cell carcinoma in patients under 20 years of age is extremely rare, the alveolar gingiva being the most frequently affected site. The metachronous occurrence (eight years later) of a dentinogenic ghost cell tumour of the anterior maxilla in the same patient has never been previously reported. The patient had been diagnosed with a poorly differentiated squamous cell carcinoma of the ventral tongue when 12 years of age and was treated by en-block resection with functional neck dissection. Eight years later, he showed a dentinogenic ghost cell tumour in the anterior maxilla, associated with an impacted central incisor, which was treated by conservative surgery with wide margins; the patient has remained free of disease, without recurrence, at the one-year follow-up. Though extremely rare, malignancies should be considered in the diagnostic work-up of paediatric/adolescent patients with head and neck neoplasms. Additional difficulties arise when dealing with multiple tumours, both under a differential diagnostic point of view and for the surgical management, due to the lack of unanimously accepted guidelines for treatment and follow-up. To the best of our knowledge, oral squamous cell carcinoma and dentinogenic ghost cell tumour are unrelated to each other and may represent a casual combination, which raises additional questions as to the clinical management.

To the Editor,

Oral squamous cell carcinoma (OSCC) is the most frequent tumour of the head and neck, usually occurring in individuals over 40 years of age, while being extremely rare in paediatric patients (1-3). In a recent review on 217 head and neck squamous cell carcinomas in young patients, including laryngeal cancers, the authors reported a median age at presentation of 15 years, with a 0-20 age range, a clear-cut prevalence in males, but with a poorer outcome regardless of therapy (4). In addition, a

delayed diagnosis of OSCC in paediatric-adolescent patients, together with the absence of guidelines on the best fitting treatment, in view of the rarity of such lesion, may be responsible for increased difficulties in the management of such malignancy (5, 6).

Dentinogenic ghost cell tumour (DGCT) also is a very rare neoplasm, formerly identified as the solid variant of calcifying epithelial odontogenic cysts (CEOC), occurring peripherally or centrally (intraosseously) in the jaw bones, the latter also being considered more aggressive due to high recurrence

Key words: oral squamous cell carcinoma; dentinogenic ghost cell tumour; odontogenic tumours; jaw bone tumour

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rates (7-10). DGCT may present over a wide range (12-75 years), with a slight prevalence in the anterior regions of the jaws (7).

The metachronous occurrence of OSCC and DGTC in an adolescent has never been reported previously.

Case report

A 12-year-old male patient was referred to our observation for a persistent white lesion of the tongue. Intraoral clinical examination highlighted a wide irregular white patch of the ventral right tongue (Fig. 1); his medical history was uneventful, but retention of the central incisor (2.1) in the maxilla was noted. Following an incisional biopsy, the diagnosis of poorly differentiated OSCC was performed (Fig. 2); the patient underwent imaging studies, was staged T2N0M0 and underwent surgical excision of the lesion with cutaneous-muscle flap and functional neck dissection. The patient had remained disease-free at subsequent follow-up for eight years.

Subsequently, panoramic and direct radiographs (Fig. 3, a,b) showed the impacted central incisor,

associated with an osteolytic lesion which included the tooth crown, surrounded by calcified material. Computed tomography (CT) scans demonstrated an irregular, multi-lobate and partly cystic mass, adjacent to but incompletely merging into the impacted tooth follicle (Fig. 3c).

With the provisional diagnosis of CEOC, after elevation of a muco-periosteal flap, the lesion was removed with bone margin curettage but without tooth extraction. Histological examination showed a well demarcated solid-cystic mass, including large amounts of calcified material with woven bone and cementum-like features, wide clusters of tubular dentine and small strands of odontogenic epithelium (Fig. 4, a,b). The latter were composed of small cuboidal cells intermingled with larger cells with well demarcated but lightly eosinophilic cytoplasm, and pale nuclei (ghost cells) (Fig. 4c). Based on these morphologic features, the diagnosis of DGCT was given.

At the one-year follow-up, the patient has remained free of disease and the impacted incisor tooth seems to be responsive to orthodontic extrusion.



Fig. 1. Wide irregular white patch of the ventral right tongue.

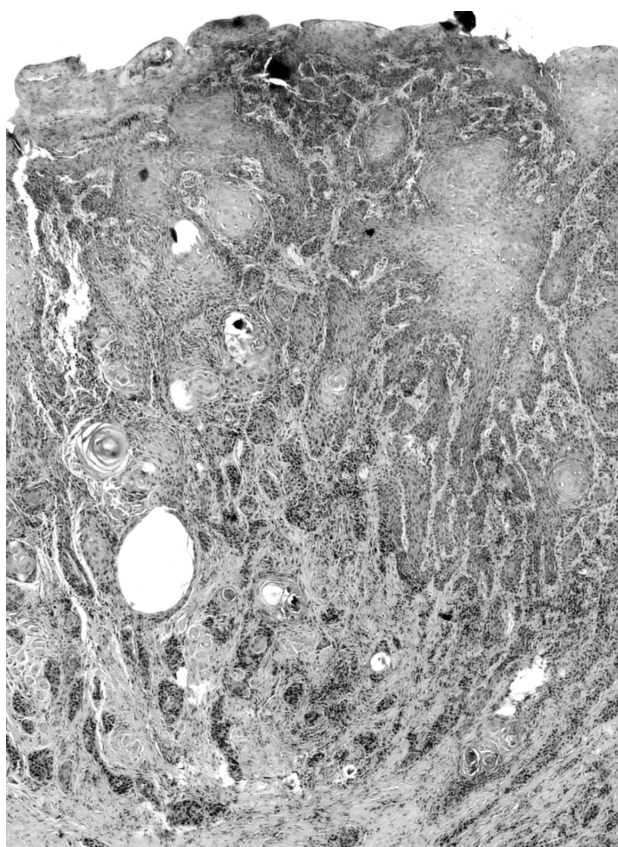


Fig. 2. Low power view of the histopathological features of the invasive squamous cell carcinoma. (Haematoxylin-Eosin, original magnification x2)

The patient gave signed informed consent to report this case.

DISCUSSION

DGCT is a very rare neoplasm which, when occurring in a central (intra-osseous) location, seems to follow a more aggressive clinical course with tendency to recur. In view of its rarity and of the lack of well-defined clinico-radiological features, the pre-operative of DGCT diagnosis may be difficult and the occurrence of such neoplasm in younger individuals and in close proximity with vital teeth may also represent a therapeutic challenge (11-14). In fact, based on the clinical and radiological features, the presence in the anterior maxilla of an irregularly radio-opaque lesion, surrounded by an osteolytic border, displacing the teeth roots, associated or not with an impacted tooth and occurring in a young individual, leads to consider a very wide range of differential diagnoses. These may include inflammatory/developmental odontogenic cystic lesions (radicular or follicular cysts), odontogenic neoplasms (calcifying epithelial odontogenic tumour - CEOT, CEOC, odontoma, ameloblastoma), and



Fig. 3. Panoramic radiogram showing the impacted central incisor; associated with an osteolytic lesion, including the tooth crown and surrounded by calcified material (a). Radiological appearance of the lesion on direct radiogram (b). CT scans showing an irregular, multi-lobate and partly cystic mass, with dentin-like density, adjacent to but incompletely merging into the impacted tooth follicle (c).

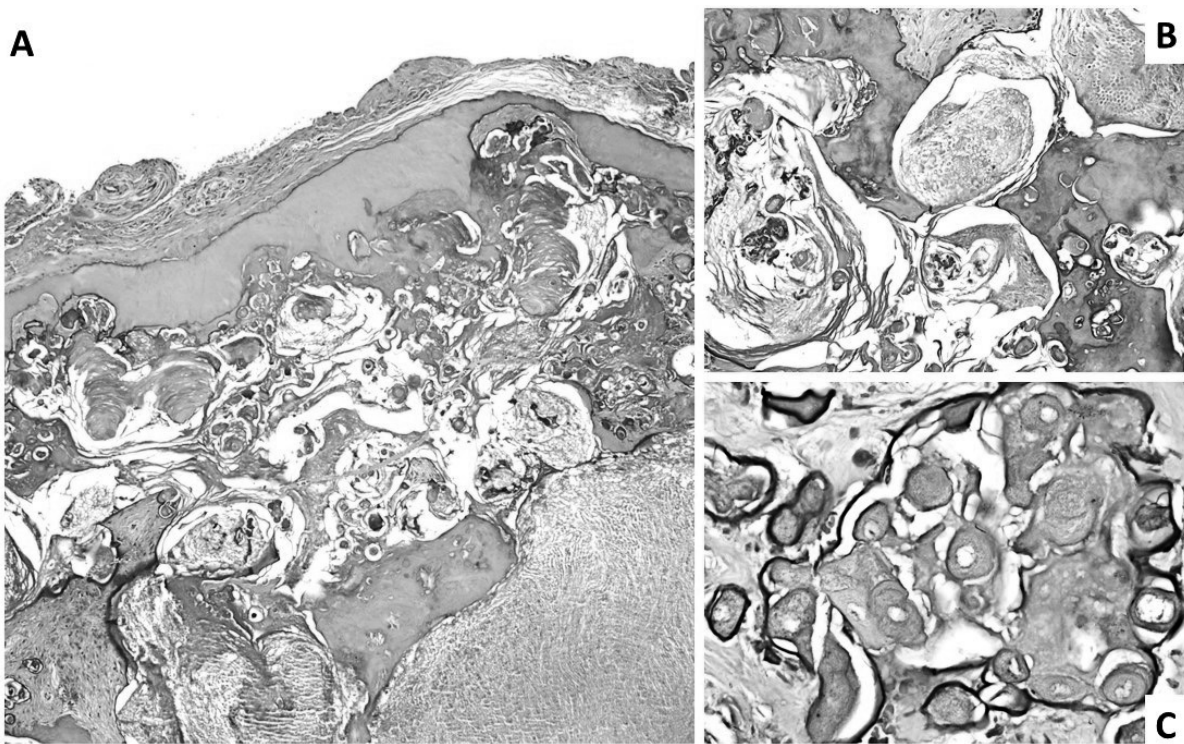


Fig. 4. Well demarcated solid cystic mass with residual cortical bone, including large amounts of calcified material with woven bone and cementum-like features (H&E stain, original magnification $\times 4$) (a). Wide clusters of tubular dentine and small strands of odontogenic epithelium (H&E stain, original magnification $\times 10$) (b). The odontogenic epithelium is composed of small cuboidal cells intermingled with larger cells with well demarcated but lightly eosinophilic cytoplasm, and pale nuclei (ghost cells) (H&E stain, original magnification $\times 40$) (c).

both benign and malignant bone tumours (giant cell tumour, osteosarcoma). In the current case, the absence of relevant symptoms, of associated dental caries and/or radicular damage, the association with a normal-appearing impacted tooth, and the presence of only focal (incomplete) cortical bone erosion, pointed to a long-standing lesion of odontogenic origin, possibly CEOT, CEOC or DGCT (13-14).

Based on these premises, in consideration of the young age of the patient, we opted for a conservative surgical approach with accurate perilesional curettage, with preservation of the adjacent tooth, to possibly promote orthodontic eruption, while achieving the final diagnosis, which could only derive from histopathological examination. Once the final diagnosis of DGCT was formulated, strict clinic-radiologic follow-up was advised, in view of possible recurrences of such neoplasm.

More intriguing is the occurrence of DGCT in

a patient who had previously been diagnosed with OSCC; its occurrence in paediatric/adolescent patients is a rare event (1-6), although a more aggressive clinical course has been observed, in comparison with older individuals. Obviously, metastatic deposits from OSCC into the maxilla were considered in the differential diagnosis but the radiological findings and, specifically, the lack of frankly invasive features of the bone lesion did not point to malignancy. Extensive search of the literature did not reveal previously reported similar cases, therefore, it is our opinion that the association of OSCC with DGCT should be considered a casual event, given the distinct localisation and histogenesis of the two lesions; nevertheless such combination surely makes the clinical work-up of the patient more laborious and requires attentive evaluation of the surgical approach, before the final diagnosis is formulated, to possibly avoid unnecessary aggressive treatments.

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LETTER TO THE EDITOR

BIO-MORPHOLOGICAL EVALUATION OF PERIODONTAL LIGAMENT FIBROBLASTS ON MINERALIZED DENTIN GRAFT: AN *IN VITRO* STUDY

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Regenerative dentistry aims to restore the volumes and tissues lost during traumas or periodontal disease. In the past, research focused on the development of new materials which could constitute the best graft for tissue regeneration. Dentinal matrix has been considered one of the materials that can be used for this purpose. The aim of this study is to evaluate the *in vitro* biocompatibility of a mineralized dentinal graft material. Human periodontal ligament fibroblast (HPLF) cell line was used for subculture, according to the manufacturer's instructions. XTT essay was performed to evaluate the cell survival, and histological and scanning electron microscopy (SEM) analyses were performed to evaluate the morphological changes in response to the new material. The dentinal particles were obtained according to the Smart Grinder protocol and Bio-Oss material was used as positive control. The response was evaluated after 24 hours, 72 hours and 7 days from the seeding. The 24-h results showed a cell survival of 96.7% in the sample tested with the dentin and of 98.9% in the sample tested with the Bio-Oss. The 72-h results showed a cell survival of 105% in the sample tested with the dentin and of 140% in the sample tested with the Bio-Oss. The 7-day results confirmed the trends of the cellular growth: the cell survival rate of the sample tested with the dentin was 152% and of 186% in the sample tested with the Bio-Oss. The morphological data confirmed the biological essays, showing the perfect biocompatibility and the "cell-friendly" properties of the tested material. HPLF cells showed a very promising growth reaction to the tested material, comparable to the considered standard material (Bio-Oss). *In vivo* studies, RCTs and systematic reviews are necessary to validate the use of this material for regenerative periodontal interventions in clinical practice.

To the Editor,

Inflammatory and destructive processes, originated by traumas or infections, guide the tissue healing with a re-modelling of the injured sites (1). These molecules intervene in the cellular chemotaxis and apoptosis of neutrophils which play a central key role in the initiation and the resolution of inflammation (1).

Failure in the healing cycle can lead to a persistent lengthy process with loss of tissue, such as in periodontal disease, where slow inflammation is the main cause of the pathology (1). Periodontitis is a chronic multifactorial inflammatory disease associated with dysbiotic plaque biofilms, characterized by progressive destruction of the tooth-

Key words: autogenous particulate dentin graft; undermineralized dentin; biocompatibility; scanning electron microscopy

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supporting apparatus (2). Regenerative medicine aims to improve the healing and the restoration of lost tissues by means of barriers, scaffolds and other biomaterial grafting (3). In dentistry, for example, the restoration of alveolar bone defects is required to allow the placement of a proper length implant fixture (4, 5). Among the biomaterials used in regenerative dentistry, particles derived from dentin have been studied. The advantages of the use of this biomaterial are its autogenous nature and its similarity to the bone tissue, as well as the cost (6).

The aim of this study was to assess the biocompatibility *in vitro* of autogenous mineralized dentin particles obtained from freshly extracted teeth through a dedicated grinder device (Smart Dentin Grinder™, KometaBio, Cresskill, NJ, USA).

MATERIALS AND METHODS

The study design included the evaluation of the biocompatibility of the particles derived from the Smart Dentin Grinder™ (SDG) using human periodontal ligament fibroblast (HPLF) cell line. The timing of evaluation was set at 24 h, 72 h and 7 days. The cell viability was evaluated with XTT essays, together with their morphology, studied by means of optical and scanning electron microscopy (SEM).

Tested and control materials

The test material was obtained according to the SDG protocol. Briefly, a caries-free upper third molar was extracted and remnants of periodontal ligament and calculus were reduced by tungsten bur (Komet Dental, Gebr. Brasseler GmbH & Co. KG, Lemgo, Germany). The tooth was dried by means of air syringe, put into a grinding sterile chamber of the SDG facility. The tooth was ground into particles measuring <1200 µm (3 seconds); then by a vibrating movement of the grinding chamber (20 seconds) the particles fell through a sieve to a lower chamber. The particles less than 300 µm (considered as a non-efficient particulate size for bone grafting) fell into a waste drawer. In the collecting drawer chamber, dentin particles between 300-1200 µm were collected and immersed in basic alcohol solution (0.5M NaOH and 30% alcohol (v/v)), for 10 min, in a small sterile glass container. After decanting the basic alcohol cleanser, the particulate was

washed twice in sterile phosphate buffered saline (PBS). As control material, deproteinized bovine bone mineral (DBBM) (Bio-Oss, Geistlich Pharma AG, Wolhusen, Switzerland) was employed.

Cell culture

Human gingival fibroblast cell line from ScienCell research Laboratories was cultured according the manufacturer's instructions and as described previously (7). Briefly, the initial vial containing 5×10^5 cells in 1 ml of volume was cultured in three plastic culture dishes in fibroblast medium (basal medium, fetal bovine serum, fibroblast growth supplement and penicillin/streptomycin solution) and incubated under standard cell culture conditions (37°C in 5% CO₂). When the cells reached sub-confluence, they were detached using 0.05% trypsin, and subcultured at a density of 110 cells/mm². The cells were used at subculture levels 3 or 4 for all experimental assays.

Cell viability assays

Cells were grown in 96-well plates under standard cell culture conditions. Two micrograms of the tested and control material were plated into the 96-well microplates. After 24 h, 72 h and 7 days, the XTT Assay (Cayman Chemical, USA) was used to assess the cell viability, and the microplates were read at an absorbance wavelength of 450 nm (7).

Morphological analysis

Cells were plated in 35-mm diameter plastic culture dishes with the tested and the control material and incubated under cell culture conditions. After 24 h, 72 h and 7 days from the seeding, three dishes containing the tested material and Bio-Oss material were fixed by means of 10% solution of formaldehyde and processed for optical microscopy using Azan Mallory staining (Bio-Optica SpA, Milan, Italy), following the instruction of the manufacturer.

After 24 h, 72 h and 7 days from the seeding, three dishes, with a cover glass inside which the cell solution containing the tested material or Bio-Oss material were seeded, were fixed by means of 2% solution of glutaraldehyde and processed for the scanning electron microscopy (SEM) as stated previously (7-9). Briefly, after the postfixation in 1% osmium for 1 h, the samples

were dehydrated in ethanol ascending solutions of 70%, 80%, 90% and three times 100% for 10 min each. The samples were then air dried by the evaporation of hexamethyldisilazane (HDMS), after which they were immersed for 3 min in 100% HDMS (Sigma-Aldrich srl, Milan, Italy) and transferred into a desiccator for 25 min to avoid water contamination. The samples were mounted on metal stubs, gold stained and observed by SEM (XL30CP, Philips, Amsterdam, The Netherlands).

Statistical analysis

The outcomes of the XTT analysis were analyzed firstly with descriptive statistics tests (means and SD). One-way ANOVA was used for the evaluation among the different timing group and among the tested and control materials, setting a p value <0.05 for statistical significance.

Statistical tests were performed using SAS University Edition software.

RESULTS

Cell viability assays

The XTT assays showed positive results in both groups of tested materials. Although the survival trend was slightly higher in the DBBM group, the

growth curve was positive in both (Fig. 1) with no statistical differences.

In particular, at 24 h from the seeding, the cell survival rate was 96.7% in the sample tested with the dentin, and 98.9% in the sample tested with the DBBM. In the 72-h groups, the XTT assays showed a cell survival of 105% in the sample tested with the dentin, and of 140% in the sample tested with the DBBM. In the 7 incubation days, the data confirmed the trends of cellular growth: the cell survival rate in the dishes tested with dentin was 152%, and in the samples tested with the DBBM was 186%.

Morphological analysis

The optical microscopy observations showed a very good reaction of the cells to both biomaterials. In particular, the cells tested with the DBBM showed morphological signs of phagocytosis of the particles of the biomaterials and a positive metabolic reaction to it (Fig. 2). SEM (Fig. 2) confirmed the cell-friendly nature of both materials at all three of the observation times (24 h, 72 h and 7 days of contact).

Regarding the tested material, the optical microscopy showed a very good morphological cell reaction to the particles, with less frequency of

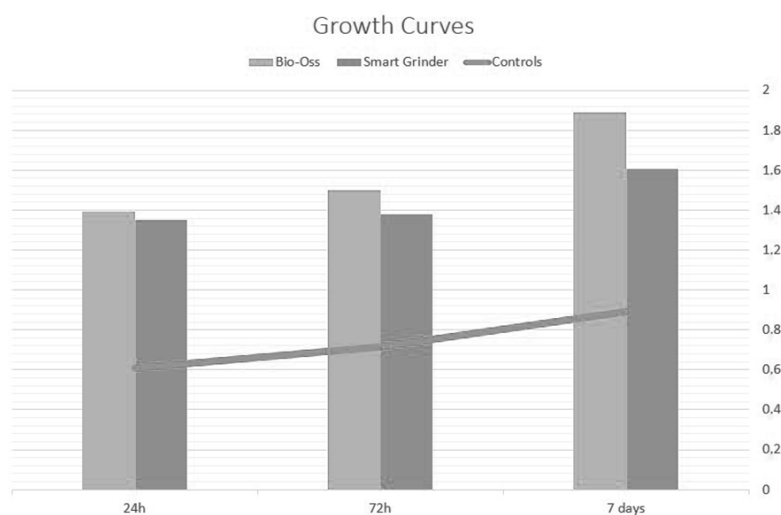


Fig. 1. Graphical representation of the growth curves of the HFLP cells in contact with the two biomaterials and the control cells. The X axis indicates the timing of evaluation. The Y axis indicates the detected absorbance values. The trend of the growth is respected and no statistical significance was revealed between the groups.

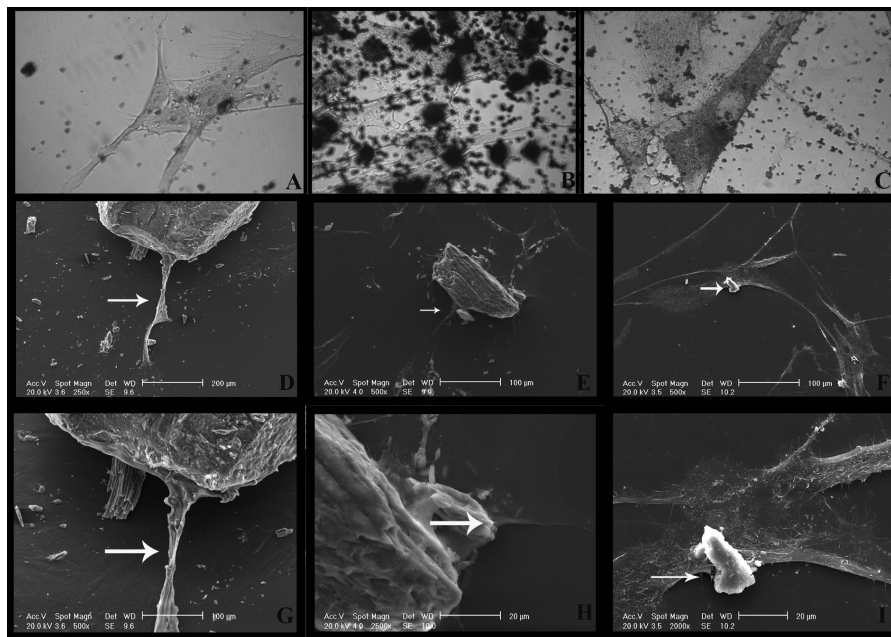


Fig. 2. Morphological analysis panel. The optical microphotographs at 40x of HPLF in contact with the DBBM, at 24 h (A), 72 h (B) and 7 days (C) show an optimal cellular reaction to the material. Azan Mallory staining. SEM microphotograph at different magnification of HPLF cells in contact with DBBM material, respectively at 24 h (D-G), at 72 h (E-H) and 7 days (F-I). In all of the pictures it is possible to appreciate how the cells are attracted by the materials, as indicated by the white arrows.

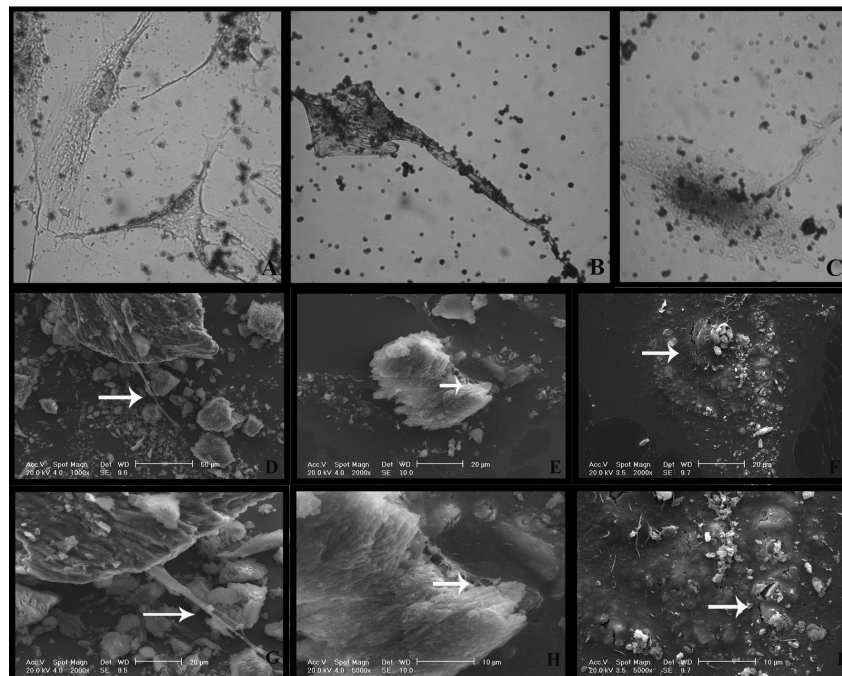


Fig. 3. Morphological analysis panel. The optical microphotographs at 40x of HPLF in contact with the dentine particles at 24 h (A), 72 h (B) and 7 days (C) show an optimal cellular reaction to the material, as well as in the control material, Azan Mallory staining. SEM microphotograph at different magnification of HPLF cells in contact with DBBM material, respectively at 24 h (D-G), 72h (E-H) and 7 days (F-I). In all the pictures it is possible to appreciate how the cells are attracted by the materials and how the growth around the particles is stimulated, as indicated by the white arrows.

cytoplasm inclusions (Fig. 3).

In the 24-h dishes, the SEM images frequently showed fibroblasts trying to cover the dentine particles.

In the 72-h incubated groups, the scenario remains similar at SEM observations, showing many more cells growing attached to the dentine material.

At 7 incubation days, the SEM revealed the cells developing numerous protrusions, aimed to cover and to include the particles completely (Fig. 3).

DISCUSSION

The bio-morphological compatibility *in vitro* of dentin material resulted almost overlapping for both HPLF and DBBM material grafts. Grafting biomaterials have been developed to guide and control the repair and the regenerative process. In dental medicine, the use of these type of biomaterials is required especially in periodontal and pre-implant surgeries, such as guided tissue regeneration and guided bone regeneration to re-gain the hard tissues lost due to inflammatory or traumatic processes (3).

The molecular components of dentin are qualitatively similar to those of bone tissue, indeed, the inorganic structure of the hydroxyapatite crystals from dentin is overlapping the same crystals of bone tissue. Collagen type I is present in both tissues and the water content (10%) is equal both in dentin and in bone (10).

The dentin matrix has been reported to express bone morphogenetic proteins (BMPs) which have osteoconductive properties. Research is ongoing to evaluate the benefit of use of autogenous dentin tissue as grafting material to alveolar ridge preservation and to correct bone defects, evaluating different protocols and degrees of dentin matrix mineralization.

Therefore, the dentin matrix is classified in undermineralized dentin (UDD), partially demineralized dentin matrix (PDDM) (70% decalcified) and demineralized dentin matrix (DDM) (6). As reported by Calvo-Guirado et al., UDD is very useful to maintain mechanical stability and to allow an early implant loading after grafting in fresh sockets and bone defects (11). RCTs and systematic

reviews are necessary to validate the use of this material for regenerative periodontal interventions in clinical practice.

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LETTER TO THE EDITOR

**CYCLIC ADENOSINE MONOPHOSPHATE PHOSPHODIESTERASE:
A POSSIBLE MARKER OF THYROID PATHOLOGY?**

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The aim of this study was to evaluate whether or not the expression of cAMP-phosphodiesterases (cAMP-PDE) varies in different thyroid pathologies and to elucidate the relationship between the expression of cAMP-PDE and clinic-pathologic factors. Forty thyroid biopsy samples, excised to perform the biopsy, were microscopically examined and classified. Then cAMP-PDE expression was assessed using high performance liquid chromatography. The expression of cAMP-PDE showed different patterns according to the histological features of the samples ($p < 0.001$). A strong expression of cAMP-PDE was observed more frequently in papillary and follicular carcinoma than in controls and benign pathologies. We did not detect any level of endogenous cAMP. cAMP could be used as additional diagnostic or prognostic biomarker in patients suspected of thyroid disease, however further studies are needed to confirm these results.

To the Editor,

Adenosine and guanosine 3',5'-cyclic monophosphates (cAMP and cGMP) are important second messengers that mediate the response of cells to a wide variety of hormones and neurotransmitters and modulate many metabolic processes. Degradation and inactivation of cAMP and cGMP are mediated by no fewer than 11 distinct families of phosphodiesterases (PDEs) (1). This enzyme activity is provided by a large multigene family which includes PDE4 and PDE7 (cAMP-specific enzymes) (2). However a great c-AMP affinity in thyroid has been found for PDE8 that is able also at low concentrations to influence the TSH signalling

(3), and PDE5 that has been found overexpressed in papillary thyroid carcinomas (4). In a previous study we recorded an increased level of cGMP-PDE in malignant thyroid biopsies and hypothesized a possible use of this marker for diagnosis (5). However, the distinction between adenomas and carcinomas is based on histopathological criteria such as the invasion of neoplastic cell into the capsule that surrounds the neoplasm, or in vessels, or in extra-thyroidal tissues and the presence of lymph nodal or systemic metastases (6). However, morphology alone is not always able to make a correct diagnosis and immunocytochemistry (ICC) and molecular biology are helpful in differentiating low- and high-

Key words: thyroid, cAMP-Phosphodiesterase, cAMP, carcinoma

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malignant risk lesions (7).

The aim of this early phase study was to evaluate the variations in the concentration of cAMP-phosphodiesterases (cAMP-PDE) in thyroid samples in both physiologic and pathologic state, in order to verify whether cAMP-PDE could be used as a marker of thyroid condition.

MATERIALS AND METHODS

The endocrinology specialists for biopsies of the thyroid gland referred 40 patients (31 females and 9 males; mean age 46 ± 12 years) to the Department of the Anatomy and Histopathology, Dental School, University of Ancona, Italy. Before proceeding to thyroidectomy, medical records and informed consent were signed by patients. A total of 40 human thyroid samples, excised in order to perform the biopsy, were microscopically and biochemically examined. After removal, the lesions were randomly divided in two parts: one was sent for histological examination to the Section of Pathologic Anatomy of the same University and the other was frozen and sent to the University of Chieti for the cAMP-degrading activity.

Histopathological analysis

Tissue samples were fixed in 10% formalin for 24 h at room temperature and embedded in paraffin. Morphologic analysis was performed on 4-6 μm thick tissue sections stained with hematoxylin and eosin (H&E). After the pathological classification, the samples were frozen and transported to the laboratory of medical chemistry of the University of Chieti, (Italy) where cAMP-PDE level was evaluated by a blind operator.

Partial purification of phosphodiesterases

The frozen samples were thawed and centrifuged for 10 min at 1000 rpm with a centrifuge model MIKRO22R (Hettich Zentrifugen), after which they were washed twice with isotonic PBS.

In order to purify the enzyme, the samples were homogenized with a phosphate buffer K1-K2 10 mM pH 7.00 in a 1:10 (w/v) ratio, using a Potter (T25 BASIC model - Ika Laborpechnik), three times for 20 s with 30 s rest; a progressive speed up gradually to red was used, always keeping the test tube on ice. Samples were then sonicated

three times with an ultrasonic bath for 5 s with one minute rest in ice. The homogenate was centrifuged for 30 secs at 1000 rpm with a centrifuge model MIKRO22R (Hettich Zentrifugen). The withdrawn supernatant was then used for the phosphodiesterase analysis in order to determinate the protein quantity, enabling the evaluation of the level of endogenous cyclic nucleotides, and to calculate the cAMP-PDE amount at protein level (8).

cAMP-PDE assay

The enzymatic analysis was carried out using the method of Spoto et al. (8) with minor modifications: 0.1 M TRIS-HCl buffer (pH 8,3), 10 mM of MgCl_2 , 0.1 M of KCl at 37°C .

The reaction was initiated by the addition of 44 μM of cyclic AMP. The mixture was incubated for 60 min at 37°C , then the reaction was terminated by adding HCl 2M for a final concentration of 0.02M and leaving the samples in a boiling water bath for 5 min. The samples were then centrifuged at 12000 rpm in a MIKRO 22R (Hettich Zentrifugen) model centrifuge and the supernatant was filtered through a nylon-66 0.22 μm filter (Rainin Corporation). The clear filtrates obtained were used for analysis in reverse phase HPLC. The system utilized during this experimentation consisted of two pumps LC-9A model (Shimadzu), one spectrophotometer at variable wavelength SPD-10AV vp (Shimadzu) which measures at 254 nm, an autosampler SIL-10AXL (Shimadzu) and a LICHROSORB RP-18 (5 μm) column. The mobile phase used for the separation of the nucleotides consisted of 350 mM ammonium acetate (pH 6.0) with 0.6% of acetonitrile (v/v), previously degassed in an ultrasonic bath model SONIC 18-35 (Simply - Italy) in order to avoid bubbles. Cyclic endogenous nucleotide evaluation kit EIA (BIOMOL GmbH, Germany) that uses a competitive immunoenzymatic analysis confirmed the quantitative determination of cyclic compounds, as previously described (8).

Protein-determination

A kit for protein determination with bicinchoninic acid (Sigma-Aldrich) and albumin of bovine serum as the standard of proteinic reference quantified the protein content in the sample. A spectrophotometric measurement at 280 nm and 350 nm determined the protein concentration of the supernatant, using a dilution 1:20.

Data processing

We followed World Health Organization (WHO) classifications of thyroid disease (9) to record and organise the data. The presence of statistically significant differences in the HPLC experimental data were tested with analyses of variance (ANOVA) applying the Bonferroni/Dunn correction for multiple testing (Stat View 4.0 software, Abacus Concepts, USA).

RESULTS

After histopathological verification, the samples were classified as follow, based on the WHO criteria (9):

- 4 healthy thyroids
- 6 benign struma
- 4 chronic thyroiditis
- 4 adenomatous hyperplasia
- 12 benign adenoma
- 3 follicular carcinoma
- 7 papillar carcinoma

Average cAMP-PDE activity of all samples is summarized in Fig 1. Phosphodiesterase activity in healthy thyroids was 24.37 (± 18.67) nmol/

mg. Compared to healthy controls, thyroiditis and benign struma were characterized by a light decrease of cAMP-PDE activity, 13.56 (± 8.90) nmol/mg and 12.16 (± 13.77) nmol/mg respectively. On the contrary, a more important decrease of cAMP-PDE activity was found in adenomatous hyperplasia 4.02 (± 4.37) nmol/mg. Samples of adenoma were characterized by a mean cAMP-PDE activity of 13.16 (± 11.06) nmol/mg. The level of these was increased in malignant neoplastic lesions and these differences were statistically significant. Mean values of cAMP-PDE activity from samples of papillar and follicular carcinoma were 94.33 (± 30.8) nmol/mg and 90.6 (± 60.72) nmol/mg, respectively. When the thyroid was affected by a non-neoplastic pathology (Benign Struma, Adenomatous hyperplasia and Chronic Thyroiditis), the activity of cAMP-PDE seemed lower in respect to healthy samples, although no statistically significant differences were found.

The calculated ratio between cAMP-PDE activity in healthy samples vs benign neoplasia was 2.7; on the contrary, the ratio between healthy and malignant ones was 0.2 (Fig. 2). No levels of endogenous cAMP were detected.

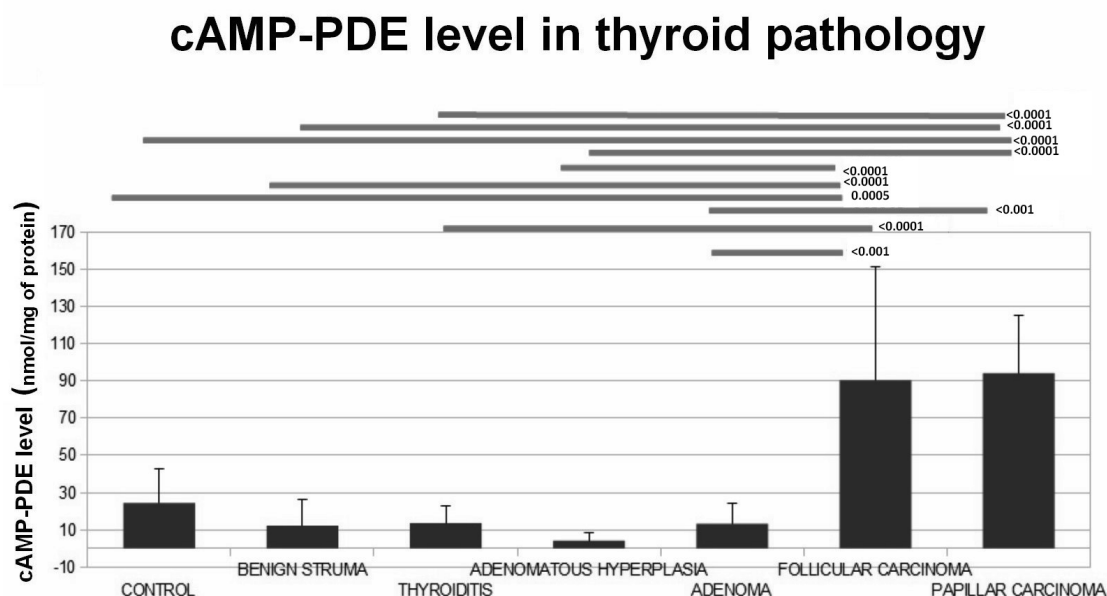


Fig. 1. Levels of cAMP-PDE in different thyroid pathologies. These values are directly proportional to the severity of the pathologies: they are low in healthy samples, slightly increased in benign pathologies and higher in carcinoma. Bonferroni showed statistically significant differences among the groups.

DISCUSSION

The expression of cAMP-PDEs through the quantification of cAMP-degrading activity showed a statistically significant increase in papillar and follicular carcinoma respect to healthy samples. On the contrary lymph nodal results were not significant for benign pathologies. These results are in accordance with our previous data on human carcinoma of salivary glands, gingiva and colon (10-12).

Although we included a broad range of T1-T4 tumours in our study, we lacked precise information on the tumour staging for adenomas, follicular and papillar carcinomas, which calls for validation of the results in an independent cohort. Data (Fig. 1) indicate that the constitutive activation of the cAMP pathway in autonomous thyroid adenomas is constantly associated with an up-regulation of cAMP-PDE activity, in accordance with studies of GH-secreting adenomas (11).

Although the HPLC method used in this study did not discriminate between the different type of phosphodiesterases (PDEs), this could have been accomplished by using antibodies-based methods; the obtained data are objective and sound because they have an internal modality of control, measuring

both the substrate degradation and the end-product increase. At the time of this study, antibody-based methods were not available at our institute. The increased cAMP degradation exerted by PDEs could represent an intracellular mechanism counteracting the phenotypic expression of two types of oncogene (12). Although a protective role of cAMP analogs against medullary thyroid cancer cell line has been shown in literature, we found no detectable levels of endogenous cAMP, contrarily to the strong expression of endogenous cGMP that we had found in similar samples of thyroid cancer (7). For this reason, we do not support the hypothesis that cAMP could exert a role in the autophagic stimulus, as for cGMP.

In conclusion, in this exploratory study, we found a sharp increase of the cAMP-PDE activity in malignant neoplasms of thyroid. Confirmation of this association in future studies is warranted, and may include an evaluation of different antibody-based methods, covering all families of phosphodiesterase. In addition, an evaluation of the distribution of the enzymes across different tumour stages and distinct functional status of the thyroid would be valuable. With confirmation of the current results in other early phase studies, later phase studies may follow

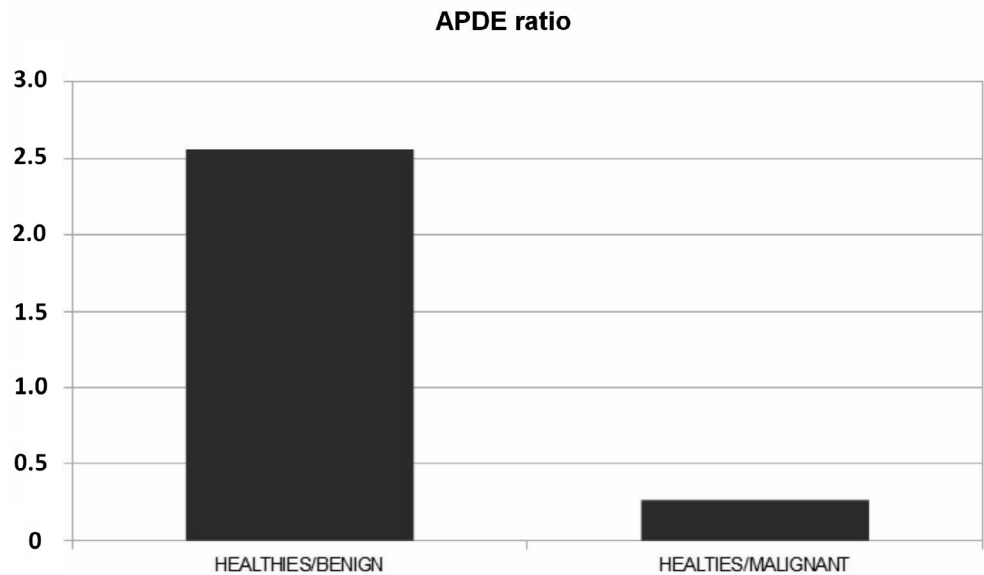


Fig. 2. *The ratio of camp-PDE level between healthy samples versus benign lesions and healthy samples versus malignant neoplasia.*

to test whether cAMP-PDE can be used as additional diagnostic or prognostic biomarker in patients suspected of thyroid disease. It is conceivable that the enhanced cAMP-PDE degrading activity in the tumours characterized by a constitutively adenylyl cyclase may be necessary for tumor progression by determining intracellular cAMP concentrations that are compatible with the initiation of cell replication.

A further development will be distinguish between the different families of phosphodiesterases involved in thyroid disease with selective inhibitor; then specific antibodies can be used in order to obtain highly selective responses.

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LETTER TO THE EDITOR

IMPLANT CONOMETRIC CONNECTION THROUGH INNOVATIVE PROSTHETIC ABUTMENT: BIOLOGICAL, CLINICAL, SURGICAL, AND PROSTHETIC ASPECTS

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The aim of this work was to describe the rehabilitation of a superior monoedentulia by positioning a conometric connection implant and a prosthetic crown using CAD/CAM technique. Surgical steps are explained and the biological aspects of this particular morse conometrical connection are evaluated, with particular attention to the possibility of removing the crown by means of an innovative attachment hole in the abutment. This type of monoedentulia can now be solved by implant-prosthetic replacement, and the dentist must choose the most suitable implant for each individual case, the most favorable connection and the most appropriate prosthetic material with regard to bone and soft tissue biology.

To the Editor,

In cases of edentulism there are different therapeutic possibilities, among which the implant choice is definitely an option widely used.

The systems supplied by various companies may differ in shape (1) and surface treatment (2), and there are different clinical protocols in terms of fixture positioning time and prosthetic load (3).

The choice of the implant-abutment connection (4) is also of great importance. The conical, conometric, internal or external hexagonal connections described in the literature are still the focus of study. In a correct therapeutic planning the clinician must choose between the various connections, exploiting their individual peculiarities. The prosthetic rehabilitation must be related to the choice of the implant, the connection, the ceramic material and the processing technique in order to obtain the most predictable

and esthetic result. Particular interest is dedicated to prosthetic rehabilitation (5) which can be made with different types of ceramics and using new computer aided design computer aided manufacturing (CAD CAM) technologies (6).

The purpose of this article is to present a case report that illustrates the implant-prosthetic rehabilitation of a monoedentulia by positioning a conometric connection implant and a prosthetic rehabilitation with CAD CAM technique, describing the surgical steps and the construction of the prosthetic components.

MATERIALS AND METHODS

A 61-year-old male patient, was observed with a monoedentulia in region 14. Following radiographic evaluation and clinical examination, different rehabilitative

Key words: dental implant; platform switching; implant abutment; morse cone; CAD CAM

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solutions were presented to the patient. After the patient having been correctly informed about the treatment, it was decided for an implant-prosthetic rehabilitation of monoedentulia with delayed loading at 4 months, as the immediate resolution of the aesthetic defect was not a priority for the patient. The treatment plan was defined by opting for a cone-shaped implant system, a titanium abutment, a temporary resin and the final in stratified zirconium. After loco-regional anesthesia, a crestal incision was made from element 13 to element 15, and the implant site was skeletonised and calibrated. The implant fixture (Biokrone M12) of 3.75 mm diameter and 10.3 mm length was positioned with controlled insertion torque at 40 Ncm and the warning to keep 1 mm below the bone profile as a surgical protocol. The incision was made with detached stitches in 6/0 nylon. After 4 months the implant was uncovered transposing the portion of the crestal mucosa vestibular to increase the volume of the tissues and at a period of 14 days the conventional impressions were taken with the pick-up technique in polyether (7) for the production of the temporary crown. The temporary crown in acrylic resin with a hole in the palatal side was glued to the peak abutment. The crown was placed with the hole of the abutment below on the same line, in order to allow a simple positioning and an atraumatic removal. Once the prosthetic device was positioned, connecting the complex

crown abutment to the implant, the convex design of the vestibular side of the provisional starts to give a shape to the soft tissues. After 2 month the final impressions were taken. The laboratory steps involved the acquisition of a virtual model (Fig. 1) which was mounted on a digital articulator (Fig. 2). Using dedicated software, the crown was designed using CAD, creating a palatal hole in the final crown that will meet the hole in the abutment. Once the design had been completed, the crown was manufactured using CAM and finished in the laboratory where the abutment was glued. The definitive crown in characterized stratified zirconium was delivered after verifying the correct adaptation to soft tissues, interproximal spaces, occlusal relationship and evaluating the aesthetic result. The palatal hole was closed in composite material.

RESULTS

All the procedures proved to be simple, predictable and without any complications. In each phase it was possible to respect what had been decided in the treatment plan and all the planned times were respected. The clinical and radiographic controls were performed at a distance of 1, 3, and 6 months and at 2 years after the loading (Fig. 3).

The peri-implant bone showed no signs of distress



Fig. 1. *Virtual project of crown on CAD system.*



Fig. 2. *Digital articulator to control the function.*

and no signs of bone remodeling were found. The peri-implant soft tissues are stable, there are no signs of suffering and the vestibular parabola created in the provisional phases remained unchanged. No movements of the stump-crown complex occurred. The prosthetic crown remained well integrated with the other dental elements.

DISCUSSION

Pre-operative analysis and the definition of each step is the key to success and predictability in implant-prosthesis.

It is necessary to plan how to manage soft tissues, to choose suitable sizes and shapes of the implants (8), to determine the type of connection in relation to the prosthetic crown and to define the most suitable ceramic material in relation to the position of the tooth to exploit its aesthetic and resistance characteristics in relation to the quadrant to be treated. The use of digital techniques (9), from intraoral scanning to the CAD CAM method of the prosthetic device simplifies the finalization of the case. International literature offers important considerations before drawing up the treatment plan. The choice of the type of implant should be made considering bone quality and available volume (10). It is common knowledge that a cylindrical-conical shape offers the highest probability of a correct osteointegration (11).

Numerous clinical studies have dealt with

establishing the performance of the different connections that various companies propose, and the choice falls unequivocally on internal hexagon connections in the anterior area to guarantee an optimal aesthetic result. We can make different assessments on the choice of using screwed abutments or conical abutments. From analysis of the literature it is clear that the platform switching connections (12) show less inflammation of the peri-implant soft tissues and lower bone loss rates (13) and that the conometric connections are not subject to unscrewing or derotation, movements that negatively affect the system. The conometric connections, however, unlike the screwed ones, prove to be less manageable when they have to be removed from the implant during the prosthetic test phases or in case of accidental fractures of the prosthetic device in which dedicated extractors are needed. The use of this implant system gives the opportunity to have an atraumatic removal of the abutment even if the conometrical connection does not normally provide this type of feature. The latest evaluation is on the choice of the material to be used for the abutment, titanium or zirconium (14), which is established in relation to the material chosen to make the prosthetic crown. In the proposed clinical case, a definitive crown in stratified zirconium was chosen, the characteristics of which make it possible to mask the color of the titanium abutment without having to use a zirconium one.



Fig. 3. *Two-year radiographic check.*

Implant-prosthetic rehabilitations require careful pre-operative evaluations and careful planning. The choice of materials influences the final result, both from a functional and aesthetic point of view, and the clinician should be aware of the characteristics and differences of all the materials.

Choosing implants with conometric connections and abutment platform switching, especially in single-tooth replacement, guarantees the stability of the prosthetic crown and prevents rotation during the masticatory load. The implant used in this case, and in particular the use of the hole created on the abutment, makes the prosthetic steps simple, both in the provisional and the final phases. Finally, a correct choice of ceramic material and its processing are important elements in the therapeutic success.

Evaluation of the predictability of the proposed technique also requires further clinical cases, which we are in the process of carrying out, as well as a longer follow-up.

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LETTER TO THE EDITOR

A NEW INJECTIVE PROTOCOL FOR THE TREATMENT OF FRONTAL LINES WITH BOTULINUM TOXIN TYPE A

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Botulinum toxin type A injection is a well established procedure for the treatment of frontal lines. In the present letter the authors propose to hyperdilute the toxin and to perform a gentle massage after the injections to let the drug to easily spread into the muscle, reducing the amount of BoNTA units required and achieving a natural result.

To the Editor,

Botulinum toxin type A (BoNTA) is a well established drug for the treatment of a variety of neuromuscular disorders such as strabismus, blepharospasm, hemifacial spasm and torticollis; although for cosmetic purposes it is also widely used to reduce rhytides of the forehead, glabellar lines and crow's feet (1). The American Society for Aesthetic Plastic Surgery statistics released in 2017 highlighted BoNTA injections were the most performed in the "top 5 non-surgical procedures" with 1,548,236 procedures carried out (2).

The injections of BoNTA to reduce frontal lines is a well-established procedure. A vial of 50 units (U) is reconstituted with 1.25 mL of saline to obtain 4 U each 0.1 mL of solution (3); once reconstituted, to reduce frontal lines BoNTA is injected with a 31G needle, 4 mm length, and 2 units per points spaced 1 cm apart are injected along frontal muscles. On average the units used to treat frontal

lines range between 12 and 24, based on gender, muscle trophism and so on.

We propose a new approach of BoNTA injections to treat forehead lines based on a different dilution of the drug and with a different modality of injections.

A vial of 100 U of Incobotulinum toxin A is reconstituted with 5 mL of saline, 2 times the volume is usually required (we never used a 50 U vial, however this could be used by reconstituting it with 2.5 mL), thus obtaining 2 U of BoNTA each 0.1 mL of solution. The injections are not performed as previously described (2 U per point spaced 1 cm along the frontal muscles) but with an intramuscular injection of 0.1 mL (2 U), about 1 cm lateral to the midline and a second one of 0.2 mL (4 U) spaced about 2.5-3 cm laterally; the site injections were identified by asking the patients to voluntarily contract the muscles in the upper third of the face. As soon as the injection is performed, the

Key words: Botulinum toxin; forehead lines; non surgical facial aesthetic; aesthetic medicine

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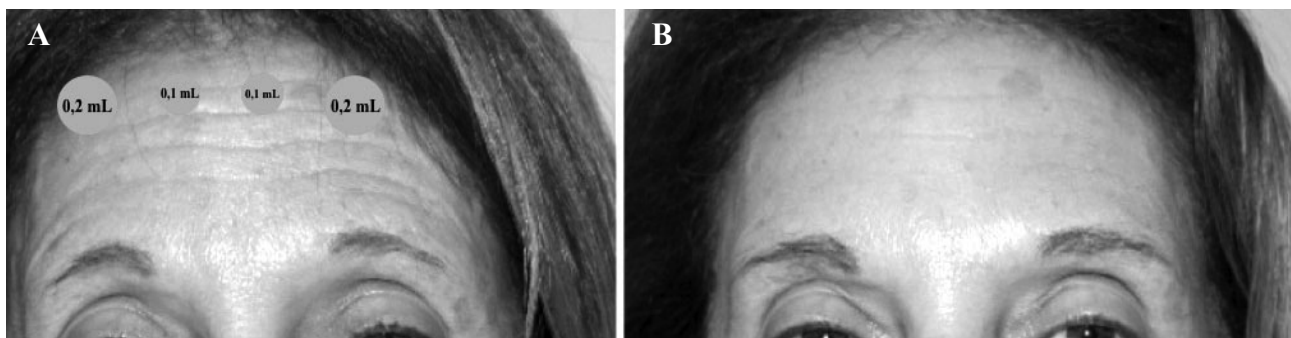


Fig. 1. *A) pre-treatment picture with the amount of volume injected per each point. B) Control after 1 week.*

physician starts with a gentle massage of the bolus injected to spread the solution along the muscle (Fig. 1).

Although the recommended BoNTA reconstitution is 1.25 mL of saline each 50 U, we used two times the volume of saline usually required, however in scientific literature it has already been shown that a larger volume for BoNTA reconstitution is not associated with a higher percentage of complications (4).

After BoNTA injections, it is usually recommended to avoid any massage in the injected area for at least the first 6 hours in order to avoid diffusion of the drug along surrounding muscles and obtaining unfavorable results characterized by brow and/or frontal line asymmetries; however, in the last 5 years, we treated more than 500 patients with this protocol, characterized by a gentle massage after 0.1 and 0.2 mL injections in a single point, and asymmetries were never observed.

BoNTA injection points for frontal lines were identified asking the patients to voluntary contract the muscles in the upper third of the face, as recently reported by Abramo et coll., firstly in 2016 and then in 2018, who performed anatomical dissections of fresh cadavers to identify the shape and cutaneous insertions of the muscles in the upper third of the face used as guidelines for botulinum toxin application (5, 6).

We believe that a gentle massage of hyperdiluted BoNTA lets the drug easily spread into the injected

muscle, thereby reducing the amount of BoNTA units to inject and achieving a more natural result, however, case control and blinded randomized studies are needed to scientifically support this new BoNTA injection technique for forehead line reduction.

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LETTER TO THE EDITOR

USE OF PHOTOBIOMODULATION INDUCED BY POLARIZED POLYCHROMATIC NON-COHERENT LIGHT IN THE MANAGEMENT OF ADULT CHRONIC PERIODONTITIS

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The aim of the study was to evaluate the efficacy of a polarized, polychromatic, non-coherent light (Bioptron), in addition to full mouth scaling and root planing, in chronic periodontitis. Forty adult patients affected by chronic periodontitis were enrolled in the study and were randomly divided into two groups. The control group received only full mouth scaling and root planing (FMSRP), while in the test group, Bioptron light treatment was added to FMSRP. The Full Mouth Plaque Score (FMPS), Full Mouth Bleeding Score (FMBS), Gingival Index (GI) and Probing Pockets Depth (PPD) were recorded in both groups at the first examination, after 1 month (T1) and after 3 months (T2). Both groups showed a significant reduction of FMPS at T1 and T2 ($p < 0.05$). Both groups showed a significant reduction of FMBS at T1 and T2 ($p < 0.05$), but a higher improvement ($p < 0.05$) was recorded in the study group at T1. Study group GI was significantly reduced at T2 ($p < 0.05$), differently from the control group ($p > 0.05$). PPD showed a reduction in both groups with no statistically significant differences ($p > 0.05$) between them. Polarized, polychromatic non-coherent light seems to be an effective additional therapy in the management of adult chronic periodontitis, reducing inflammatory clinical indexes.

To the Editor,

Periodontitis is an infective-immune-inflammatory disease characterized by the loss of tooth-supporting structures, including the connective tissue attachment and the alveolar bone (1). It is reported that 10-15% of the worldwide adult population is affected by periodontitis (2). This condition can be chronic or acute, generalized or localized, and can lead to gingival inflammation and bleeding, progressive attachment loss with probing pocket depth.

Full mouth scaling and root planing (FMSRP) represent the gold standard in periodontal therapy in order to remove plaque and subgingival bacterial biofilm, which play the major role in the disease initiation and progression (1). Periodontal therapy is also aimed to provide long-term improvement in clinical parameters, including inflammation relief, reduction of gingival bleeding and probing pocket depth, and gain in attachment level (3). Providing patients with customized care protocols to be carried out at home, is a key aspect

Key words: light therapy; chronic periodontitis; periodontal therapy

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of modern periodontal treatment (4). Increased health awareness and improvements in preventive dentistry and in minimally invasive therapies have led to a decrease in tooth loss. Increased life expectancy and greater health expectations lead to changes in demand of individuals of all age groups for periodontal treatment and a substantial increase in periodontal therapy. New aims can be reached in terms of accelerating wound healing, reducing more clinical indexes and improving patient comfort. Different adjunctive therapies to treat periodontal patients have been proposed in the literature (5, 6). Polarized, polychromatic, non-coherent light is used in medicine for the treatment of a wide range of medical conditions, including carpal tunnel syndrome (7), epicondylalgia (8), and chronic ulcers (9, 10).

Polarized, polychromatic, non-coherent light induces activation and modulation of biological processes, promoting anti-inflammatory, antibacterial and regenerative endogen mechanisms.

The increase of nitric oxide production, modulation of cytokine secretion, and effects on the vessels and on collagen metabolism are the key biological aspects controlled by the light application (10).

MATERIALS AND METHODS

Inclusion criteria

Forty consecutive adult patients referred to a private practice in Rome were included in this study. All the patients enrolled had received a diagnosis of generalized chronic periodontitis (more than 30% of total sites with no reversible attachment loss). The study was conducted in accordance with the Declaration of Helsinki, and all participants provided written and informed consent before participation.

Exclusion criteria

Patients assuming antibiotics or NSAIDs in the previous month, pregnant or nursing, and patients with previous diagnosis of melanoma were excluded.

The included patients were randomly divided in a test group and a control group using a randomization software (<https://www.randomizer.org>).

Periodontal protocol

Full Mouth Plaque Score (FMPS) was collected at the

beginning of the study (T0). According to Dental Biofilm Detection Topographic Technique (D-Biotech) protocol, fluorescein plaque detector was applied and highlighted with VALO LED curing light to facilitate FMPS recording and plaque removal. After FMPS recording, a deplaquing procedure was performed with Chlorhexidine (CHX) 0.2% mouthwash (Corsodyl®, GSK) for one minute. Each tonsil was reached with one CHX spray (Corsodyl®, GSK). Airpolishing (Mectron® Combi touch) was applied only where plaque was highlighted with a 90° angled tip for occlusal and buccal surfaces and with a 120° angled tip for palatal and lingual surfaces.

Full Mouth Bleeding Score (FMBS), Gingival Index (GI) and Probing Pocket Depth (PPD) were collected with a PCP 15 UNC periodontal probe.

FMSRP was performed using Mectron® Combi touch and cures. S1 ultrasonic universal tip and manual scaler M23 slim was used for supragingival scaling. P10 ultrasonic periodontal tip and Queen of Hearts universal curette were used for all PPD ≥4 mm sites. Periopolishing with glycine powder was performed.

Once the FMSRP was completed, the test group patients received an application of polarized polychromatic non-coherent light (Bioptron B2 device, Bioptron AG, Menfi Industry S.P.A.). Bioptron light was held at 90° at 10 cm in front of the buccal area of the incisors, as this is reported to achieve maximal penetration of light, for exactly 10 min. A transparent cheek retractor (Directa®) was used to keep gums well exposed. Bioptron light had the following output characteristics: rated power of halogen = 90 W; light wavelength = 480–3400 nm; degree of polarization = 95%; specific power density = 40 mW/cm²; and energy density = 2.4 J/cm². Treatment duration was 10 min every 7, 14, 21 days. Control group patients received no additional treatment at FMSRP.

Each patient received the same periodontal domiciliary indications, according to the Tailored Brushing Method (TBM). For the first 7 days, patients were asked to brush twice per day for two min with a soft bristles toothbrush (Gum Technique Pro Compact Soft) and a specific toothpaste for bleeding gums (Parodontax®). The tongue was brushed for at least 15 s and oral hygiene was completed with a 0.2% CHX mouthwash for 1 min. After 7 days, patients were asked to brush twice per day with a medium bristle toothbrush (Gum Technique Pro Compact Medium) for two min at least with a protective toothpaste

for exposed roots (Sensodyne Repair and Protect®). The tongue was brushed for at least 15 sec. Clinical scores (FMPS, FMBS, GI, PPD) were recorded after 1 month (T1) and after 3 months (T2).

Statistical analysis

Student's *t*-test was used to compare clinical indexes of the Study group and the Control Group at T0, T1 and T2. Graph Pad software T Test Calculator was used to perform the analyses.

RESULTS

Forty patients (20 in the study group and 20 in the control group) were enrolled, 17 women and 23 men. Overall mean age was 53±11 years (47±4 for women and 54±10 for men). At baseline, no statistically significant differences were found between the mean clinical indexes of both groups. None of the patients enrolled dropped out the study. In Table I are resumed the clinical indexes collected in the study. The study flow chart is reported in Fig. 1.

FMPS in the study group was 31.7% at T0, 13% at T1 and 14.3% at T2. The same index in the control group was 34.5% at T0, 14% at T1 and 17.3% at T2. The FMPS reduction was statistically significant in both groups at both times of observation ($p=0.001$). There was no

statistically significant difference between the two groups at T1 ($p>0.05$) and at T2 ($p>0.05$).

FMBS in the study group was 38.1% at T0, 16.3% at T1 and 13.7% at T2. The FMBS reduction was statistically significant both at T1 and T2 ($p<0.05$). FMBS in the control group was 41% at T0, 15.7% at T1 and 16.3% at T2. The FMBS reduction was statistically significant both at T1 and T2 ($p<0.05$). The difference between the two group was statistically significant only at T1 ($p<0.05$).

GI in the study group was 2.7 at T0, 2 at T1 and 1.5 at T2. The reduction was better at T2 than at T1 ($p<0.05$). GI in the control group was 2.9 at T0, 2.1 at T1 and 2.8 at T2. The reduction was statistically significant only at T1 ($p<0.05$), while at T2 the index was higher compared to T1. There was a statistically significant difference between the 2 groups at T2.

PPD in the study group was 5.6 at baseline, 4.9 at T1 and 4.2 at T2. PPD in the control group was 5.9 at baseline, 5.1 at T1 and 4.3 at T2. The reduction was statistically significant in both groups only at T2 ($p<0.05$), but there was no statistically significant difference between the 2 groups ($p>0.05$). No side effects were reported.

DISCUSSION

Adjunctive treatment with Bioptron light in periodontal patients can accelerate the healing process, with a faster

Table I. Comparison of clinical indexes for test group and control group.

		T0	T1	T2	T0-T1		T0-T2	
FMPS					value	p	value	p
	Study group	31.7%	13%	14.3%	-18.5	0.001	-17.4	0.001
	Control group	34.5%	14%	17.3%	-20.5	0.001	-17.2	0.001
	p	Ns	Ns	Ns	Ns		Ns	
FMBS	Study group	38.1%	16.3%	13.7%	-21.8	0.001	-24.4	0.001
	Control group	41%	15.7%	16.3%	-25.3	0.001	-24.7	0.001
	p	Ns	Ns	Ns	0.001		Ns	
GI	Study group	2.7	2	1.5	-0.7	0.001	-1.2	0.001
	Control group	2.9	2.1	2.8	-0.8	0.001	-0.1	Ns
	p	Ns	Ns	0.001	Ns		0.001	
PPD	Study group	5.6	4.9	4.2	-0.7	Ns	-1.4	0.001
	Control group	5.9	5.1	4.3	-0.8	Ns	-1.6	0.001
	p	Ns	Ns	Ns	Ns		Ns	

Ns: not significant

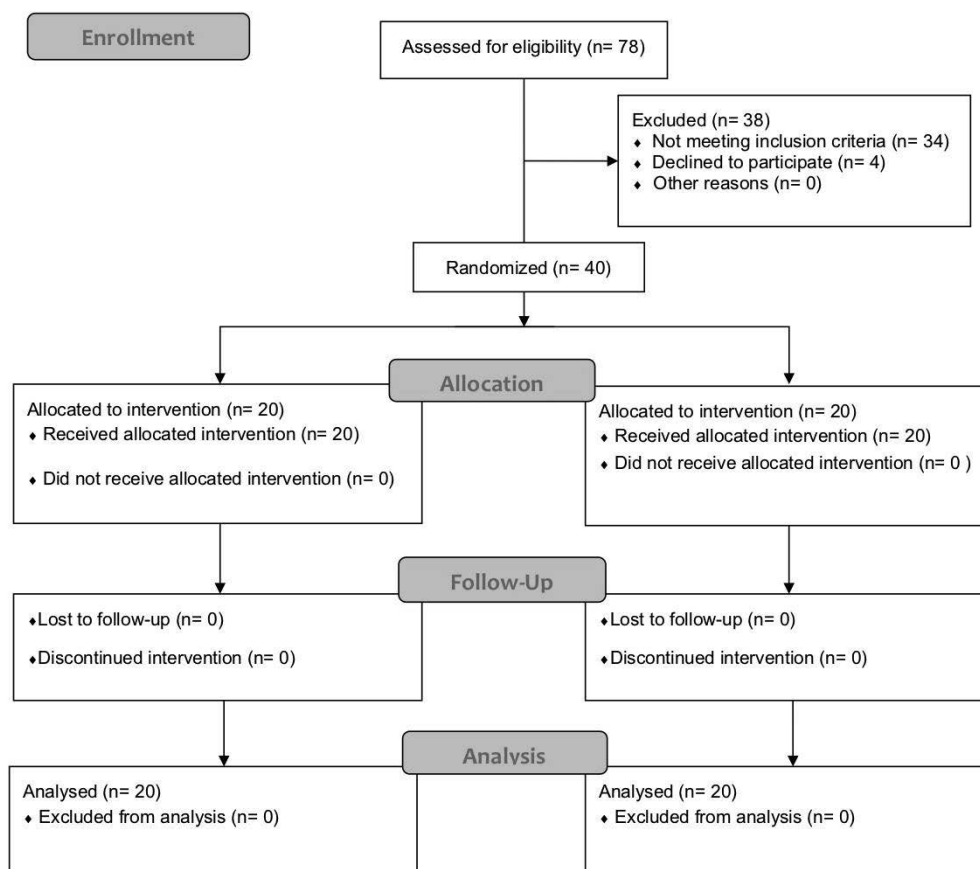


Fig. 1. Study flow-chart.

improvement of inflammation clinical indexes. FMBS and GI were already improved in Bioptron light group after 1 month. The wavelengths of Bioptron light, including the visible and the infrared spectrum, are compatible with the acceleration and improvement of blood supply and with the release of macrophage secreted growth factor (MSGF) that can stimulate fibroblast proliferation (11). The current literature (7-10) demonstrates the advantage of visible light therapy for skin injuries and diseases with the photobiomodulation, in which light at low energy levels modulates intra- and extra-cellular photoreceptors by molecular and cellular processes that can stimulate both anti-inflammatory mechanisms and cell proliferative homeostasis. To the best of our knowledge, no similar studies have been conducted to evaluate the use of polarized, polychromatic, non-coherent light for periodontal treatment.

Nevertheless, low level laser therapy (LLLT) has been demonstrated to enhance mechanical periodontal

treatment; LLLT in addition to FMSRP led to significantly lower periodontal and bleeding indexes and reduced PPD, and was proved to inhibit some subgingival bacteria (especially *Porphyromonas gingivalis*) (12).

Differences exist between these two adjunctive physical treatments. Like LLLT, Bioptron is also a low-power light source, but contrary to that it is polychromatic and incoherent rather than monochromatic and coherent. In fact, LLLT commonly employs red and near infrared light (600 nm-1000 nm), while polychromatic light wavelength is far wider (480-3400 nm). Bioptron delivers a power density of 0.04 W/cm², in line with LLLT devices (0.005-50 W/cm²). The Bioptron light beam has a wide diameter, allowing the exposure of larger anatomical areas simultaneously, otherwise, LLLT needs to be applied on site-by-site to be effective, requiring longer sessions and a steeper learning curve.

The improvements reached in our study confirm that SRP remain the gold standard therapy for periodontitis, as

all the clinical indexes were reduced, and almost all were statistically significantly reduced. The results confirm also the importance of a customized home maintenance protocol to improve oral health. Moreover, Bioptron light application is an effective adjunctive therapy combined with FMSRP, as our test group had a highly significant reduction of important clinical outcomes such as the GI and the FMBS.

Despite the accuracy of sample selection, data collection and statistical analysis, this study has the limit of a small sample and a short follow-up period, but the results encourage further studies on this technology applied to oral cavity.

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LETTER TO THE EDITOR

POSTMORTEM DIAGNOSIS OF 21-HYDROXYLASE DEFICIENCY CONGENITAL ADRENAL HYPERPLASIA: MEDICO-LEGAL ISSUES

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Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders due to decreased activity of enzymes necessary for cortisol biosynthesis. Each of the CAH disorders represents a clinical spectrum which reflects the consequence of a specific mutation. The Salt Wasting (SW) form of CAH due to 21-hydroxylase deficiency (21-OHD) is a life-threatening disease when untreated. It is easily diagnosed with newborn screening. Early detection of the defect prevents severe complications and an adrenal crisis that often leads to death. The authors report a case of a 20-day-old male infant, seemingly in good health, who died suddenly despite early cardiopulmonary resuscitation. The forensic autopsy showed the presence of enlarged adrenal glands. Analysis of a blood spot sample using tandem mass spectrometry revealed high serum levels of 17 hydroxyprogesterone (17-OHP), consistent of 21-OHD. The cause of death was a hypovolemic shock heart failure due to dehydration, caused by 21-OHD CAH. The report highlights the importance of extensive newborn screenings that would have prevented this death. The case evokes two relevant medico-legal issues, firstly for the hospital where the child was born, due to the lack of adequate information about the limited and inhomogeneous screening panels routinely adopted by different hospitals. Secondly, for suspicious clinical signs appearing after the discharge from hospital that could have been neglected by the pediatrician.

To the Editor,

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders due to decreased activity of the enzymes necessary for cortisol biosynthesis. The prevalence of CAH disorders ranges from 1:15,000 to 1:19,000 (1). CAH due to 21-hydroxylase deficiency (21-OHD) accounts for 90–95% of cases of CAH (2, 3). It is classified into classic (severe) or nonclassic (mild-

severe) forms. Classic 21-OHD is divided into Salt Wasting (SW) and Simple Virilizing (SV) forms. The SW form accounts for 75% of classic cases (4) and it is characterized by cortisol and aldosterone deficiencies, with a potential fatal outcome caused by adrenal crisis within the first 2-3 weeks of life (5). Males affected by classic 21-OHD CAH usually have normal genitalia, even if hyperpigmentation of the scrotum and enlarged phallus might be present

Key words: congenital adrenal hyperplasia; 21-hydroxylase deficiency; newborn screening; forensic autopsy

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at birth (3). The SV form leads to adequate levels of aldosterone and overproduction of adrenal androgens that cause variable degrees of genital virilization in females at birth (6). The nonclassic form can manifest later in life, with signs due to androgen excess, including premature pubarche, acne, rapid somatic growth and accelerated skeletal maturation (7). The diagnosis of 21-OHD CAH is performed by measuring blood serum level of 17 hydroxyprogesterone (17-OHP), which is elevated in affected infants, and sensitive and specific newborn screening tests are available. In populations with clinical awareness and standard care for 21-OHD, the lack of systematic newborn screening implies a probability of death from neonatal adrenal crisis that varies from 0% to 4% of the affected patients (8).

The authors report the case of a 20-day-old male infant, whose sudden death was a result of undiagnosed 21-OHD CAH. This case highlights the importance of extensive newborn screening as well as evoking relevant medico-legal issues.

Case presentation

The case is related to a sudden unexpected infant death of a 20-day-old male. Informed written consent was given by the family. He was the third child of Pakistani parents, who were blood relatives (first cousins). The family history was unremarkable, apart from the intra uterine growth restriction (IUGR) of an unknown cause of the first son (1,890 g at birth), despite the term pregnancy. The third infant was delivered by vaginal birth at 40 weeks of gestation. The APGAR index was 9 at 1st minute and 10 at 5th minute; the weight was 3,740 g. On hospital

discharge, no pathological conditions were reported, other than a weight loss of 160 g. No signs or symptoms were noted over the following few days. On 11th day following birth, during the first pediatric evaluation, postnatal growth restriction was noted (3,650 g) (Table I) and infant formula was prescribed by the pediatrician.

The child died 20 days after birth. The parents reported he had slept through the night without waking to eat. He was not hungry the following morning and appeared somewhat lethargic. After the midday feeding, the baby's condition suddenly deteriorated with vomiting, respiratory insufficiency and cardiovascular arrest. Physicians tried to resuscitate him, but all attempts failed.

A complete post mortem forensic examination was performed within 24 hours of death, according to the recommended protocol for the investigation in case of SIDS (9).

At thorough external examination, skin dehydration, increased facial and limb hair growth, and genital hyperpigmentation were noted. The forensic autopsy showed enlarged adrenal glands (Fig. 1) that weighted 7 g and 9 g, respectively whilst the normal weight range is 1.2-1.7 g. The adrenal cortices showed increased thickness and hyperpigmentation. No other pathological signs were found from the autopsy. Hematoxylin-eosin staining of adrenal gland samples revealed an adrenal hyperplasia, a thickened zona fasciculata, with polygonal cells showing prominent eosinophilic staining cytoplasm and a cytoplasmic lipid depletion.

Suspecting CAH, blood spot samples collected during autopsy were analyzed with tandem mass

Table I. *Post-natal anthropometric measurements.*

	Birth	Hospital discharge (3 days after birth)	Pediatric examination (11 days after birth)	Post mortem examination (within 24 h of death)
Length	52 cm	-	54 cm	57 cm
Cranial Circumference	36 cm	-	36 cm	36 cm
Weight	3740 g	3580g	3650 g	3240 g



Fig. 1. *Enlarged adrenal glands.*

spectrometry, revealing a high level of 17-OHP, demonstrating the presence of 21-OHD. Owing to the results of the blood examination and the remarkable adrenal hyperplasia detected by forensic pathologists, no further genetic or electrolytes analysis were performed. The cause of death was attributed to a fatally severe loss of enzyme activity due to 21-OHD CAH that eventually resulted in hypovolemic shock heart failure from dehydration.

DISCUSSION

The case describes a male infant, seemingly in good health, who quickly developed typical signs and symptoms of untreated classic SW 21-OHD. Carrying out a screening test based on analysis of 17-OHP and subsequent early treatment could have prevented death. Usually 17-OHP serum levels are above 10,000 ng/dL in classic 21-OHD, while the level in healthy newborns is <100 ng/dL (10).

At the time of the reported case, CAH screening was available in only four of the 20 regions in Italy. In 2013, a national law encouraged the adoption of extended screening panels in all regions. However,

because of specific regional laws, only a few did so. Finally in 2016, a national law (167/2016 law) established the adoption of a uniform extended panel nationwide. CAH, however, was not included. This law also established that information sheets must be provided to parents in order to obtain consent, but there is not yet a national standard of counseling for parents about their options for expanding newborn screenings. The delay in adopting a national uniform screening panel raises medico-legal questions for hospitals and pediatricians in Italy at least for the information duty and consent. From a medico-legal standpoint, every Italian hospital should offer complete information to families about how many and what diseases are screened for, but they should also clarify that all screening panels are limited and uneven assorted through the different Regions (11).

Furthermore, in the present case, the medical records of the pediatrician who visited the baby after the hospital discharge could have revealed whether some early clinical signs of CAH, such as growth restrictions and hyperpigmentation of the scrotum, were manifest after 10 days. Missing these signs could raise suspicions of pediatrician negligence.

Therefore, the case illustrates the importance for countries to adopt extended and uniform newborn screening programs. Early detection of 21-OHD CAH and other congenital metabolic diseases reduces morbidity and mortality and generates considerable health care savings (12). Clear and explicit information about the limited and uneven assorted screening panels should be provided to parents by hospital personnel and pediatricians who follow children after hospital discharge in order to prevent legal claims.

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LETTER TO THE EDITOR

EFFECT OF SILVER VITELLINATE, HYALURONIC ACID AND SODIUM BENZOATE NASAL SPRAY AFTER SEPTOPLASTY

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The aim of the study was to evaluate the postoperative endoscopic score and the quality of life (QoL) in two groups of adults undergoing septoplasty with turbिनoplasty and who were treated after surgery with a nasal spray containing silver vitellinate, hyaluronic acid and sodium benzoate, or saline solution alone. This single-blind randomized study was carried out on 54 patients (30 males, and 24 females, mean age 33.5±2.7 years) undergoing septoplasty and volumetric tissue reduction of inferior turbinates for nasal obstruction. All subjects underwent the Visual Analogue Scale (VAS) questionnaire and the nasal endoscopy score by Lund and Kennedy (LK) at baseline before surgery (T0), 15 days (T15) and 21 days (T21) after treatment. All subjects were randomized into 2 groups, the experimental arm, group I (GI), and the control arm, group II (GII). Patients were given the treatment, a nasal spray containing silver vitellinate, hyaluronic acid and sodium benzoate for group I and a saline solution alone (sodium chloride 0.9%) for group II. After therapy, better VAS and LK scores were found ($p < 0.05$) in the GI in GII. No patients reported adverse reactions or complications. Our findings suggest that silver vitellinate, hyaluronic acid and sodium benzoate nasal spray improved not only the endoscopic score, but also the quality of life (QoL) in the early postoperative period after septoplasty. In addition, the absence of adverse reactions or complications pointed out the tolerability and safety of the treatment.

To the Editor,

Septoplasty with concomitant turbिनoplasty, is one of the most frequent surgical procedures performed on adults by otolaryngologists (1) to correct a deviated septum and to restore nasal patency. To date, surgeons have developed a variety of surgical techniques (2) that have changed over time, from the concept of a wide resection to that of preservation and regularization of the deviated septum (3, 4). However, regardless of the surgical technique used and despite the consolidated use

of different postoperative treatments, the early postoperative period after nasal surgery is often compromised by edema, crust formation, mucosal alterations, and excessive secretions (5).

Furthermore, despite accurate postoperative medications and the use of available treatments as nasal irrigation, inhalations, sprays, and ointments, postoperative discomfort often persists and no established gold standard treatment exists (5). To this end, otolaryngologists are continually searching for devices able to promote wound healing and

Key words: septoplasty; turbinate surgery; QoL; postoperative outcomes; nasal spray

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postoperative regeneration of nasal mucosa (5).

Intriguingly, previous studies reported that silver-based medical preparations exert not only an antimicrobial effect in the airway of a murine model of asthma, but also a role in decreasing the inflammation (6). Moreover, silver-based preparations as well as quartz seem to modulate the proliferation rate of fibroblast cells, the main characters of wound healing process (7). For instance, silver vitellinate (silver proteinate) is a powerful germicidal and decongestant, that has been used for a long time to treat infection of nasal mucosa (8) of viral or bacterial origin, in children. In addition, hyaluronic acid, the main component of the extracellular matrix, is a glycosaminoglycan, that may exert reparative, anti-inflammatory and immune-modulating activities (5, 9-12).

Hence, an alternative local postoperative therapy after nasal surgery could be the application of nasal spray containing silver vitellinate and hyaluronic acid (Rinostil plus, DECA, Milan, Italy). For this purpose, we evaluated the postoperative quality of life (QoL) in non-allergic adults undergoing septoplasty with turbinoplasty, treated after surgery with a nasal spray containing silver vitellinate, hyaluronic acid and sodium benzoate.

MATERIALS AND METHODS

This randomized study was carried out at the ENT Department of Cardarelli Hospital in Naples on 54 non-allergic patients (30 males, 24 females, mean age 33.5 ± 2.7 years) undergoing septoplasty and volumetric tissue reduction of inferior turbinates by Vesalius Quantum (Quantum Molecular Resonance Cautery) for nasal obstruction due to septal deviation and inferior turbinate hypertrophy.

The enrolled subjects gave their informed consent to the study, which was approved by the local Board of Medical Ethics. The study was carried out according to the Declaration of Helsinki.

All subjects underwent:

- Skin prick test (SPT) using a standard allergen extract panel [(Stallergenes Company, Anthony, France). Positive (histamine) and negative (distilled water) controls were also performed. Aeroallergens

included house dust mite, pollens, alternaria, aspergillus, cladosporium, grasses, weeds, wheat, cockroach] (13).

- Nasal endoscopy by a 4-mm 30-degree rigid endoscope (Storz, Tuttlingen, Germany) at baseline before surgery (T0), 15 days (T15) and 21 days (T21) after treatment. The nasal endoscopy was performed, without decongestant or local anesthesia and scored as previously described by Lund and Kennedy (LK) (14).
- the Visual Analogue Scale (VAS) at baseline before surgery (T0), 15 days (T15) and 21 days (T21) after treatment. The VAS included questions about nasal obstruction (VASo), and rhinorrhea (VASr). The disease was divided into mild, moderate, and severe based on total severity VAS scores (mild, VAS = 0-3; moderate, VAS = 4-7; and severe, VAS = 8-10). A VAS > 5 affected patient QoL (15).

Patients suffering from systemic diseases, allergy, acetylsalicylic acid (ASA) sensitivity, cystic fibrosis, primitive ciliary dyskinesia, or with a history of previous nasal surgery were excluded from the study. Subjects who were being treated with antibiotics, steroids, antihistamines, and local vasoconstrictive decongestants during the previous month were also excluded.

The enrolled subjects underwent the septoplasty as described by Sulenti under general anesthesia by the same experienced consultants (16). The maxilla-premaxilla approach combining mobilization and/or removal of any deranged portion of the bony and/or cartilaginous septum, followed by reconstruction of the septum support (16) was performed. The septum was fixed through trans-septal sutures and the septal caudal incision was closed.

In addition, turbinoplasty was performed by using the Vesalius Quantum (Quantum Molecular Resonance Cautery), which aims to create a decongested submucosa. The nose was packed, and the packing was removed 48 h after surgery, after which subjects were randomized into 2 groups and assigned the treatments. The investigational arm, group I (GI), included 28 subjects, and the control arm, group II (GII), also included 26 subjects.

GI subjects received topical therapy with a nasal spray (2 puffs for each naris 5 times a day for 21 days) containing silver vitellinate, hyaluronic acid and sodium benzoate (Rinostil plus, DECA, Milano, Italia).

GII subjects received topical therapy with a nasal spray (2 puffs for each naris 5 times a day for 21 days)

containing saline solution (sodium chloride 0.9%) alone.

Continuous baseline characteristics were described as mean and standard deviation, whereas differences between groups were tested with the Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

At baseline, no statistically significant differences were observed between groups (Table I).

After treatment at T15 and T21, we observed a statistically significant ($p < 0.05$) improvement of both VASo, VASr and LK scores in GI (Table II), whereas we did not find improvement ($p > 0.05$) in either the VAS scores or in the LK scores in the GII at T15 (Table II). Conversely, in GII we observed improvement ($p < 0.05$) in the VASo, VASr and LK only at T21. After therapy at T21, the VASo, VASr and LK scores were better ($p < 0.05$) in the GI than in the GII (Table II). No patients reported adverse reactions or complications.

DISCUSSION

Comfortable nasal breathing is a condition directly related to the QoL, and the nasal obstruction is one of the most common symptoms observed in ear, nose and throat (ENT) practice. Consequently, septoplasty is of the utmost importance in the field of otorhinolaryngology (2,18).

Since the QoL is a subjective evaluation of the effect of a disease or of treatment on a patient's health, it represents an important assessment in clinical interventions. Interestingly, several patients with the same objective conditions may have different QoL (19).

Currently, there is evidence that the patient's perspective on treatment outcomes is a crucial element for improving high-quality care and patient-rated therapeutic outcomes in terms of symptoms, and that it can provide a much more realistic feedback of the effectiveness of a treatment than those of objective outcomes (5). For these reasons, our study aims at evaluating postoperative treatment outcome in terms of QoL.

Despite the consolidated use of different topical

treatments after septoplasty, the early postoperative period is often painful and uncomfortable, seriously affecting the patient's QoL. Indeed, crusting, mucosal changes, ventilation disorders due to excessive secretion and edema, hemorrhaging, or the development of synechia with possible re-obstruction are critical factors that can lead to postoperative complications (20). In addition, the final postoperative result depends on a rapid wound healing of the nasal mucosa, which considerably reduces the risk of infections.

Our study evaluated the effect of a nasal spray containing silver vitellinate, hyaluronic acid and sodium benzoate on adults undergoing septoplasty with turbinoplasty for nasal obstruction. We investigated whether the local treatment influenced the postoperative status of the nasal mucosa upon endoscopic evaluation and the QoL.

Silver vitellinate, which has been used for a long time to treat upper airways phlogosis is a powerful antimicrobial and decongestant (6-8). In particular, this molecule blocks the metabolism of bacteria, reducing their replication, implementing the lysis of the microbial cell wall, damaging bacterial DNA and bacterial proteins, and inducing the blockage of the respiratory enzyme system. It also has mucolytic and decongestant properties (6, 7).

Recently, mounting evidence suggests that hyaluronic acid - the major component of extracellular matrices of the respiratory mucosa - serves important biological roles beside its function as a structural component of interstitial and connective tissues. Furthermore, it seems to promote wound healing, repairing mucosal surfaces, and cell motility. Previous studies showed that topical application of hyaluronic acid may promote the healing process of the nasal mucosa, preventing extensive crusting, and leading to the recovery of smell function and cooling sensation (5, 9-12). Furthermore, sodium benzoate has bacteriostatic and antimycotic properties (21).

In our study, the impact of the postoperative treatment was assessed with the endoscopic score and QoL questionnaire, VAS at T15 and T21. At T0 we did not find differences between groups (Table I).

After treatment, we observed a statistically significant ($p < 0.05$) improvement in all parameters

Table I. Differences in both groups at baseline.

GROUP T0	LK	VASo	VASr
I	5.8±2.2	7.3±2.0	7.5±1.3
II	6.2±1.2	6.8±1.0	7.0±1.3
p	>0.05	>0.05	>0.05

No statistically significant differences were found in the two groups at baseline.

LK: endoscopic score; VASo: obstruction; VASr: rhinorrhea.

Table II. Differences in both groups after treatment at T15 and T21.

	GI			GII			
	LK	VASo	VASr	LK	VASo	VASr	
T0	5.8±2.2	7.3±2.0	7.5±1.3	6.2±1.2	6.8±1.0	7.0±1.3	
T15	3.4±1.3	1.1±1.2	0.8±1.0	5.5±1.8	6.2±1.3	6.5±1.0	P:GIvsGII<0.05
P	<0.05	<0.05	<0.05	>0.05	>0.05	>0.05	
T21	0.7±1.3	0.7±0.8	0.45±1.0	5.0±1.2	5.2±1.0	5.6±1.0	P:GIvsGII<0.05
P	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	

After treatment at T15 and T21, a statistically significant ($p<0.05$) improvement was observed of both VAS and LK scores in GI, whereas at T21 we did not find improvement either in the VAS scores or in the LK scores in GII. In GII we observed improvement in the VAS and LK parameters only at T21. After therapy only at T21, the VAS and LK scores were better ($p<0.05$) in GI than in GII. LK: endoscopic score; VASo: obstruction; VASr: rhinorrhea.

studied in GI (Table II). Conversely, in GII at T15 we did not observe improvement in the VAS and LK scores, but at T21 we observed improvements in all considered scores (Table II).

These observations suggest that the nasal spray containing silver vitellinate, hyaluronic acid and sodium benzoate improved not only clinical/endoscopic parameters reducing the formation of crusts and edema, but also the QoL.

Furthermore, in GI, the improvement of VAS and LK scores after 15 days already demonstrated that the effect of the nasal spray containing silver

vitellinate, hyaluronic acid and sodium benzoate might reduce the healing time after nasal surgery. Indeed, in GII, we observed an improvement of endoscopic and QoL parameters only 21 days after surgery. However, this could be the result of the physiological post-operative healing process *per se*.

In addition, although the results were statistically significant in both groups, they were better in GI than in GII. Eventually, the absence of adverse reactions or complications confirmed the tolerability and safety of the treatment.

In conclusion, our findings suggested that a

nasal spray containing silver vitellinate, hyaluronic acid and sodium benzoate improves not only the clinical parameters, but also the QoL in the early postoperative period.

Moreover, given the large number of septoplasties performed by otolaryngologists, the use of the spray containing silver vitellinate, hyaluronic acid and sodium benzoate could represent an economic advantage, particularly in terms of reduction of indirect costs (disability, reduced school performance and absence from school) related to the duration of the convalescence.

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LETTER TO THE EDITOR

APPLICATION OF STEM CELLS IN ORAL SCIENCE:
A LITERATURE REVIEW

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The objective of this work was to provide a review of the literature on studies of stem cells and their potential applications in dental practice. Research included every article published between 2000 and 2018 in PubMed PMC, Scopus, Cochrane Trial Library, and Web of Science databases featuring the keywords: regenerative dentistry, stem cells, tissue engineering. Papers that did not directly address the subject were excluded. Emphasis was given to scientific studies that showed possibilities of stem cell therapy in the oral and maxillofacial region. The methodological quality of selected papers was scored using the “Swedish Council on Technology Assessment in Health Care Criteria for Grading Assessed Studies” (SBU) method. Two review authors independently assessed eligibility, extracted data and verified the quality of the studies.

To the Editor,

Stem cells are considered a useful tool against oral diseases and represent a therapeutic option for replacement of oral maxillofacial structure. They are defined as a type of undifferentiated cells with particular properties as precursor with the capacity of processing different types of cells with different functions, the most important being the self-renewal. Basically, the most important type of cells are the embryonic stem cells (ESCs) and the adult stem cells (ASCs). The first ones derived from the interior cell mass of a blastocyst and are classified as totipotent: they can form any type of tissue found in the adult body. Adult stem cells are classified as pluripotent: they are undifferentiated cells that remain quiescent in tissues; if they are stimulated it is possible to create

types of cells compatible with the tissue where they reside. Adult stem cells are relatively easier to isolate and do not have any ethical issues (1). Mesenchymal stem cells (MSCs) are a type of ASCs, and can be extracted from different locations, such as bone marrow, umbilical cord, adipose and dental tissues. Bone marrow stem cells (BMSCs) can be harvested from sternum or iliac crest. The advantage of bone marrow is the larger volume of stem cells and the possibility to be differentiated into a wide variety of cells. Umbilical cord blood contains cells that are able to develop totipotent cells and isolates in all three of the germ layers: endoderm, ectoderm and mesoderm. Adipose derived stem cells (ADSCs) include a group of pluripotent mesenchymal stem cells that exhibit multilineage differentiation. The principle advantage

Key words: regenerative dentistry, stem cells, tissue engineering

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of adipose tissue is that it is abundant in many individuals and easily accessible (2). One study on the influence of polylactide and polyglycolide (PLPG) plates on the bone gene RUNX2 in ADSCs demonstrated the immediate activation of the gene and consequently the osteogenesis while reducing osteoclastic activity (3).

In oral and maxillofacial area, five types of dental stem cells (DSCs) were identified and characterized. They included dental pulp stem cells (DPSCs), stem cells from exfoliated deciduous teeth (SHEDs), stem cells from apical papilla (SCAPs), periodontal ligament stem cells (PDLSCs), and dental follicle progenitor cells (DFPCs). DSCs are easy to obtain and present a great potential of differentiation, so there is growing interest in regenerative medicine (4). The aim is to regenerate both bone and dental tissues, including the periodontal ligament, dental pulp and enamel. This article reviews the innovative technologies in regenerative dentistry with the involvement of stem cells. Furthermore, we gave relevance to the articles regarding the effectiveness of applying stem cells for regeneration of oral tissue and the achievements obtained compared to traditional techniques.

MATERIALS AND METHODS

Articles published from 2000 to 2018 were selected using PubMed, PMC, Scopus, Cochrane Trial Library, and Web of Science databases, with the key words regenerative dentistry, stem cells, and tissue engineering. *In vitro* and *in vivo* studies on humans and on animals were analyzed. In particular, articles on the application of stem cells in the oral maxillofacial region were selected for their importance in tissue regeneration. Articles that demonstrated the importance of the topic were included (Table I). The SBU method divided the articles selected into three groups: A includes articles with a high value of evidence (only one article at level A); B those of moderate value (at least 14 articles were estimated at level B); C are those with low value of evidence (most of the research was included at this level). When the score was completed we stated the review in one of these grades: level 1 - strong scientific influence; level 2 - moderate scientific evidence; level 3 - limited scientific evidence; and level 4 - insufficient scientific evidence (Table II).

The purpose of this paper is to present a review of the literature on stem cells in dentistry, focusing on their application in regenerative therapy.

Table I. Selection criteria of articles.

INCLUSION CRITERIA	EXCLUSION CRITERIA
• <i>In vivo/in vitro</i> studies, human/animals • Applications of stem cells in dentistry • Application of stem cells in maxillofacial regeneration • Bone and tissue regeneration • Published in English language • Dental pulp stem cells (DPSCs), stem cells from exfoliated deciduous teeth (SHEDs), periodontal ligament stem cells (PDLSCs), stem cells from apical papilla (SCAPs) and dental follicle progenitor cells (DFPCs), adipose stem cells (ADSCs), bone marrow stem cells (BMSCs)	• Case report, case series, commentary / letter to the editor, abstract only, conference paper • Articles not in English language • Articles about umbilical stem cells, amniotic stem cells, non-bone stem cells (cardiac, neuronal, retinal) • Application of stem cells not in dentistry area

RESULTS

Excluding articles that do not concern the orofacial complex, from the initial 499 articles, 44 were selected (Fig. 1). Among the advantages of stem cells derived from the oral and maxillofacial area we can underline high plasticity, cryopreservation for longer periods, and good interplay with scaffold and growth factors. In some cases the stem cells can create progression of pathogenic cells and an immunosuppressive effect on the recipient subject, therefore, the best option is autologous stem cells. Dental stem cells have MSC-like qualities, such as self-renewal and differentiation potential (5).

Dental pulp stem cells (DPSCs) were successfully isolated by Gronthos et al. in 2000. In their studies on samples of immunocompromized mice, they showed that odontoblast from DPSCs produce ectopic dentin. In an *in vitro* study, these cells have the capacity to differentiate into osteoblasts, adipocytes, chondroblasts, neural cells and melanocytes (4, 6).

Stem cells from exfoliated deciduous teeth (SHED) were identified as a type of cells with higher proliferation capacity, with increased population doublings, immature multipotent clonogenic cells extracted from temporal teeth that can differentiate into many types of cells. In comparison with DPSCs, SHEDs have a higher cell proliferation rate, shorter population doubling time and increased clonogenic potential (7).

Periodontal ligament stem cells (PDLSCs) were

isolated from periodontal ligaments of 25 human third molars by Seo et al. They demonstrated that this type of stem cell can differentiate into cementoid cells and adipocytes when transplanted into immunocompromized rodents. PDLSCs exhibit a particular self-renewal capacity and express cell surface markers similar to bone marrow-derived MSCs (8).

Stem cells from human apical papilla (SCAP) were isolated by Sonoyama et al. in 2008. These cells are able to create odontoblast-like cells *in vivo*. Moreover, these cells have an important higher proliferation and mineralization potential in comparison with DPSCs (9).

Nowadays, regeneration in oral and maxillofacial areas can be carried out using stem cell therapy which has gained momentum. Tissue samples with stem cells were placed in a screw-top vial containing an appropriate media, which nourished it during transport. In the laboratory, the samples were trypsinized and passed to yield colonies of stem cells. Cultured stem cells must be passed through stem cell markers, such as Oct4, Nanog, SSEA4, TRA-1-60 and TRA-1-81, before they are administered to patients to discover the lineage of the cell. Stem cells are charged in an appropriate carrier called “scaffold” to repair the defects of human structures or replace an organ. Scaffold can be made of different shapes, pattern and biomaterials, however, depending on the necessity, it can be made up of natural or artificial materials.

Table II. Swedish Council on Technology Assessment in Health Care (SBU) Criteria for Grading Assessed Studies.

Swedish Council on Technology Assessment in Health Care (SBU) Criteria for Grading Assessed Studies
Grade A: high value of evidence. All criteria should be met: randomized clinical study or a prospective study with a well defined control group, defined diagnosis and endpoints, diagnostic reliability tests and reproducibility tests described, blinded outcome assessment
Grade B: moderate value of evidence. All criteria should be met: cohort study or retrospective case series with defined control or reference group, defined diagnosis and endpoints, diagnostic reliability tests and reproducibility tests described
Grade C: low value of evidence. One or more of the conditions below: large attrition, unclear diagnosis and endpoints, poorly defined patient material

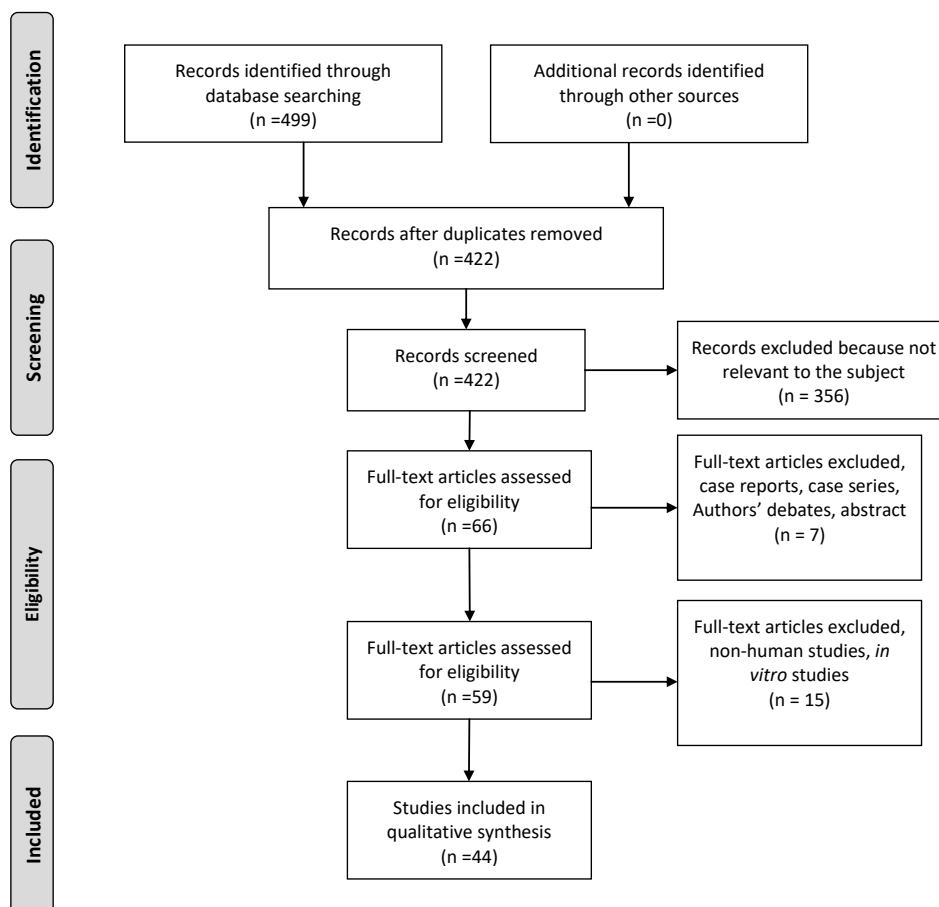
DISCUSSION

DSCs had great importance in the studies of periodontal treatment, pulp regeneration after necrosis, repairing maxillofacial bone disease, as well as the development of new teeth. Recently, two strategies have been investigated for pulp regeneration after endodontic treatment: DPSCs autologous transplantation associated with scaffolds, and pulp canal revascularization. However, histological analysis shows that there is no tissue formation similar to pulp tissue. Periodontitis is an inflammatory pathology that causes injuries in the cementum, periodontal ligament and alveolar bone. In humans, it is possible to repair dental structures affected by periodontal disease. Chen et al. performed autologous transplant of PDLSCs in 30 patients and demonstrated significant improvement in the injured area. Thus, the use of different colonies

of DSCs in the treatment of periodontal disease can be an interesting approach (10). Stem cells can be useful for bone regeneration and to correct stronger craniofacial defects due to cyst enucleation, tumor resection, and trauma. The closure of a bone defect is commonly carried out with the addition of new tissue, which has some disadvantages: it is not able to restore the unique function of the lost part; the donor site has a large morbidity, accompanied by scarring, infection and loss of function. Stem cells from oral and maxillofacial regions can be combined with bone marrow stem cells to correct larger defects. Oral maxillofacial bone tissue repaired with stem cells was performed with collagen sponge scaffold and dental pulp stem cells harvested from third molars of the same patient.

The regeneration of adult teeth will be possible in the future with the newer advancement in stem cell therapy and tissue engineering. Regenerative

Fig. 1. Selection and screening of articles.



procedures would be an alternative therapy to dental implants. Experimental studies with animal models have shown that the tooth crown structure can be regenerated using tissue engineering techniques that combine stem cells and biodegradable scaffolds.

In future, this technique can be applied to regenerate other bones in the oral maxillofacial area (11). Engineered bone graft, engineered temporomandibular joint and craniofacial structure would be developed with stem cells, combined or not with natural bone.

Dental patients often forget that the loss of teeth has important consequences for health, nutrition and oral hygiene, not only for the aesthetic appearance. Tissues reconstruction raises hope for dental patients worldwide and the research should be continued. Stem cell therapy will have a key role in regenerative dentistry as a primary modality to solve maxillofacial disease. The broad spectrum of MSCs creates significant prospects in the research for dental regeneration and the progress has given substantial confirmation that therapy based on stem cells is a realistic option. Research on stem cells is aimed at refining prevention and care in the field of health, through new vanguard technologies that can encompass and improve all areas of dentistry such as orthodontics, periodontics, surgery, and prosthodontics (12-14).

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