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EDITORIAL

ASPROSIN AND TYPE 2 DIABETES MELLITUS: A NOVEL POTENTIAL THERAPEUTIC IMPLICATION

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Diabetes is a group of metabolic disorders that is characterized by hyperglycemia which increases the risks of cardiovascular, microvascular, and macrovascular complications. Innovative therapeutic trials regarding diabetes control and management are continually being undertaken. The present review was aimed to explore the potential effects and mechanisms that lead to the pathogenesis of type 2 diabetes mellitus (T2DM) and its relation with asprosin. Asprosin is a newly discovered hormone that is encoded by protein fibrillin 1 (FBN1 gene), secreted by white adipose during fasting conditions at 5–10 nM levels, which acts on the liver through cell membrane receptors and activates the G protein cAMP- PKA pathway. Asprosin secretion is increased during fasting as the compensatory mechanism in hypoglycemia. Asprosin concentration is higher in patients with T2DM and impaired glucose regulation compared to healthy subjects. Genetic deficiency of asprosin may cause problems of poor appetite and extreme leanness in humans. Attenuating asprosin activity or depleting asprosin may serve as a novel therapeutic innovation for the treatment of T2DM and obesity. Hence, asprosin may serve as a beacon for the target of a future therapy in diabetes management.

Asprosin is a newly discovered hormone and is correlated with insulin secretion; a lot is still to be revealed about it. The primary objective of the present review was to assess plasma asprosin concentrations in the case of T2DM, and to explore the potential effects and mechanisms that lead to the pathogenesis of T2DM and its relation with asprosin. Fig. 1 shows the conceptual framework of the review.

Asprosin may serve as an ideal for future therapy in the management of diabetes which would help clinical practitioners (especially endocrinologists and

laboratory technicians) to consider novel hormone asprosin as a reference for diabetes diagnosis and its subsequent management.

Asprosin biology

Asprosin is encoded by protein fibrillin 1 (FBN1 gene) (140 amino acid residues 2732-2871, molecular weight 30 kDa), and this gene also encodes fibrillin. The encoded pre-protein is processed by proteolysis to generate two proteins-fibrillin-1 and asprosin (1-4). Fibrillin-1 insufficiency interferes with

Key words: asprosin; adipokine; protein hormone; type 2 diabetes

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wound healing in the connective tissue damaged by inflammatory diseases. Asprosin has a prime role in hepatic glucose synthesis and in the regulation of glucose homeostasis and is also linked to wound healing (2). The ultimate two exons of FBN1 are responsible for the encoding: exon 65 encodes 11 amino acids, while exon 66 encodes 129 amino acids (3). Romere et al. identified a fasting-induced protein hormone modulating hepatic glucose release to maintain glucose homeostasis in the body and termed this C-terminal cleavage product of profibrillin as 'asprosin' (3, 5-10).

Mammalian-generated asprosin (plasma half-life ~145 min) is about twice the molecular weight of the bacterially-generated asprosin (plasma half-life ~20 min) due to the glycosylation of the mammalian-generated protein. They found that 60 mcg subcutaneous injections produced a peak plasma level of 40 nM. There was a latent phase of a

few hours after its injection before the appearance of the orexigenic effect, differentiating it from the more acute-acting orexigenic peptide hormone ghrelin (secreted from the stomach) (8, 9). Duerrschmid et al. reported that asprosin level in cerebrospinal fluid (CSF) is increased by overnight fasting in a similar way to that of plasma asprosin in 6- to 12-week-old male wild-type mice, but CSF concentrations of asprosin were four to five times below the plasma concentrations (9).

Asprosin discovery and chemistry

Asprosin is a novel adipokine discovered by Romere et al. (under the guidance of medical geneticist Dr. Atul Chopra at Baylor College of Medicine, Houston, USA) as a fasting-induced glucogenic protein hormone. They discovered it whilst analyzing the genome of patients with the extremely rare Wiedemann-Rautenstrauch

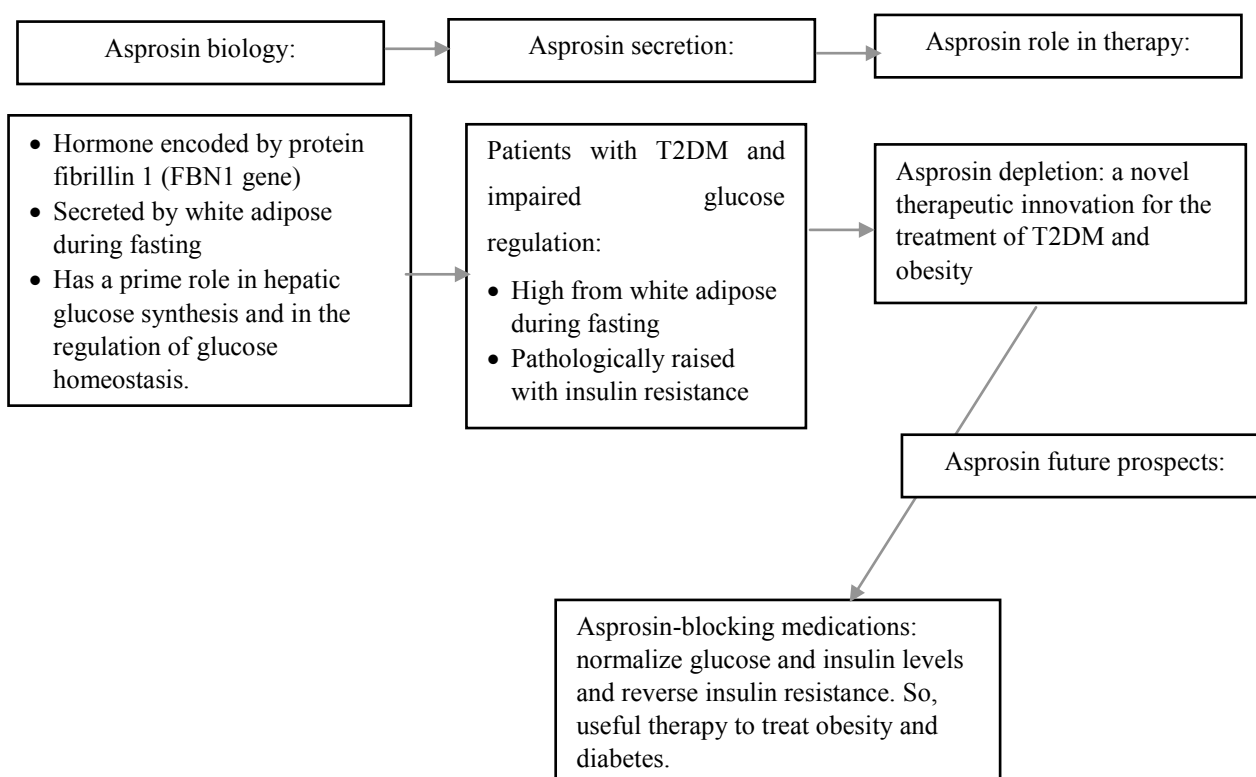


Fig. 1. Conceptual framework of role of asprosin in diabetes therapy.

syndrome (i.e., NPS) (3, 11). Romere et al. further assessed daily fluctuations in circulating asprosin concentrations. They concluded that glucose has a negative influence on plasma asprosin levels in a negative feedback manner (3).

Asprosin and diabetes mellitus

Asprosin has the potential therapeutic intervention for diabetes mellitus. Protein hormones are mainly the byproduct of the cleavage of a larger proprotein and circulate to the target organ on secretion, where they bind the target cell using the cell-surface receptor and stimulate rapid signal transduction via the second messenger system, and ultimately result in a measurable physiologic effect. This is the principle under consideration for asprosin in the diagnosis and management of diabetes. Hepatic glucose release into the blood circulation is essential for the smooth regulation of the brain function and survival during periods of fasting (3). The detection range for asprosin is 1–32 nmol/L with ELISA, and levels are high in obese and T2DM patients (11).

Romere et al. reported that the absence of asprosin in humans results in metabolic dysregulation (namely partial lipodystrophy) along with reduced plasma insulin. Asprosin is pathologically raised with insulin resistance in mammals including humans and mice. The reduction of asprosin protects against hyperinsulinism (3). Wiecek et al. also concluded that asprosin concentration is higher in patients with T2DM and impaired glucose regulation compared to healthy subjects (11).

A recent study by Li et al., reported that OLFR734 is a receptor of asprosin and promotes hepatic glucose production during fasting and in obesity. This study also highlighted that deficiency of Olfr734 decreases gluconeogenesis and increases insulin sensitivity (12).

Asprosin and neonatal progeroid syndrome patients

Romere et al. found that mutations in the gene encoding profibrillin (FBN1) in patients with neonatal progeroid syndrome (NPS) disrupted the expression of asprosin. NPS is a rare genetic condition (i.e., caused by a genetic deficiency of asprosin), first described in humans in 1977 and

is characterized by congenital, partial lipodystrophy, poor appetite and extreme thinness (mainly affecting the face and extremities) (2, 4, 13). NPS patients have mutations in the FBN1 gene which is responsible for controlling asprosin, therefore, they have low levels of asprosin and are devoid of the fatty layer under their skin due to the defects in asprosin (2). NPS patients generally look progeroid due to facial dysmorphic features and reduced subcutaneous fat (3).

Romere et al. found that the overnight-fasted plasma insulin levels in NPS patients were two-fold lower than the unaffected subjects. They concluded that NPS mutations may reduce plasma insulin levels in humans (3). Duerrschmid et al. found orexigenic property in asprosin because it crosses the blood-brain barrier (BBB) and activates the orexigenic agouti-related neuropeptide (AgRP⁺) neurons (hypothalamic neurons that promote hunger) via a cAMP-dependent pathway. They also found that genetic deficiency of asprosin may cause problems of poor appetite and extreme leanness in humans (4, 6, 7, 9, 11, 13–15).

Asprosin plasma concentration

Asprosin is secreted by white adipose during fasting conditions (i.e., glucose deprived condition) at 5–10 nM levels and acts on the liver through cell membrane receptors and activates the G protein cAMP- PKA pathway (cyclic adenosine monophosphate-protein kinase A) (2, 3, 5, 8). This leads to the rapid release of glucose into the blood circulation and insulin secretion as the compensatory mechanism. This is the nexus of asprosin with diabetes management, for which it is being investigated (3). Romere et al. also observed that asprosin-binding antibodies in diabetic mice normalized their glucose and insulin levels but asprosin did not alter the serum concentration of glucagon, epinephrine, norepinephrine and cortisol in those mice. They also presented that asprosin is present in the plasma of humans, mice, and rats at consistent nanomolar levels by calculating the plasma levels with the help of a standard curve using recombinant asprosin (rAsprosin) (3).

Asprosin secretion is increased through fasting in humans as the compensatory mechanism in hypoglycemia. Glucose serum levels below 20 mg/

dL lead to cerebral seizures or coma. Subcutaneous administration of the rAsprosin in mice increases both blood glucose and insulin (5, 8, 9). Duerrschmid et al. found that the circulating concentration of asprosin rises with fasting and acutely declines with refeeding in a biocircadian rhythmic pattern (9). Beutler et al. found that circulating asprosin levels increased with overnight fasting in mice, rats, and humans, but rapidly declined after food intake, suggesting the role of asprosin as a signal for hunger (8).

Inhibition of asprosin secretion

Duerrschmid et al., in their study on wild-type mice, concluded that AgRP⁺ neurons are essential for stimulation of appetite by asprosin. They confirmed its necessity by comparing the appetite level with and without AgRP⁺ neurons. In the latter group, where these neurons were excised, they found a complete absence of asprosin orexigenic stimulus even after feeding the mice with a normal diet. They concluded that asprosin-mediated activation of AgRP⁺ neurons can be completely prevented by pretreating the cells with suramin (an inhibitor of heterotrimeric G proteins), NF449 (an inhibitor of the Gas subunit of heterotrimeric G proteins), NKY80 (an inhibitor of adenylate cyclase), and PKI (an inhibitor of protein kinase A, PKA) (8). These all may be exploited for future clinical trials.

Therapeutic implications of asprosin

Various studies have reported that asprosin might be a novel therapeutic target for diabetes management (4, 16). Alan et al. found a raised level of asprosin in T2DM patients compared to their matched controls and its levels were positively correlated with insulin resistance in those subjects (15). Asprosin can also play a potential therapeutic target for obesity management because uncontrolled adiposity is a culprit for the dysfunction of adipokines and may lead to obesity-related disorders (1, 5, 9). Zhang et al. hypothesized that asprosin may have a link to the pathogenesis of lipid disorders because insulin resistance can lead to dyslipidemia (5).

Asprosin, when injected intravenously, crosses the BBB to activate the hypothalamic feeding circuit and this stimulates appetite and maintains adiposity

in long-term applications (9). Researchers have also explored its potential implications in polycystic ovarian syndrome (PCOS), a metabolic disorder with insulin resistance (13, 15). Li et al. found the correlations of glucose, insulin, and lipid metabolism profiles in the PCOS group with the plasma asprosin level (13). Lee et al. also found that therapeutic application of rAsprosin induced inflammation and insulin resistance in skeletal muscle (7).

Romere et al. derived an interesting conclusion that asprosin treatment has no effect on the plasma levels of catabolic hormones (glucagon, catecholamines, and glucocorticoids) which are responsible for the induction of the hepatic glucose release (3). Donma et al. also reported that mice with insulin resistance and humans with pre-diabetes have elevated levels of asprosin. Hence, the administration of an asprosin-blocking medication may be a useful therapy to treat obesity and diabetes as it may normalize glucose and insulin levels and reverse insulin resistance (2). Acara et al. studied the relation of asprosin in unstable angina pectoris (UAP) patients by measuring its levels in the blood serum of the patients and the control subjects with the microELISA method. They recommended asprosin as a relevant biomarker for UAP diagnosis and treatment (17).

Asprosin mechanism of action in diabetes mellitus

Under hyperlipidemic conditions, β -cells can be a source of asprosin, and asprosin induces β -cell inflammation, dysfunction, and apoptosis through TLR4/JNK-mediated signalling. Asprosin promotes inflammation through TLR4/JNK-mediated pathway (Toll-like receptor4/cJUN N-terminal kinase) in β -cells. JNK is a serine/threonine kinase that falls under the mitogen-activated protein kinase family (6). This novel adipokine has a diabetogenic role in the body as it stimulates hepatic gluconeogenesis by elevating cellular cAMP levels, and raises blood glucose levels, which ultimately helps to develop diabetes (7) (Table I).

Duerrschmid et al. observed that mammals respond to fasting by activating the cascade of interconnected and hormone-coordinated processes. Such coordinated processes during early fasting

conditions are stimulation of appetite and subsequent release of hepatic glucose into the circulation, and these processes together provoke the mammals to take or search food to derive the nutrition for the functioning of the brain (9). Lee et al. also observed

that asprosin contributes to palmitate-mediated inflammation, cellular dysfunction, and apoptosis in β -cells and human islets of Langerhans (7). Duerrschmid et al. reported that asprosin acutely inhibits ~85% of the proopiomelanocortin (POMC⁺)

Table I. Comparative review of asprosin and DM.

References	Level of evidence ^a	Grade of evidence ^a	Findings
Zhang et al. (2017) (5)	Ila	B	Attenuating asprosin activity or depleting asprosin may serve as a novel therapeutic innovation for the treatment of T2DM and obesity.
Wang et al. (2018) (4)	Ila	B	Circulating asprosin might be a predictor of early diagnosis of DM and serve as a potential therapeutic intervention for prediabetes and T2DM.
Lee et al. (2019) (7)	Ib	A	Asprosin plays a significant role in preserving pancreatic β -cells and act as the target for the treatment of obesity-associated insulin resistance and diabetes.
Donma et al. (2018) (2)	Ia	A	Mice with insulin resistance and humans with prediabetes have elevated levels of asprosin. Administration of an asprosin-blocking medication may normalize glucose and insulin levels and reverse insulin resistance.
Romere et al. (2016)(3)	Ila	B	Circulating asprosin maintains glucose homeostasis in the body at the time of low dietary glucose by releasing liver glucose stores. Glucose has a negative influence on plasma asprosin levels in a negative feedback manner. Hence, asprosin may be a therapeutic target in T2DM.
Beutler et al. (2018)(8)	IV	C	Circulating asprosin levels increase with overnight fasting in mice, rats, humans, but rapidly decline after food intake, suggesting the role of asprosin as a signal for hunger.
Duerrschmid et al. (2017) (9)	Ib	A	Circulating asprosin rises with fasting and acutely declines with refeeding in a biocircadian rhythmic pattern.
Wiecek et al. (2018)(11)	III	B	Asprosin concentration is higher in patients with T2DM and impaired glucose regulation compared to healthy subjects.
Alan et al. (2018)(15)	IIb	B	Asprosin level is raised in T2DM patients compared to their matched controls and its levels are positively correlated with insulin resistance in those subjects.

^aWHO. General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine. World Health Organization 2000: 1-74. [Grade A: Evidence levels quality Ia, Ib; Grade B: Evidence levels Ila, IIb, III; Grade C: Evidence level IV].

neurons (anorexigenic neurons within the arcuate nucleus of the hypothalamus, ARH) that perform in a coordinated way with the AgRP⁺ neurons (9).

Asprosin future prospects in diabetes therapy

Diabetes care is continually evolving as innovative research, technology, and therapeutic approaches, such as checking serum testosterone in men with diabetes and signs and symptoms of hypogonadism, are being reported (18). Innovative therapeutic trials regarding diabetes control and management are still continually being undertaken. Holden et al. reported that patients with T2DM on simultaneous therapy with insulin plus metformin had a reduced risk of death and major adverse cardiac events (MACE) compared with those treated with insulin monotherapy (19). Glucose control remains the all-time major desired therapeutic endpoint in the management of T2DM and reduction of hyperglycemia decreases the progression of microvascular complications (20, 21), and asprosin may open a new venture in diabetes management.

Davies et al. also highlighted that diabetes self-management education and support (DSMES) program be applied to promote adherence, to adopt a healthy lifestyle, and minimize therapeutic inertia by emphasizing the coordinated chronic care model (20). Recent advancements in diabetes therapy showed promising results on cardiovascular outcomes with glucagon-like peptide 1 (GLP-1) analogues liraglutide and semaglutide (16, 22) and empagliflozin (23).

Alan et al. explored that asprosin research was mainly focused on exploring its potential application in metabolic disorders related to insulin resistance (15). Lee et al. suggested that asprosin may have therapeutic implications as a target for the treatment of obesity-associated insulin resistance and diabetes, as it plays a significant role in preserving pancreatic β -cells (7). Duerrschmid et al. described chronic asprosin loss-of-function using a pharmacological entity (9). Romere et al. developed an asprosin-specific monoclonal antibody and validated its specificity for asprosin using FBN1 wild-type and null cells (3).

Zhang et al. confirmed that information about the

potential role of asprosin in T2DM is still lacking. That study also concluded that asprosin concentrations are increased in T2DM adults, suggesting it as one of the risk factors in the pathogenesis of T2DM, and attenuating asprosin activity or depleting asprosin may serve as a novel therapeutic innovation for the treatment of T2DM and obesity (5). More extensive prospective or randomized controlled trials based on these findings may be conducted in the future to establish the conclusive causal association between asprosin and T2DM, obesity.

CONCLUSION

Asprosin is a fasting-induced hormone secreted from adipose tissue that enhances glucose production in the liver and stimulates appetite regulation in the hypothalamus by initiating the cAMP signaling pathway. Asprosin concentrations are pathologically expanded in T2DM, insulin resistance and obesity patients, and a decrease of its bio-level may improve these metabolic disorders. Asprosin is a predictor for early determination of DM and may be a potential therapeutic target for prediabetes and T2DM.

Lessening asprosin action or draining asprosin may serve as a novel therapeutic innovation for the treatment of T2DM and obesity. Subsequently, a newly found protein hormone asprosin may serve as a target for future therapeutic options in the management and treatment of diabetes.

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EDITORIAL

**MAST CELLS CONTRIBUTE TO CORONAVIRUS-INDUCED INFLAMMATION:
NEW ANTI-INFLAMMATORY STRATEGY**S.K. KRITAS¹, G. RONCONI², AL. CARAFFA³, C.E. GALLENGA⁴, R. ROSS⁵ and P. CONTI⁶

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Coronavirus, which can cause respiratory syndrome, to date has affected over seventeen thousand individuals, especially in China. Coronavirus is interspecies and can also be transmitted from man to man, with an incubation ranging from 1 to 14 days. Human coronavirus infections can induce not only mild to severe respiratory diseases, but also inflammation, high fever, cough, acute respiratory tract infection and dysfunction of internal organs that may lead to death. Coronavirus infection (regardless of the various types of corona virus) is primarily attacked by immune cells including mast cells (MCs), which are located in the submucosa of the respiratory tract and in the nasal cavity and represent a barrier of protection against microorganisms. The virus activates MCs which release early inflammatory chemical compounds including histamine and protease; while late activation provokes the generation of pro-inflammatory IL-1 family members including IL-1 and IL-33. Here, we propose for the first time that inflammation by coronavirus may be inhibited by anti-inflammatory cytokines belonging to the IL-1 family members.

Viral infections are certainly increasing globally; they can cause an epidemic spread, but also a pandemic one in different countries, as was the case with the avian influenza virus H5N1 (mortality 35%), as well as severe acute respiratory syndrome (SARS) (mortality 10%) (1). Middle East respiratory syndrome caused by coronavirus first appeared in 2012, and since then over seventeen thousand patients have been infected worldwide (especially in China) of whom about 900 have died (a number destined to increase), according to the World Health Organization (WHO) (2). There are several types of coronavirus,

they can be transformed and become interspecies and have an incubation ranging approximately from 1 to 14 days (1). They are of unknown origin, although may originate from various animals such as snakes, bats, pigs, or dromedary camels, which are the main host, and in these animals can cause acute diarrhea, gastroenteritis, villous atrophy, poor absorption and death (2). Human-to-human virus transmission also occurs in healthcare facilities and communities. Coronaviruses often have highly pathogenic activity in animals, but today it has been seen that, with their particular pathogenic proteins, they can cause

Key words: coronavirus; immunity; infection; inflammation; mast cells

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more or less serious respiratory diseases both in adults and in children. Moreover, it has now come to the fore that coronavirus is one of the major respiratory pathogens causing serious inflammatory outbreaks of acute pneumonia in individuals (3). Therefore, human coronavirus infections can induce not only mild to severe diseases, but also systemic inflammation, high fever, cough, acute respiratory tract infection and dysfunction of internal organs that can lead to death. Coronavirus is classified as a Ribonucleic Acid (RNA) virus, with a genome that can often escape the innate immune system, especially if it is malfunctioning (4). Regardless of the various types of coronavirus, the entry of the virus into the organism activates innate immunity, which intervenes in the first instance to engulf the

invader. The severity of the disease lies in the ability of innate immune cells to stem viral infection (5). The stronger the innate immune system, the less the ability of the virus to replicate itself, suppress the immunity and, therefore, to induce the pathological state. In the case of innate immune suppression by the virus, adaptive immunity is also inhibited. The coronavirus is capable of producing viral enzymes and proteases that damage the immunity and inhibit the signaling pathways of type I interferon (IFN), along with the nuclear factor- κ B, facilitating innate immune evasion (6).

In light of all the above, new anti-coronavirus strategies are needed to fight the disease, in order to avoid infection. Laboratory experiments on rodents show that inoculating various different types of coronavirus, all induce an innate and adaptive immune response, but also an acute encephalomyelitis with invasion of the virus throughout the brain (7). Therefore, the virus mainly settles in the white matter, causing chronic neuroinflammation and demyelination. The disease causes the activation of T and B cells which are attracted to the place of infection by specific chemokines (8). Coronavirus infection is primarily attacked by immune cells, but the virus has developed viral proteins over time that counteract the innate immune system (9). Some viral proteins antagonize interferon (IFN) and stimulate inflammatory proteins, including IL-1 family member cytokines (10). The abnormal production of cytokines aggravates the inflammatory state and pathogenesis of the disease, which also takes place in SARS. There are several types of coronaviruses with variable morphogenesis of the envelope and production of different proteins (11). The protein E of the envelope is very small but capable of influencing the replication of the viral genome, accentuating the pathogenicity, and may cause strong inflammation and even death of the patient. In addition, protein E activates the inflammasome in the tissues and causes increased edema, with lethal results for humans. Immune Treg cells expressing IL-2 receptor, CD4 receptor and FoxP3 transcription factor, are cells that regulate immune status tolerance in humans and are generated in the thymus (12). Corona virus-

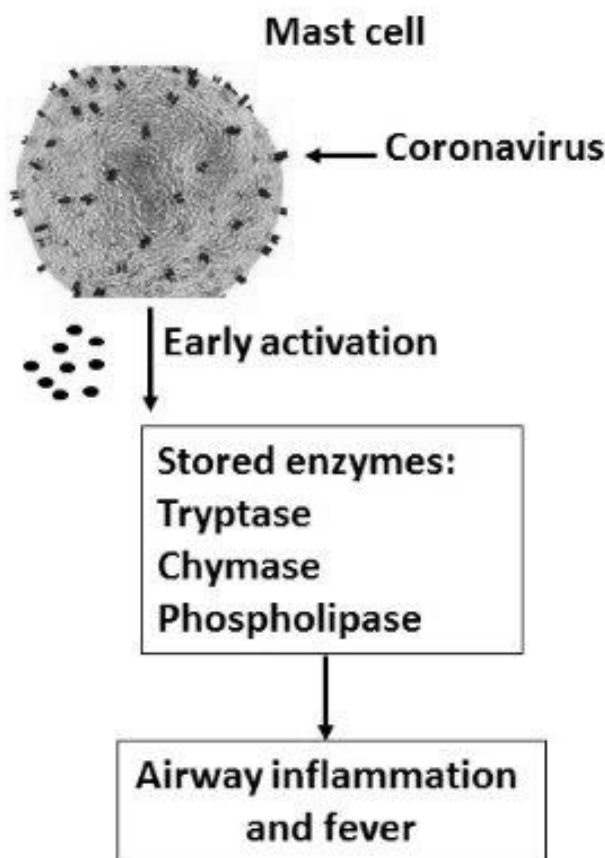


Fig. 1. Coronavirus activating mast cell which early releases proteases and other compounds, and late release IL-1 family members, which both contribute to airway inflammation and fever.

induced type 1 Treg cells produce anti-inflammatory IL-10, protecting the body from the most aggressive forms of infection. The development of therapeutic anti-viral agents based on strengthening the immune system has been one of the main objectives of modern medicine. The immune response to the virus can be enhanced with the administration of antigens, adjuvants and vaccines. The vaccines that normally protect us from bacterial and viral infections, in the case of coronavirus, are not currently available and the experimental ones have not proved to be effective, although some vaccines have exerted a slight protective effect (13). We believe that coronavirus infection, as well as other pathogenic microorganism infections, can be combatted by preventing contagion and boosting the immune system.

Mast cells

Mast cells (MCs) are immune cells derived from

hematopoietic precursor cells (bone marrow CD34⁺), which mature and reside in virtually all vascularized tissue (14). MCs are located perivascularly in proximity to neurons, and their differentiation and proliferation are regulated by the stem cell factor (SCF), which binds the surface kit-receptor. After activation, MCs produce inflammatory mediators, including proteases and pro-inflammatory cytokines, participating in host response, for example, in skin diseases, and asthma (14). MCs are involved in the innate and adaptive immune systems, playing a role in autoimmunity, infections, tissue damage, and inflammatory signals (15). MCs express TLR which can be quickly recognized by pathogens, including viruses that activate them in the airways and respiratory tract, causing serious damage. When properly activated, MCs generate biologically active substances including chemokines and cytokines without degranulation. MCs are responsible for

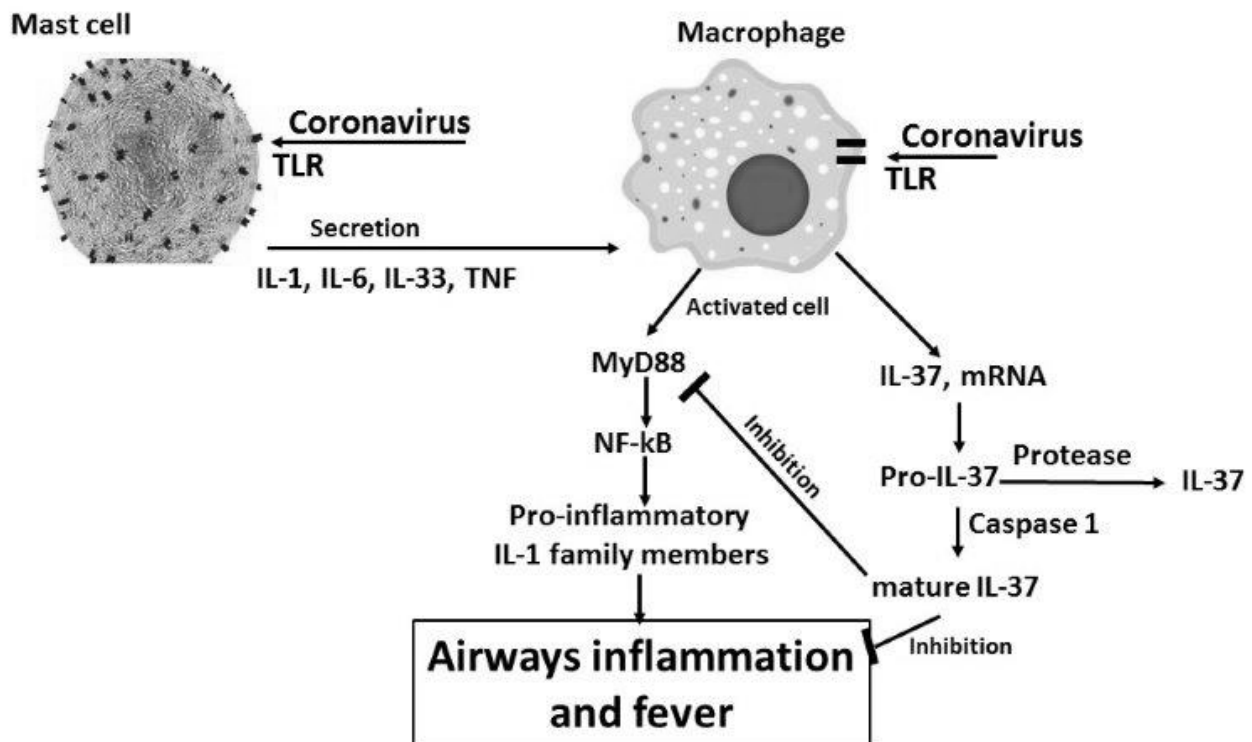


Fig. 2. Coronavirus binding Toll-like receptor; activating mast cell which secretes IL-1 family members (IL-1, IL-33), IL-6 and TNF. These cytokines along with coronavirus activate macrophages. This stimulation provokes dual effects: one being airway inflammation and fever, and the other production of IL-37 which inhibits airway inflammation and fever.

allergic reactions, but also participate in inflammation and defend the body against bacterial helminthic and viral infections. MCs can be classically activated by IgE and specific antigen, but also by bacteria and viruses. IgE stimulates MCs via FcεRI high-affinity receptor ($1 \times 10^{10} \text{M}^{-1}$), which bind to the FcεRI and cause receptor aggregation and a series of biochemical events (14). Activated MCs immediately release chemical mediators and/or late production of pro-inflammatory proteins such as cytokines and chemokines (16). Therefore, virus-activated MCs may provoke the release, after seconds, of stored chemical mediators such as histamine, tryptases and chymase. In addition, after hours of incubation, activated MCs secrete synthesized inflammatory cytokines including IL-6, IL-1, IL-31, IL-33 and TNF, and chemokines CC5, CCL2, MCP-1 and CXCL8 which attract white blood cells to the inflammatory sites (16). All these compounds have a major role in inflammation. MCs produce, store and synthesize highly inflammatory TNF, both intra-cellular and extra-cellular. MC innate immune cells are real sentinels of the human body, ready to immediately attack the external invaders which can be harmful to cells, tissues and organs. MCs release IL-33, a member of the IL-1 family of cytokines, which is considered a cytokine called “alarmin”. MCs are peripherally located near dendritic cells, in communication with sensory nerves that cross-talk with brain cells, including microglia. MCs protect the body from infections caused by bacterium and viruses (17). These cells, communicating with the external environment, neutralize various pathogens, but in doing so they also produce pro-inflammatory compounds which can be harmful for the body. Stressful and chronic conditions predispose humans to pathogenic infections including those caused by coronavirus. In fact, neurotransmitters, including substance P, that are generated in stress, can activate immune cells such as MCs to produce pro-inflammatory cytokines and chemical mediators of inflammation (18).

Experiments with coronavirus in mice have shown that this pathogenic RNA microorganism can activate the cells of both innate and adaptive immune systems, including MCs. These cells are located in

the submucosa of the respiratory tract and in the nasal cavity and represent the protective cells that are at the forefront in fighting viral and bacterial agents (19). They are involved in many biological processes where they induce the secretion of a broad spectrum of cytokines and chemokines. In addition, viruses activating MCs cause the release of some specific chemokines, such as the ligand 5 (CCL5), which attracts the CD8 T immune cells that defend the lung tissue and fight viral infection (20). Epithelial cells represent a physical barrier to the entry of microorganisms including viruses. The cross-talk between viruses and epithelial cells causes the production of immune mediators in order to defend the body. Viruses develop particular mechanisms to invade the body and immune cells including MCs. The host response to RNA virus invasion activates TLR3 on MCs with an anti-viral IFN production (positive effect) (21); but often the virus only causes sensitization of MCs with the synthesis of IgE that bind to the FcεRI receptor and trigger a violent inflammatory reaction (negative effect). Therefore, the function of MCs towards viral infections is not yet clear. However, RNA viruses stimulate MCs to produce IFN gamma e CXCL8 chemokines, resulting in the recruitment of NK cells that also produce type I interferons (IFNs) which are anti-viral cytokines. In addition, type I IFNs enhance the cytotoxic activity of NK cells against virus-infected cells (22). So, in this case, MCs are anti-viral immune cells through the above mechanism. On the other hand, viruses stimulate mucosa MCs to release pro-inflammatory cytokines such as TNF, IL-1, IL-6 and proteases, which aggravate the inflammatory state.

Virus-activated MCs produce histamine, prostaglandin D2 (PGD_2), and leukotriene C4 (LTC_4) which induces acute bronchoconstriction and lung inflammation. Therefore, we can conclude that viral infections can activate MCs which respond with dual effects, a positive one helping the immune system to fight infection, and a negative one causing the release of chemical mediators of inflammation and the secretion of pro-inflammatory cytokines such as IL-1, IL-33, IL-18, and TNF, aggravating the pathological state of the patient.

IL-37 is a member of the IL-1 family of cytokines,

which is activated by caspase-1 and suppresses transcription of pro-inflammatory genes and immune responses. Since IL-37 is an inhibitor of IL-1, a potent pro-inflammatory cytokine (23), we believe that this anti-inflammatory cytokine may suppress fever and inflammation provoked by coronavirus, so as to reduce the number of deaths.

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ANTI-PSORIASIS ACTIVITY OF SHIKONIN, A PLANT CELL NATURAL COLORANT NAPHTHOQUINONE, VIA THE JAGGED1/NOTCH1 PATHWAY

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Shikonin, a naphthoquinone compound isolated from *Lithospermum erythrorhizon* Siebold & Zucc. (Pinyin: zPiny), has been reported to exert protective roles against psoriasis. However, the molecular mechanism remains largely unknown. The current study aimed to explore the mechanism of Shikonin in keratinocyte differentiation and apoptosis. It was found that overexpression of Jagged-1, Notch-1, Hes-5, but not Hes-1, on psoriatic lesions is involved in the pathogenesis of psoriasis. *In vitro*, the application of exogenous recombinant human Jagged-1 (rh-Jag1, ligand of Notch-1) promotes proliferation and suppresses apoptosis of keratinocytes from psoriasis, which could be blocked by DAPT (γ -secretase inhibitor). Additionally, in recombinant human Epidermal Growth Factor (rh-EGF)-stimulated HaCaT cells (to mimic hyperproliferation condition of psoriasis), Shikonin was effective on anti-proliferation and pro-apoptosis of keratinocytes with down-regulation of Jagged-1, Notch-1, Hes-5, Bcl-2, and up-regulation of caspase-9, inhibitor of caspase-activated deoxyribonucleases (ICAD). Taken together, we propose that Jagged1/Notch1/Hes5 signaling involved in the pathogenesis of psoriasis could be suppressed by Shikonin.

Psoriasis is a common, chronic, relapsing, immune-mediated systemic disease, which affects approximately 2-4% of the general population (1) and causes a heavy psychosocial burden (2). It is known that both genetic predisposition and external stimuli, including infection, stress, injury, and drugs, contribute to the development of psoriasis (3). Currently, there is no cure, however, the symptoms can be alleviated by various medications. In clinical practice of Traditional Chinese Medicine (TCM), dried-red-root of *Lithospermum erythrorhizon*, is widely used for the treatment of skin diseases,

especially for ecchymosis and erythema induratum. Intriguingly, such skin lesions are similar to psoriasis vulgaris (PV), usually owing to the outburst of acute inflammation. Recently, the efficacy of zǐcǎo and its extract (Shikonin) in the treatment of psoriasis has been evaluated and the clinical application of Shikonin had been approved (4). Shikonin has been reported to improve the severity index (PASI) scores and prolonged remission duration (5).

Shikonin is a promising naphthoquinone compound (6), and its anti-inflammatory effect has been confirmed in many studies of various diseases.

Key words: psoriasis; Jagged1/Notch1/Hes5 signaling; Shikonin; proliferation; apoptosis

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Shikonin also functions to enhance wound healing by inhibiting NF- κ B signaling (7). In psoriasis, Shikonin has been shown to suppress IL-17-induced angiogenesis via blockage of JAK2/STAT3 signaling (8). Furthermore, Shikonin was capable of modulating cell proliferation and apoptosis of immune cells (10). In the current study, we aimed to elucidate the function of Shikonin on keratinocytes of psoriasis.

The Notch gene was first identified in 1919 by genetic pioneer Thomas in the fruit fly *Drosophila melanogaster*. Notch signaling plays an important role in cell differentiation, tissue homeostasis, and individual developments (11). The rapid turnover of skin keratinocytes, from stem cells to desquamation, as well as the balance between cell renewal and loss, is regulated by Notch signaling. Dysregulation of notch signaling results in uncontrolled differentiation of keratinocytes, and the onset of various skin diseases, including skin cancer (12), psoriasis (13), and atopic dermatitis (14). In the pathogenesis of psoriasis, up-regulation of Notch-1 has been reported (15), while the impact on its target Hes gene expression is still unknown.

In mammals there are four Notch receptors (Notch 1~4, transmembrane proteins) and five ligands (Delta-like 1, 3, and 4, and Jagged 1 and 2). The activation of Notch signaling requires the crosstalk of Notch ligands and receptors among juxtaposed cells, generation of the Notch intracellular domain (NICD) via proteolytical cleavage of Notch receptors, as well as the translocation of NICD into the nucleus which form a complex with transcription factor recombinant binding protein suppressor of hairless (RBP-J). Activated Notch signaling further regulates target gene expression, including Hes-like genes, members of basic helix-loop-helix transcription factors which play essential roles in development decision (16). In this process, DAPT (γ -secretase inhibitor) was effective in blocking Notch signaling by inhibiting active NICD release. It is known that blockade of Notch-1 signaling usually interferes with Th17-mediated disease, such as psoriasis (17).

In this study, we explore the molecular mechanism of the protective effects of Shikonin on psoriatic using keratinocytes from psoriatic lesions (KCps)

and to find out whether Shikonin functions through modulation of the Jagged1/Notch1/Hes5 signaling pathway.

MATERIALS AND METHODS

Biopsy samples collection

From February 2017 to May 2018, at the Department of Dermatology of Zhejiang Hospital of Traditional Chinese Medicine, patients diagnosed with PV clinically (n=18, 7 females, 11 males, age range 25-59) with no anti-psoriasis therapy 1 month prior to skin biopsy, were enrolled in the study. All patients signed informed written consent. The healthy controls (gender and age-matched, n=12) were obtained from the cosmetic surgery department of the same hospital. This study was performed under the guidelines of the institutional review committee of Zhejiang Chinese Medical University, which is in agreement with the Declaration of Helsinki. Full thickness skin biopsies from two groups were divided into two parts, one part was immersed in 10% neutral-buffered formalin for immunohistochemistry, and the other part was immediately collected in sterile Eppendorf tubes with 0.5% dispase (Invitrogen, Carlsbad, CA, USA) for later use.

Tissue isolation and cell culture of epidermal keratinocytes

The skin tissues were incubated overnight at 4°C in 0.5% dispase, then epidermis was peeled off from the dermis and incubated in 0.25% trypsin (Invitrogen) for 10 min at 37°C and terminated by the addition of fetal bovine serum (18), then dispersed into a single-cell suspension by filtering through 200 mesh cell strainer, and centrifuged at 1,200 rpm for 5 min. After discarding the supernatant, keratinocytes were cultured in SFM (Gibco BRL, Grand Island, USA) supplemented with rh-EGF (10 ng/mL, no.100-15, PeproTech, RM, USA). HaCaT cells (human immortal keratinocytes) were obtained from the Chinese Academy of Sciences Kunming cell bank, and cultured with DMEM (Gibco BRL) containing 10% fetal bovine serum (Life Technologies, UK), penicillin (100 U/mL)/streptomycin (100 mg/mL) and rh-EGF (10 ng/mL). Both KCps and HaCaT cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Immunohistochemistry

Immunohistochemistry (IHC) staining of Notch

signaling related-proteins were performed on tissue sections from formalin-fixed, paraffin-embedded tissues, as described previously (19). In brief, the paraffin blocks were sliced into 4- μ m thick sections, deparaffinized with xylene and rehydrated with decreasing concentrations of ethanol. Antigen retrieval was performed by boiling the slides in citrate buffer (pH 6.0) for 20 min, followed by cooling at room temperature with PBS. Endogenous peroxidase activity was quenched by hydrogen peroxidase treatment (15 min at room temperature). After blocking with goat serum for 2 h, slides were incubated overnight at 4°C with primary Rabbit poly-clonal antibodies against Jagged-1 (no. sc-390177), Notch-1 (no. sc-376403), Hes-1 (no. sc-166410) (Santa Cruz, CA, USA), Hes-5 (no. ab-236142, Abcam, Cambridge, UK), diluted by Lab Vision Antibody Diluent (1:100; Santa Cruz). After washing, the slides were incubated with Goat anti-Rabbit IgG (HRP) secondary antibody (Zhongshan, Beijing, China) for 1 h at room temperature. Then slides were detected by an HRP labeled Streptomyces protein and color development was achieved by applying diaminobenzidine (DAB) substrate kit (Weiao, Shanghai, China). The slides were then counterstained by hematoxylin, dehydrated through ethanol, transparented through xylene and cover-slipped.

On the basis of the intensity of immunostaining located in the cell membrane, cytoplasm or nucleus, the samples were scored as: strong positive (+++) (dark brown), moderate positive (++) (brown), weak positive (+) (light yellow), and negative (-) (non-staining). Image-Pro analysis software (Meyer Instruments, USA) was used for the quantitative analysis of the luminous intensity of the positive spot. Under a microscope ($\times 400$), 5 random fields of each section were chosen to calculate the photo permeability intensity on positive spot, and the blank areas on the same slide were used for background correction. The result was expressed as positive spot optical density (OD).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted with TRI-Reagent (Sigma-Aldrich, Darmstadt, Germany) according to the manufacturer's instructions. The cDNA synthesis was performed with 100 ng total RNA by the Prime Script RT reagent kit (Takara, Dalian, China) according to the manufacturer's protocols. Biorad Evagreen kit (Sigma-Aldrich) was used for PCR reaction (Applied Biosystems,

Thermo Fisher Scientific). Actin was regarded as the endogenous control. The relative expression level of above genes was calculated by $2^{-\Delta\Delta Ct}$ (20). The reaction condition for qPCR were as follows: 10 min at 95°C followed by 45 cycles (95°C for 15s, 59°C for 15s, 7°C for 20s). All primers were synthesized by Sangon Biological Engineering Technology (Shanghai, China) as follows: Jagged-1, forward, 5'-AGT GCC TGA ATG GAC GGA-3', and reverse, 5'-ACT CGC TGT GCC CTT TGT-3'; Notch-1, forward, 5'-TGC CAG CAG CCC CTG CGA CT-3', and reverse, 5'-TCG CAG CTG CCC CCG TTG T-3'; Hes-1, forward, 5'-TGG AAA TGA CAG TGA AGC ACC T-3', and reverse, 5'-GTT CAT GCA CTC GCT GAA GC-3'; Hes-5, forward, 5'-TGG AGA AGG CCG ACA TCC T-3', reverse, 5'-GGC GAC GAA GGC TTT GC-3'; and Actin, forward, 5'-TAA GTA GGC GCA CAG TAG GTC TGA-3', and reverse, 5'-AAA GTG CAA AGA ACA CGG CTA AG-3'.

Western-blot

Epidermal keratinocytes were harvested and collected in RIPA lysis buffer (Thermo Scientific, USA) with protease inhibitor. The protein concentrations of each sample were quantified by BCA assay (Sigma-Aldrich) according to the manufacturer's instructions. Protein samples (20-50 μ g) were separated by SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (EMD Millipore, MA, USA) (21). The membranes were blocked at room temperature for 1 h and washed with TBST, then incubated overnight at 4°C with primary antibodies: Jagged-1 (dilution, 1:200), Notch-1 (dilution, 1:250), Hes-1 (dilution, 1:100), Hes-5 (dilution, 1:150), PCNA (no. ab19166; dilution, 1:1000; Abcam), caspase-9 (no. ab32068; dilution, 1:500), ICAD (no. ab108924; dilution, 1:400), Bcl-2 (no. ab196495; dilution, 1:1000) (abcam), GAPDH (no. sc-20358, dilution, 1:1,000; Santa Cruz) on an orbital shaker. After washing with TBST, the membranes were incubated with 1:2000 diluted secondary antibodies for 2 h at room temperature. Western blots were visualized with ECL detection kit (Pierce, Beijing, China), scanned and quantified with Image Lab software (Bio-Rad, USA).

MTT assay

Keratinocytes were seeded in growth medium in 96-well plates for 24 h, and treated with corresponding different concentration of rh-Jag1 (0, 1, 5, 10, 25, 50, 100

ng/ml) (no. 044970, USBiological, Massachusetts, USA), DAPT (5 μ M) (CAS:208255-80-5, Sigma-Aldrich) and Shikonin (0, 2, 5, 10, and 15 μ M) (CAS:517-89-5, Sigma-Aldrich) for 24 h, after which 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenylterazolium bromide (MTT) was added to each well and incubated at 37°C for 4 h. The OD of each well was measured by an enzyme-linked immunosorbent assay detector (BioTek, Shanghai, China) at 570 nm. The value of the absorbance in each group was normalized to dimethylsulfoxide (DMSO) control group and the dose-effect curve of rh-Jag1 and Shikonin was generated.

Flow cytometry

Apoptosis of cells was detected by Annexin V/PI Apoptosis Detection Kit (Logji Bio, Beijin, China) according to the manufacturer's protocol. In brief, keratinocytes were plated in 6-well plates to reached a level of 70-80% confluence. After treatment for 48 h, cells were trypsin-released and Annexin-V/PI stained at room temperature for 20 min (22). Apoptotic cells were

measured by Accuri C6 flow cytometer (BD Bioscience, San Jose, CA, USA) and quantitated.

Statistical analysis

SPSS 20.0 software (IBM Corp., USA) was used for statistical analysis. Data were expressed as mean $\pm$ SD. Statistical differences between the groups were evaluated by one-way analysis of variance (ANOVA). Experiments were repeated at least three times, and P value less than 0.05 was considered statistically significant.

RESULTS

Expression levels of Jagged-1, Notch-1, Hes-5, and Hes-1 in psoriatic lesions

PV features hyperproliferative superabasal layers and a thick cornified layer. As shown in HIC (Fig. 1), Jagged-1, Notch-1, Hes-5, and Hes-1 expressions were detected in the nucleus and cytoplasm of epithelial cells reflected by different color granules.

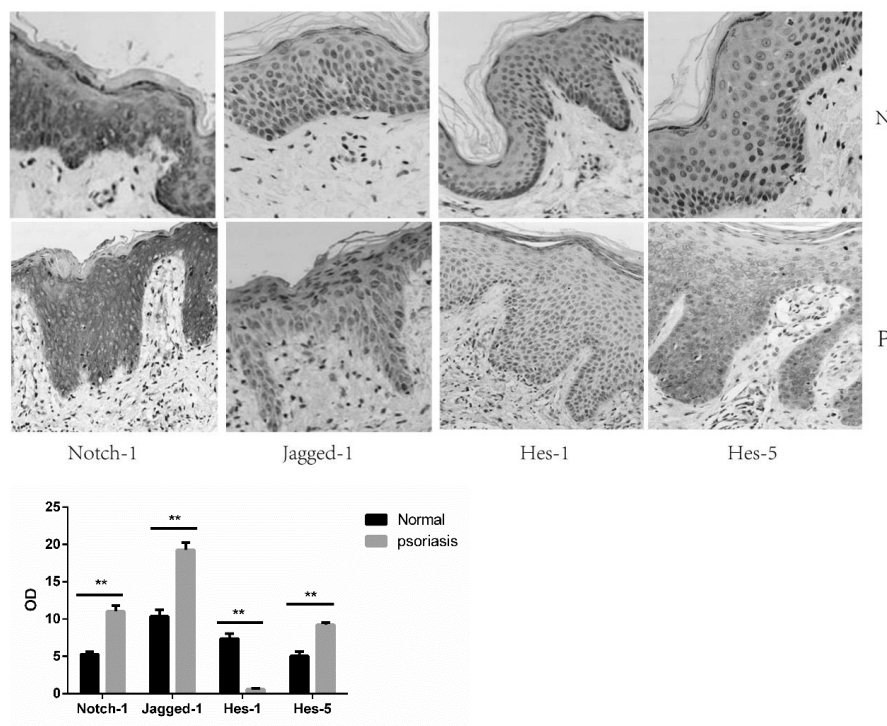


Fig. 1. A comparison of the expression levels of Notch1 signaling proteins on the normal skin of healthy and the psoriasis lesions. In IHC by DAB visualization showed that Jagged-1, Notch-1, and Hes-5 significantly increased in the pan epidermis, excluding Hes-1, which was almost negative in psoriatic lesions. N: normal healthy skin; P: psoriatic lesions. ** $P < 0.01$, the data are expressed as the mean $\pm$ SD.

In psoriasis, Notch-1, Jagged-1, Hes-5 were highly expressed in pan epidermal layer, and to a lesser extent in the suprabasal layers, where differentiation occurred, while the expression of Hes-1 was faint and even absent in some samples. In normal skin, Jagged-1, Notch-1, Hes-1 and Hes-5 protein were all expressed mainly in basal layers at different degrees, while Notch-1 was mainly expressed in basal and spinous layer (Table I).

Expression levels of Jagged-1, Notch-1, Hes-5, and Hes-1 in KCps

The mRNA expression levels of Jagged-1, Notch-1, Hes-1 and Hes-5 in KCps were detected by RT-qPCR. In comparison with normal keratinocytes, KCps showed elevated gene expressions of Jagged-1, Notch-1 and Hes-5 in KCps to a certain degree, while Hes-1 was reduced (Fig. 2A). The corresponding protein expressions of Jagged-1, Notch-1, Hes-1 and Hes-5 in KCps were further evaluated by Western-blot assay. Consistent with the gene expression, Jagged-1, Notch-1 and Hes-5 were increased, while Hes-1 was reduced in KCps compared to keratinocytes of normal skin (Fig. 2B). The mRNA and protein expression levels of Jagged1/Notch1/Hes5 signaling in psoriasis patients were increased compared to the normal control, which may result in hyperproliferation or immaturity of keratinocytes in psoriasis. Except that, Notch-1 signaling was involved in the pathogenesis of PV. One study found that Notch-1 was overexpressed in psoriatic CD34+ bone marrow cells which may mediate T cell disorders in epiderm of PV. Another study indicated that Notch-1 caused endothelial cell activation and invasion in skin lesions of psoriasis. Taken together, it is suggested that activation of Notch1

signaling may be associated with the development of psoriasis.

Promoting effects of rh-Jag1 on KCps were partially reversed by DAPT

To explore the activity of rh-Jag1 in the proliferation of KCps, MTT assay was performed, and the results showed that rh-Jag1 promoted proliferation of KCps in a dose-dependent manner. Compared with DMSO control, the dosage of rh-Jag1 at 50 ng/ml achieved the optimal dosage-effect ($P < 0.001$) (Fig. 3A). However, the pro-proliferation effect of rh-Jag1 on KCps at 50 ng/ml was significantly inhibited by DAPT (Fig. 3B). Since DAPT, a γ -secretase inhibitor, has been identified as Notch pathway inhibitor, initiation of Notch1 signaling caused by rh-Jag1 could be blocked by DAPT.

Additionally, the protein expression of PCNA in KCps was detected due to its “positive index of cell proliferation” role. We found that PCNA was up-regulated by rh-Jag1, while down-regulated by DAPT, including rh-Jag1-induced high expression of PCNA (Fig. 3C).

The result of Annexin-V/PI staining suggests that the apoptosis ratio of KCps in DMSO control was 5.3% and 3.8% in rh-Jag1 group, while in the DAPT group, it was up to 15.6%. Conversely, the apoptosis ratio was down to 9.8% in the combination of DAPT and rh-Jag1 treated group (Fig. 3D).

Shikonin regulates proliferation, apoptosis, and down-regulates Notch1 signaling in EGF-induced HaCaT cells

In the pathogenesis of psoriasis, EGF plays a pivotal role especially involved in the proliferation,

Table I. Immunolocalization of Jagged-1, Notch-1, Hes-1, Hes-5 in healthy and psoriasis epidermis

Proteins	localization of Normal (n=12)	localization of Psoriasis (n=18)
Jagged-1	Pan epidermal† (+)	Pan epidermal‡ (+++)
Notch-1	Basilar(++)	Pan epidermal(+++)
Hes-1	Basilar(+)	-
Hes-5	Pan epidermal† (+)	Pan epidermal (+++)

Note: positive expression of the above proteins in normal skins and psoriatic lesions. ‡ mainly localized in suprabasilar layers; † mainly located at tips of the dermal papillae.

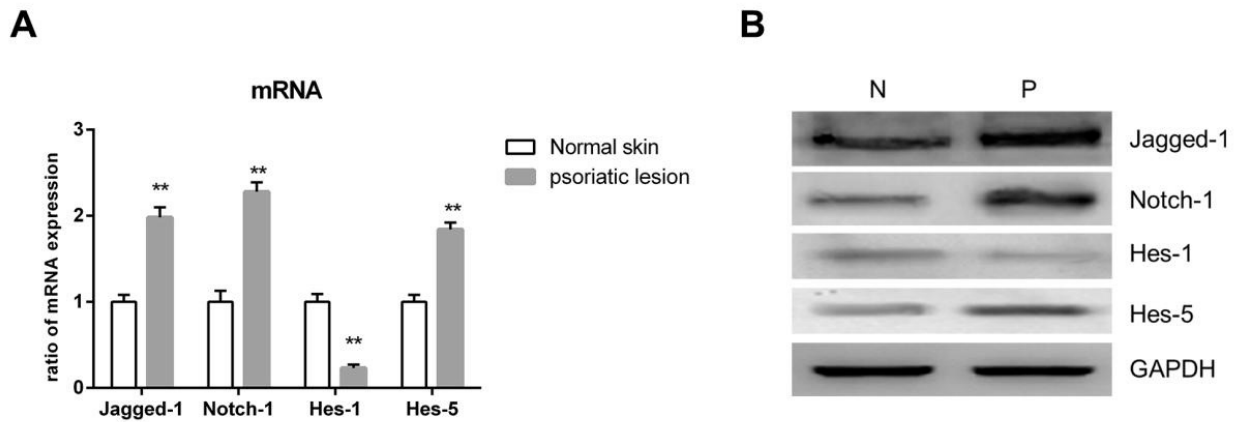


Fig. 2. Both mRNA and protein expression levels of Jagged-1, Notch-1, Hes-5, except for Hes-1, were increased in keratinocytes of psoriatic lesions. ** $P < 0.01$ vs normal control. **A)** Relative mRNA expression levels (normalized to actin) of Jagged-1, Notch-1, Hes-1, Hes-5 by RT-qPCR. **B)** Protein expressions of Jagged-1, Notch-1, Hes-1 and Hes-5 were detected by Western-blot. N: keratinocytes of normal skin; P: keratinocytes of psoriatic lesions.

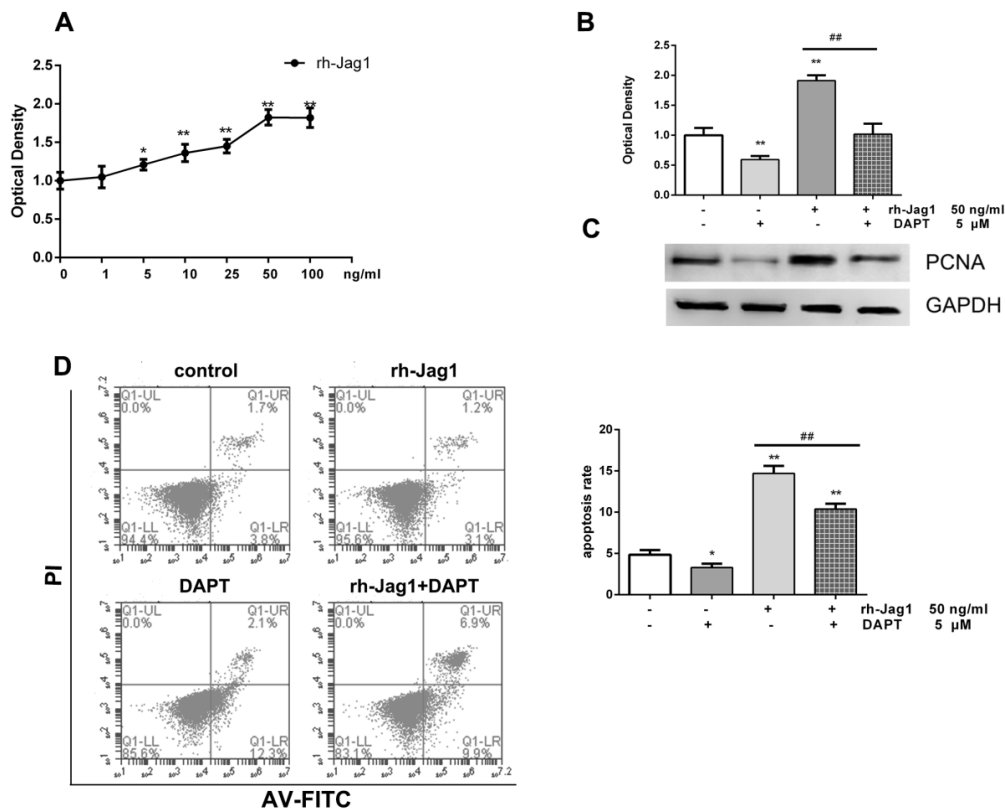


Fig. 3. rh-Jag1 promotes proliferation of KCps in a dose-dependent manner. **A)** rh-Jag1 promotes proliferation of KCps in a dose-dependent manner as determined by MTT assay. **B)** The effect of 5 μ M DAPT and 50ng/ml rh-Jag1 on the proliferation of KCps tested by MTT. **C)** Function of DAPT and rh-Jag1 on the protein expression of PCNA in KCps detected by Western-blot. **D)** Function of rh-Jag1 and DAPT on the apoptosis of KCps tested with Annexin-V and PI double staining by flow cytometry. All data are shown as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ vs DMSO control; ## $P < 0.01$ vs rh-Jag1 group.

angiogenesis and cell differentiation of keratinocytes (23). Here, HaCaT cells were stimulated by 10 ng/mL rh-EGF to mimic hyperproliferation condition of psoriasis (9). MTT method was applied to detect cell proliferation inhibition rate of different concentrations of Shikonin on rh-EGF-induced keratinocytes. The result revealed that Shikonin inhibited cell proliferation at 5-15 μ M dosage, reaching a maximum effect of $91.30 \pm 5.37\%$ at the dosage of 15 μ M when compared with DMSO control (Fig. 4A).

The effect of Shikonin on keratinocyte growth was further examined by flow cytometry which showed that different concentrations of Shikonin induced cell apoptosis rate to 4.2%, 3.9%, 4.7%, 8.6%, and 27.5%, respectively. When compared with the DMSO control, dosage at 10, 15 μ M of Shikonin significantly increased early apoptosis ($p=0.04$, $p=0.001$), while dosage at 2, 5 μ M had no effect (Fig. 4B).

To further understand the mechanism of Shikonin-induced cell growth, proteins of apoptosis-related and

Notch1 signaling were measured using Western blot. The analysis showed that Shikonin reduced expressions of Notch-1, Jagged-1, Hes-5 on keratinocytes in a dose-dependent way. It also regulated expressions of apoptosis-associated proteins, i.e. decreased Bcl-2, and increased Caspase-9/ICAD (Fig. 4C). Additionally, expressions of Notch-1, Jagged-1, Hes-5 were gradually reduced, which also inversely correlated with Bcl-2 and consistent with Caspase-9/ICAD.

DISCUSSION

Psoriasis is a chronic inflammatory disease characterized by abnormal, excessive and rapid growth of the epidermal keratinocytes (premature) of the skin, which is induced by activation of various inflammatory cascades and abnormal immune cell response in the dermis. In cutaneous epithelial cells, the Notch signaling plays a critical role in ensuring skin homeostasis. In addition, Notch signaling also

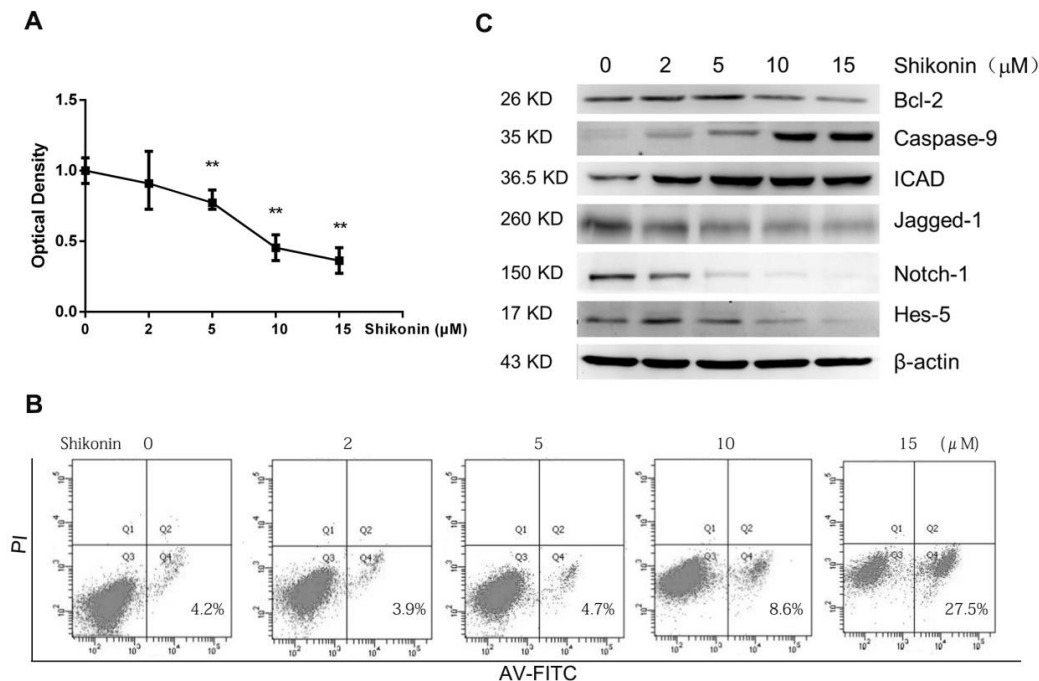


Fig. 4. Shikonin regulates proliferation, apoptosis and Notch1 signaling expression levels of rh-EGF-induced HaCaT cells in a dose dependent way. **A)** MTT method was used to measure proliferation rate of different doses of Shikonin on keratinocytes. ** $p < 0.01$ vs DMSO control. **B)** Apoptosis rate of keratinocytes under different concentrations of Shikonin tested by Annexin-V and PI double staining. **C)** Shikonin on expression of apoptosis-associated proteins and Notch1 signaling proteins in keratinocytes by Western blot.

cross-communicates with other signaling systems, i.e., Wnt signaling, Hedgehog signaling, cellular hypoxic response (HIF1 α and HIF2 α), and the p53 family member, p63 (24), which is associated with chronic inflammation reaction in psoriasis (25).

Recently, several clinical investigations found that some components (not exactly, and controversial) of the Notch pathway were dysregulated in psoriasis when compared with normal skin. The expression levels were returned to normal after receiving biological or phototherapeutic treatment (26). In the present study, we examined the localization and expression levels of Jagged-1, Notch-1 and Notch downstream Hes-like genes (Hes-1, Hes-5) in psoriatic lesions, and verified at cellular level. It was clearly indicated that expressions of Jagged-1, Notch-1, and Hes-5 were increased, while Hes-1 was decreased in both tissues and keratinocytes from psoriasis lesions. Furthermore, keratinocytes of psoriasis showed increased proliferation and reduced apoptosis when treated with rh-Jag1, which could be reversed by DAPT, a kind of Notch signaling inhibitor. These results suggest that Jagged1/Notch1/Hes5 signaling plays a promoting role in the pathogenesis of psoriasis.

Skin undergoes self-renewal throughout the entire lifetime. Keratinocytes, which are constantly producing new cells through constant mitosis, are the predominant type of cells to control skin renewing and repairing activities. Here, rh-Jag1 could be regarded as a stimulant to keratinocytes of psoriasis, and very likely antagonized by Shikonin. *In vivo*, administration of Shikonin could improve PASI in imiquimod-induced psoriasis-like disease mouse model, featured by decreased Th17 cytokines, decreased matrix metalloproteinase (27) and inhibition of dendritic cell activity (28). *In vitro*, HaCaT cell, an immortalized human keratinocyte cell line usually used for psoriasis study upon stimulation by cell growth factors and/or inflammatory cytokines, was used. In earlier studies, Shikonin has been reported to exert protective effects against psoriasis through proliferation inhibition (29), apoptosis-inducing (30), anti-inflammation (31) and modulation of regulatory T cells (32). In this study, Shikonin treatment inhibited “psoriasis-

like” keratinocyte (rh-EGF-induced HaCaT cell) proliferation while stimulating their apoptosis in a dose-dependent way. Further mechanistical study revealed that Shikonin treatment down-regulated Jagged1/Notch1/Hes5 signaling, Bcl-2, and up-regulated Caspase-9/ICAD in rh-EGF-induced HaCaT cells. Our results suggest that high expression and abnormal activation of Jagged1/Notch1/Hes5 signaling would cause aberrant proliferation and apoptosis of the epidermis in psoriasis.

In addition, inhibited apoptosis of keratinocytes are involved in the pathogenesis of psoriasis characterized by scaly patches. Apoptosis is a form of programmed cell death which is highly controlled. Apoptosis can be initiated by various stimuli through one of two pathways: the intrinsic pathway (also called the mitochondrial pathway) and the extrinsic pathway. As reported in earlier studies, apoptosis can be modulated by various mediators, including caspase-9 (initiator caspase), Bcl-2 (negative regulator) and ICAD (Inhibitor of CAD). Intriguingly, recent studies proved that caspases also participate in other cellular processes, i.e. proliferation, differentiation, and migration (33). Our findings also proved that inhibition of hyperproliferation of keratinocytes with Shikonin and DAPT impacted cellproliferation/apoptosis, characterized by decreased Bcl-2 /PCNA, and increased caspase-9/ICAD. Consequently, it showed that apoptosis inhibition of keratinocytes was related to psoriasis.

In summary, we supposed that Notch1/Jagged1/Hes5 signaling is involved in the pathogenesis of psoriasis, which could be down-regulated by Shikonin. However, the intervention of Shikonin on Notch1 signaling and inflammation in psoriasis still needs to be explored. In future studies, the protective effects of Shikonin need to be verified in animal models, for example BALB/c mice with imiquimod-induced psoriasis-like lesion. These *in vivo* studies will extend our understanding of the mechanism of Shikonin effects on psoriasis.

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microRNA-331-3p ATTENUATES NEUROPATHIC PAIN FOLLOWING SPINAL CORD INJURY VIA TARGETING RAP1A

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Neuropathic pain (NP) after spinal cord injury (SCI) leads to compromised physical and cognitive functions in a majority of patients. Aberrant miRNA expression plays vital roles in the pathogenesis of SCI. This study aims to investigate the effect of miR-331-3p in rats following SCI. Microarray assay was performed in SCI- and sham-operated rats to evaluate the expression of miR-331-3p. Assigned SCI rats were treated with miR-331-3p agomiR alone or miR-331-3p agomiR plus RAP1A-expressing lentivirus or control agomiR. Rat locomotor performance was evaluated by BBB locomotor rating scale. Neuronal tissue damage and apoptosis were detected by histological analyses and Western blot. Inflammation in spinal cord was determined by detection of the expression of inflammatory genes with qRT-PCR, and ELISA. Downstream expression of RAP1A was measured by Western blot. The results showed that SCI induced the downregulation of miR-331-3p in the spinal cord of SCI rats. Overexpression of miR-331-3p improved the locomotor performance, reduced tissue damage, neuronal apoptosis and inflammation in rat SCI model. Rap1a (Ras-related protein Rap-1A) was predicted as a downstream target for miR-331-3p, and upregulation of RAP1A impaired the beneficial effect of miR-331-3p post-SCI, which was shown as worse locomotor activity, more severe tissue damage, as well as promoting apoptosis and inflammation in SCI rats. Furthermore, miR-331-3p reduced the activation of RAP1A downstream genes via inhibiting RAP1A expression. These findings indicate a protective role of miR-331-3p in the development of SCI via the modulation of RAP1A, and may help to develop novel therapy against SCI-induced complications.

Neuropathic pain (NP) is a pathological, chronic pain resulted from somatosensory lesions or diseases, including central nervous injury and peripheral neuropathy (1). Central neuropathic pain is often caused by spinal cord injury (SCI), demyelinating diseases, and syringomyelia, whereas peripheral neuropathic pain is mainly induced by the damage to peripheral fibers, known as A β , A δ , and C fibers (2). The prevalence of NP in the general population has been estimated to be 7-8%, and the rates of NP post-

SCI as high as 53% (3, 4). A better understating of the mechanisms underlying NP following SCI is of great importance for the development of mechanism-based therapeutic approach in clinical practice (5).

MicroRNAs (miRNAs), a class of short non-coding RNAs with 18-24 nucleotides in length, are abundantly expressed in the nervous system, where they destabilize and/or degrade target mRNAs (6). The emerging role of microRNA (miRNA) as a regulator and potential therapeutic target for chronic

Key words: ERK, miR-331-3p, neuropathic pain, p38, RAP1A, spinal cord injury

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NP has already been highlighted (7). MiR-331-3p belongs to the miR-331 family, and has been widely reported to regulate cell proliferation and apoptosis in cancer cells (8). It was also shown that the expression of miR-331-3p was downregulated in a mouse model of the neurodegenerative disorder, amyotrophic lateral sclerosis (9). However, whether miR-331-3p affects the development of NP-related neuronal damage following SCI remains unknown.

Long-term plasticity in synaptic transmission is considered as one the major pathological mechanisms for NP (10). The Ras family proteins, including Ras, Rap1, and Rap2, and their downstream targets, such as extracellular signal-regulated kinases (ERK) and p38, were shown to be the key signaling linked to synaptic plasticity (11). RAP1A belongs to the Rap1 subfamily, and the knockout of Rap1 impaired the synaptic plasticity in a mouse model of fear learning (12).

In this study, we established a rat model of SCI and assessed the expression of miR-331-3p in rats following the injury. Then the effects of miR-331-3p on the functional recovery, neuronal inflammation, and the development of SCI-induced neuronal damage were investigated by the administration of miR-331-3p agomiR in SCI rats. Rap1a was predicted as a downstream target of miR-331-3p with a putative binding site. The regulation of miR-331-3p on the expression level of RAP1A and its target genes in the spinal cord were also explored. Our results suggested that miR-331-3p attenuated neuropathic pain and neuronal damage post SCI via the inhibition of RAP1A.

MATERIALS AND METHODS

HEK-293T cells were purchased from ATCC (Manassas, USA) and cultured in DMEM medium supplemented with 4.5 g/L glucose, 10% fetal calf serum, 1% streptomycin and 2 nM glutamine (Sigma-Aldrich, St. Louis, USA) in a 5% CO₂ humidified incubator at 37°C. The miR-331-3p mimics and its corresponding control (miR-NC) were synthesized by Ribobio (Guangzhou, China). To construct luciferase reporter RAP1A-WT, RAP1A 3'-UTR fragment containing the putative binding site of miR-331-3p

(5'-UCAUUUUACCCUCCACCAGGGGG-3') was amplified and cloned into the downstream of luciferase gene in the pmirGlo vector (GenePharm, Shanghai, China). The mutant 3'-UTR sequence of putative binding site (5'-UCAUUUUACCCUCCAGGUCCCCG-3') was used to construct RAP1A-MUT vector. HEK-293 cells were plated into 24-well plates (Thermo Fisher Scientific, Waltham, USA) and co-transfected with miR-NC (or miR-331-3p) and RAP1A-WT (or RAP1A-MUT) at 60–70% confluency. The luciferase activities were assessed 48 hours after transfection using Dual-Luciferase Reporter Assay System (Promega Biotech Co., Madison, USA).

Adult female Sprague-Dawley rats (220–240 g) were purchased from Charles River Laboratories China and housed in a temperature-, humidity- and light-controlled room (25°C, 50% humidity, 12-hour alternating light-dark cycle) with *ad libitum* access to food and water. All animal-handling procedures were approved by the Animal Care and Use Committee of People's Hospital of Deyang City, and were performed in accordance with the Guide for the Care and Use of Laboratory Animals (13). After one-week adaptation to the new environment, rats were anesthetized by i.p. administration of 10% chloral hydrate (3 mL/kg) and fixed on the operation table in prone position. An incision was made with T10 spinous process as the center, and the skin and muscles were separated to fully expose the vertebral laminae. The laminectomy was performed at T8 level under the microscope and then the spinal cord was vertically compressed using a smooth vascular clamp for 20 seconds (14, 15). Sham surgery was performed on control group without clamping the spinal cord. All rats received intramuscular injection of penicillin (Sigma) and manual assistance of urination after the surgery.

Assessment of SCI model. Animals were randomly assigned into an SCI group and a sham group followed by the operation procedure mentioned above. The locomotor performance was evaluated in both groups (n=5/group) at days 1, 3, 7, 14, 21, and 28 post-operation using the Basso, Beattie and Bresnahan (BBB) Locomotor Rating Scale. Rats subjected to the measurement of miR-331-3p level

were euthanized with carbon dioxide asphyxia (5.5 L/min) at days 0, 3, 7, and 14 after the operation (n=5/group/time). Spinal cord (segments L4–L6) samples were harvested from each group (n=5/group) for the assessment of spared tissue 7 days after operation.

miR-331-3p agomiR experiments. Rats were randomly divided into three groups: a SCI + NC agomiR group, a SCI + miR-331-3p agomiR group, and a sham group. SCI + NC agomiR group and sham group were operated as mentioned above. Rats in SCI + miR-331-3p agomiR group received SCI operation followed by the intrathecal treatment of miR-331-3p agomiR (20 μ M, 1mL/h) for two weeks. Then the expression of miR-331-3p in SCI + NC agomiR group and SCI + miR-331-3p agomiR group was measured at day 1, 3, 7, and 14 after the treatment (n=5/group/time). The locomotor performance was evaluated in all groups (n=5/group) at day 1, 3, 7, 14, 21, and 28 after the treatment of miR-331-3p agomiR using the BBB Locomotor Rating Scale. At day 7 after the treatment, animals (n=5/group) were euthanized with carbon dioxide asphyxia (5.5 L/min) for the collection of spinal cord (segments L4–L6) samples. The percentage of spared tissue and the number of residual neurons were assessed using cresyl violet staining. The ratio of injury area was determined by hematoxylin and eosin (H&E) staining. The apoptotic cells were detected by TUNEL staining. Immunohistochemistry, Western blot assay, quantitative real-time PCR (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA) were also performed.

Rap1 as a target of miR-331-3p. *Rap1a* was predicted as a potential target gene of miR-331-3p with a putative binding site. The mRNA and protein expressions of *rap1a* in the spine were measured in SCI + NC agomiR, SCI + miR-331-3p agomiR, and sham groups (n=5/group) using qRT-PCR and Western blot. Rats were randomly assigned into four groups: SCI + NC agomiR, SCI + miR-331-3p agomiR, SCI + miR-331-3p agomiR + LV-RAP1A and sham group. Recombinant lentiviral vector expressing RAP1A were constructed and amplified by GenePharm (Shanghai, China). In the group of SCI + miR-331-3p agomiR + LV-RAP1A, spinal cord surgery was performed followed by the injection

of 10 μ L RAP1A vector suspension at a depth of 1 mm rostral and caudal to the lesion as previously described (16). Then the intrathecal treatment of miR-331-3p agomiR (20 μ M, 1mL/h) was performed for two weeks. BBB Locomotor Rating Scale was used to assess locomotor performance in all four groups (n=5/group) at days 1, 3, 7, 14, 21, and 28 after the treatment. Seven days after the treatment, spinal cord (segments L4–L6) tissues from all animals (n=5/group) were collected after euthanasia, and analyzed for different parameters using cresyl violet staining, TUNEL staining, immunohistochemistry, qRT-PCR, and Western blot.

General locomotor behavior was evaluated at days 1, 3, 7, 14, 21, and 28 after SCI using the BBB Locomotor Rating Scale as previously described (17). The scoring scale (0 - 21) discriminates the stages of hindlimb locomotor performance and post-injury recovery.

Animals were euthanized 7 days after SCI and the spinal cord (segments L4–L6) from each rat was harvested, post-fixed and cryoprotected before sectioning into 10 μ m slices. Spinal cord sections were stained with H&E and observed under a light microscope (magnification 40 $\times$) for the investigation of the injury area. Tissue sections were also stained for caspase-3 antibody (#ab13847, Abcam, Cambridge, UK) using immunohistochemistry method (magnification 100 $\times$).

For the detection of spinal cord lesion, sections were stained with myelin basic protein (MBP, Merck Millipore, Burlington, USA) and cresyl violet (Sigma-Aldrich) for the presentations of myelin and Nissl substance, respectively, as previously described (18). Spared white matter and grey matter were defined by the morphology and the color of the stains in which the normal structure of spinal cord was preserved (19). The percentage of spared tissue on each given section was calculated as: [spared white matter + spared grey matter]/total area of spinal cord.

The residual neurons stained for Nissl substance in the spared grey matter were quantified using the Optical Fractionator probe (20). Cells were counted in 6 randomly selected fields with the size of 100 μ m $\times$ 100 μ m per field.

To detect apoptotic DNA fragmentation, spinal cord sections were stained with TUNEL reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. Apoptotic cells were identified by brown stained nucleus. Cells were counted in 6 randomly selected fields using a fluorescence microscope (magnification 100 $\times$).

Quantitative real-time PCR (qRT-PCR)

Rats were euthanized at designed time points and segments L4–L6 of the spinal cord were harvested. Total RNAs were extracted using All-in-One™ miRNA RT-qPCR Detection Kit (GeneCopoeia Inc., Rockville, USA) or Trizol (Invitrogen, Carlsbad, USA) following the manufacturers' instructions. cDNA was analyzed using 7300 Real-Time PCR System (Applied Biosystem, Foster City, USA). The sequences of the primers were: miR-331-3p: 5'-UCAUUUUACCCUCCACCAGGGGG-3'; COX-2 forward: GATTGACAGCCCACCAACTT, COX-2 reverse: CGGGATGAACTCTCTCCTCA; IL-1 β forward: CCAGGATGAGGACCCAAGCA, IL-1 β reverse: TCCCGACCATTGCTGTTTCC, TNF- α forward: GGGGCCACCACGCTCTTCTGTC, TNF- α reverse: TGGGCTACGGGCTTGTCACCTCG; IL-6 forward: GCCCTTCAGGAACAGCTATGA, IL-6 reverse: TGTCAACAACATCAGTCCCAAGA; RAP1A forward: GCGTTGGGAAGTCTGCTCTGGTAA, RAP1A reverse: TGTCTCCCACATCCACTCTGCC.

Proteins were extracted from homogenized spinal cord segments followed by the normalization of protein content using bicinchoninic acid assay, as previously described (21). Protein lysates were separated using Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis on a 12% gel under reducing conditions, and transferred to polyvinylidene fluoride membranes. The membranes were incubated with the following primary antibodies at 4°C for 12 h: cleaved caspase-3 (1:1000, #ab49822, Abcam, Cambridge, UK), cleaved PARP (1:2000, #ab32064, Abcam), Bax (1:2000, #ab32503, Abcam), Bcl-2 (1:800, #ab59348, Abcam), RAP1A (1:1000, #ab115776, Abcam), p-ERK1/2 (1:1000, #9101, Cell Signaling, Danvers,

USA), ERK1/2 (1:1000, #4695, Cell Signaling), p-MRK1/2 (1:1000, #8532773, MyBioSource, San Diego, USA), MRK1/2 (1:1000, #712891, MyBioSource), p-p38 (1:1000, #ab4822, Abcam), p38 (1:1000, #ab170099, Abcam), and β -catenin (1:2000, #ab32572, Abcam). Then membranes were incubated with goat anti-rabbit secondary antibody (1:2000, #ab6721, Abcam) and the expressions of proteins were analyzed using Alphamager™ 2000 Imaging System (Alpha Innotech, USA).

Spinal cord homogenates from designated groups were analyzed for the concentrations of COX-2, IL-1 β , TNF- α and IL-6 using ELISA kits according to the manufacturer's instructions (MyBioSource Inc., San Diego, CA, USA). The absorbance at 450 nm was detected using a Power Wave Microplate Reader (Bio-TEK, USA).

Statistical analysis

Data are presented as mean $\pm$ standard deviation and analyzed by using software SPSS (version 24.0). The difference among groups were analyzed by one-way analysis of variance (one-way ANOVA). The correlation between the levels of miR-331-3p and RAP1A was estimated by linear correlation coefficient. A value of $p < 0.05$ was considered statistically significant. All experiments in this study were performed in triplicate and repeated three times.

RESULTS

To investigate the potential involvement of miR-331-3p following SCI, we first established a rat SCI model. The locomotor activity of rats was examined using the BBB rating scale from day 1 to 28 after surgery. Results showed that SCI rats had significantly lower BBB scores compared to the sham-operated group, indicating impaired limb movements following SCI (Fig. 1A). Spinal cord segments were harvested and sectioned for cresyl violet staining at day 7 post-surgery. Compared to sham-operated rats, the SCI group exhibited significantly less spared tissues at multiple distances from the epicenter (Fig. 1B). Then microarray assay was used to detect the expression of miR-331-3p in spinal cord tissues at day 0 to 14 following SCI. We found that miR-331-

3p level was significantly downregulated in SCI rats compared to sham-operated group from 3 to 14 days (Fig. 1C). The aberrant expression of miR-331-3p in SCI model suggested the potential regulatory role of miR-331-3p in the development of SCI.

Overexpression of miR-331-3p improves the functional recovery and reduces tissue damage in rats following SCI

To further explore the effect of miR-331-3p on SCI pathogenesis, we intrathecally treated SCI rats with miR-331-3p agomiR. The expression of miR-331-3p was significantly elevated in rats which received SCI + miR-331-3p agomiR compared to the NC agomiR group from days 1 to 14 following the treatment (Fig. 2A). BBB rating scale was used to assess the locomotor performance and showed that miR-331-3p overexpression in SCI + miR-331-3p agomiR group significantly improved the locomotor activity from days 7 to 28 compared with SCI + NC agomiR rats (Fig. 2B). The loss of spared tissue, which was induced by SCI, was remarkably suppressed by the overexpression of miR-331-3p (Fig. 2C). SCI operation also led to white and grey matter deterioration as shown by H&E staining, whereas the treatment of miR-331-3p agomiR reduced necrosis, karyopyknosis, polymorphonuclear leukocyte infiltration, and the number of macrophages in the spinal cord, thereby

decreasing the size of injury area (Fig. 2D). Then the residual neurons stained for Nissl substance was calculated. The loss of residual neurons in SCI rats was significantly reduced by the overexpression of miR-331-3p (Fig. 2E). The above data indicated that miR-331-3p agomiR improved the locomotor function and reduced spinal lesion in SCI rats.

Upregulation of miR-331-3p suppresses neuronal apoptosis and inflammation in SCI rats

We next examined the effect of miR-331-3p upregulation on neuronal apoptosis using TUNEL staining. Compared to the sham-operated group, SCI + NC agomiR rats showed significantly more TUNEL-positive cells. By treating SCI rats with miR-331-3p agomiR, the number of positive cells was largely decreased (Fig 3A). Then we evaluated the levels of pro-apoptotic proteins, including cleaved caspase-3, cleaved PARP, Bax, and anti-apoptotic proteins Bcl-2 in the spinal cord samples (22, 23). Augmented expressions of pro-apoptotic proteins and downregulated level of Bcl-2 were observed in SCI rats compared with sham-operated group. The administration of miR-331-3p agomiR, however, reversed the expressions of both pro- and anti-apoptotic proteins with statistical significance (Fig. 3B). Spinal cord sections from all groups were also stained for caspase-3 using immunohistochemistry. Consistent with results obtained in Western blot, the

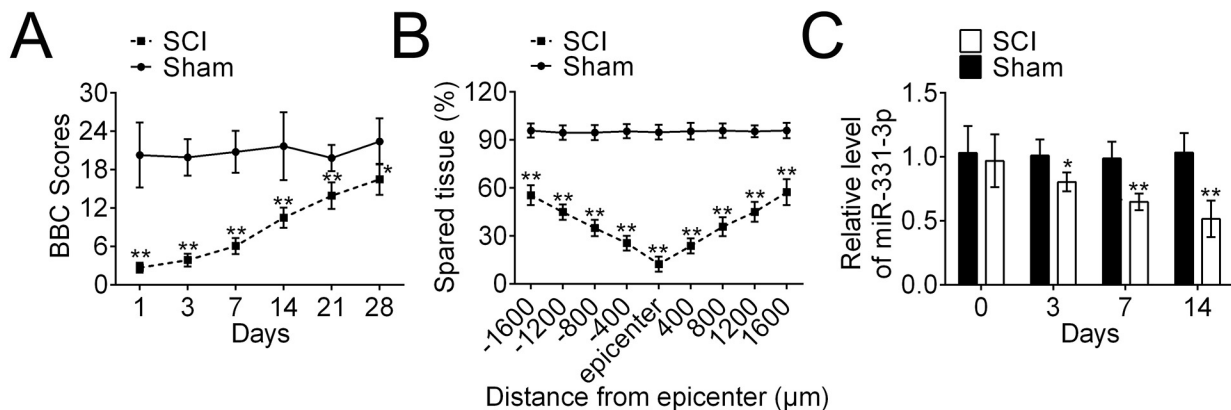


Fig. 1. Assessment of locomotor performance, spared tissues and miR-331-3p expression in SCI model. **A)** Rat locomotor behavior was evaluated at days 1, 3, 7, 14, 21, and 28 after operation using the BBB Locomotor Rating Scale in both SCI and sham groups. **B)** Spinal cord (segments L4–L6) were harvested for the assessment of spared tissue using cresyl violet staining at day 7 post operation. **C)** Relative expression of miR-331-3p in SCI and sham groups were determined by qRT-PCR at day 0, 3, 7, 14 post SCI. * $p < 0.05$, ** $p < 0.01$, SCI vs sham.

intensity of caspase-3 was significantly enhanced in rats with SCI, but dramatically suppressed by the upregulation of miR-331-3p (Fig. 3C). The levels of inflammatory factors, including COX-2, IL-1 β , TNF- α and IL-6, in the spinal cord were also suppressed by miR-331-3p treatment as measured by qRT-PCT (Fig. 4A) and ELISA (Fig. 4B). The above findings suggested the inhibitory role of miR-331-3p agomiR on neuronal apoptosis and inflammation in rats after SCI.

MiR-331-3p targets rap1a and their expression levels were negatively correlated

The bioinformatic prediction analysis on miRDB indicated that *rap1a* was a potential target of miR-331-3p with a putative binding site (Fig. 5A). To confirm that *rap1a* was a direct target of miR-331-3p, we generated luciferase reporter vectors containing wild-type and mutant *rap1a* target sequences, and co-transfected HEK-293T cells with miR-NC (or miR-331-3p) and RAP1A-WT

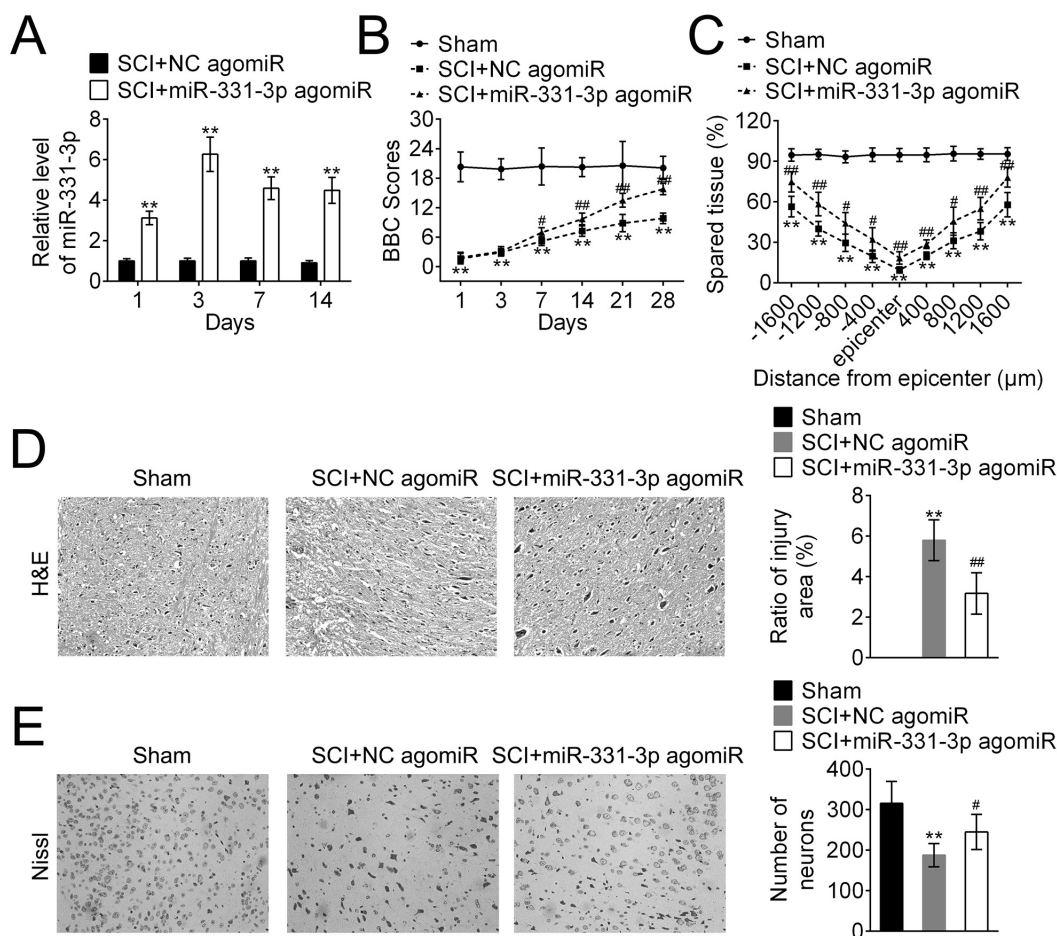


Fig. 2. Effect of miR-331-3p overexpression on functional recovery and tissue damage in SCI rats. Rats were randomly divided into three groups: SCI + NC agomiR, SCI + miR-331-3p agomiR, and sham group. SCI + miR-331-3p agomiR group received intrathecal treatment of miR-331-3p agomiR after SCI operation. **A**) Relative expression of miR-331-3p was measured at day 1, 3, 7, and 14 in SCI rats following the treatment of miR-331-3p agomiR. (** $p < 0.01$, SCI + miR-331-3p agomiR vs SCI + NC agomiR). **B**) The locomotor performance was assessed at day 1, 3, 7, 14, 21, and 28 in SCI model following the treatment of miR-331-3p agomiR in all three groups. **C**) Cresyl violet staining was performed at day 7 to detect the spared tissues areas. Spinal cord sections were stained for H&E (**D**) and Nissl substance (**E**) for the evaluation of the injury area and the number of residual neurons. ** $p < 0.01$, SCI + NC agomiR vs sham; # $p < 0.05$, ## $p < 0.01$, SCI + miR-331-3p agomiR vs SCI + NC agomiR.

(or RAP1A-MUT). The relative luciferase activity was significantly down-regulated when cells were transfected with RAP1A-WT and miR-331-3p. In contrast, the relative luciferase activity of the RAP1A-MUT reporter was not affected by miR-331-3p transfection (Fig. 5B). Then we measured the mRNA and protein expressions of *rap1a* in all groups. Results showed that both mRNA and protein levels of RAP1A were significantly decreased in SCI rats after the administration of miR-331-3p (Fig.

5C, D). Furthermore, the level of miR-331-3p was negatively correlated with the expression of RAP1A in the spinal cord (Fig. 5E). These results indicated that *rap1a* was a downstream target of miR-331-3p, and its expression was adversely regulated by miR-331-3p in SCI rats.

MiR-331-3p attenuates neuropathic pain following SCI via the inhibition of RAP1A

To further ascertain that miR-331-3p ameliorated

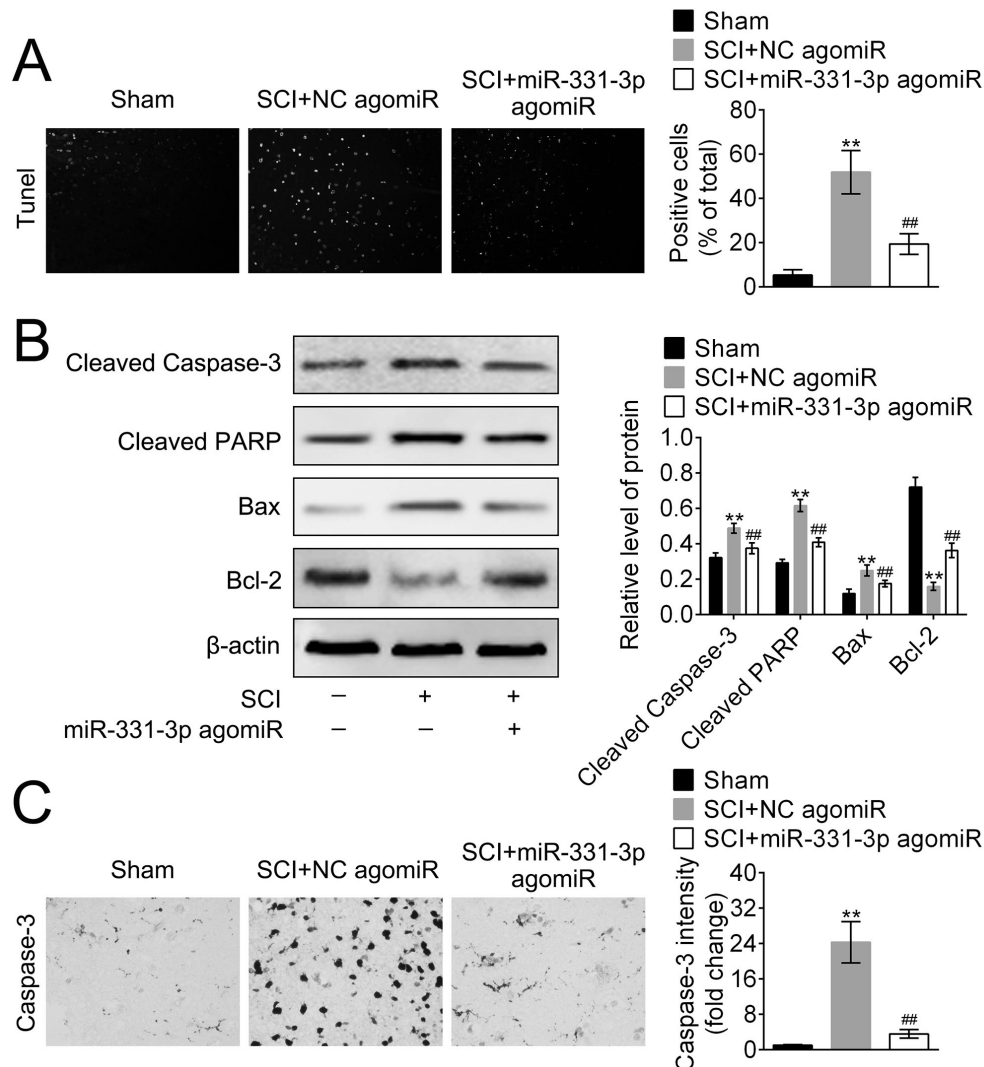


Fig. 3. Overexpression of miR-331-3p reduces apoptotic cell death in SCI model. **A)** TUNEL staining was performed to measure apoptotic cell death at day 7 following the treatment of miR-331-3p agomiR in all three groups. **B)** Protein expressions of cleaved caspase-3, cleaved PARP, Bax, and Bcl-2 in spinal cord were examined by Western blot and normalized by β -catenin expression. Representative plots (left) and bar charts (right) were shown. **C)** Spinal cord sections were stained for caspase-3 antibody using immunohistochemistry. ** $p < 0.01$, SCI + NC agomiR vs sham; # $p < 0.05$, ## $p < 0.01$, SCI + miR-331-3p agomiR vs SCI + NC agomiR.

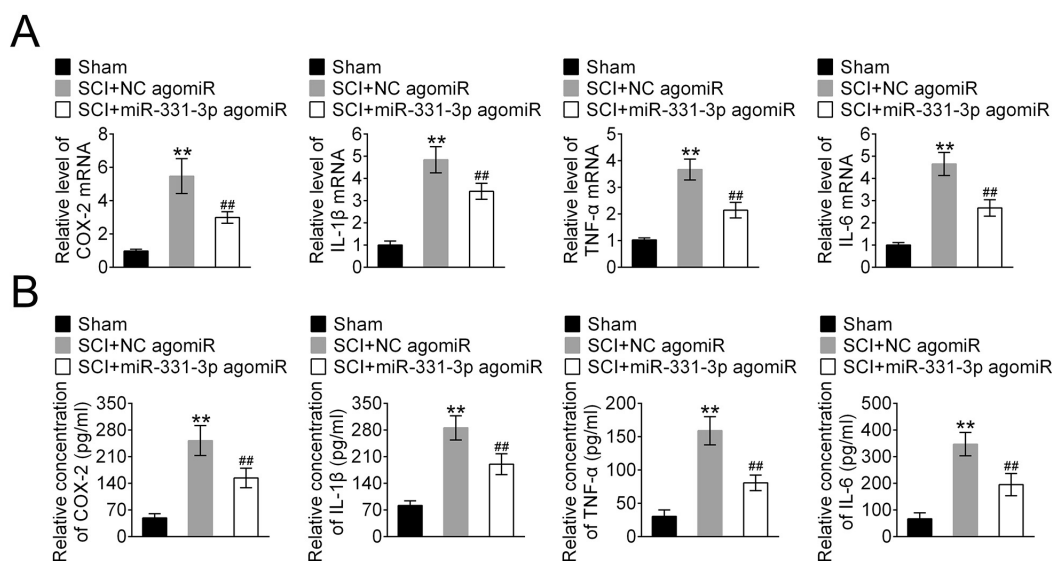


Fig. 4. Effect of miR-331-3p overexpression on neuronal inflammation. Spinal cord segments were collected from SCI + NC agomiR, SCI + miR-331-3p agomiR, and sham groups at day 7 following the treatment of miR-331-3p agomiR. The levels of COX-2, IL-1 β , TNF- α and IL-6 were measured using qRT-PCR (A) and ELISA (B). ** $p < 0.01$, SCI + NC agomiR vs sham; # $p < 0.05$, ## $p < 0.01$, SCI + miR-331-3p agomiR vs SCI + NC agomiR.

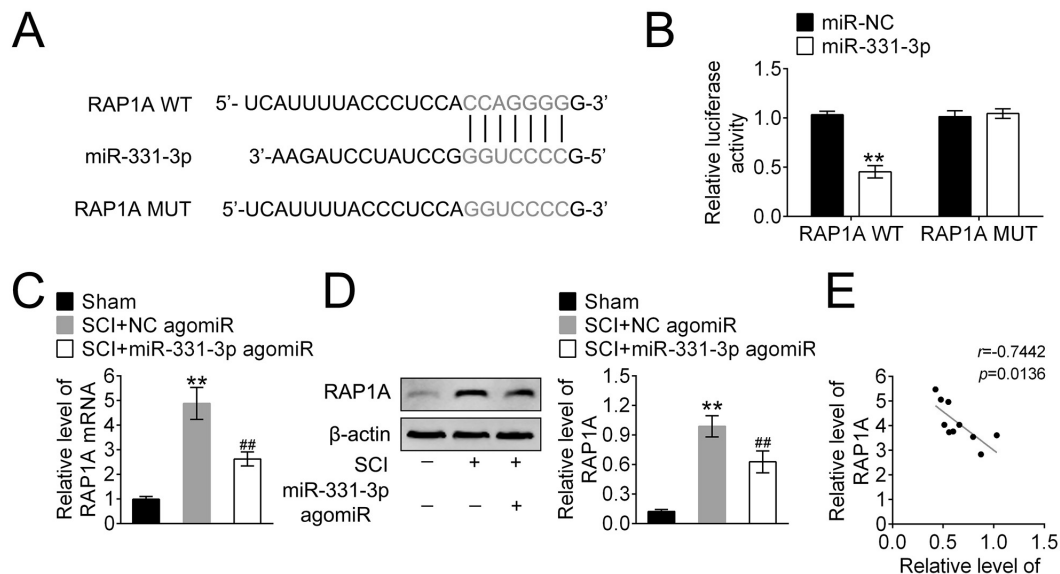


Fig. 5. RAP1A is targeted by miR-331-3p. **A)** The putative RAP1A binding site for miR-331-3p was predicted on miRDB, and the designed mutant sequence was shown as RAP1A MUT. **B)** The luciferase activity was determined by dual-luciferase reporter assay in HEK-293T cells after the co-transfection of miR-NC (or miR-331-3p) and RAP1A-WT (or RAP1A-MUT). ** $p < 0.01$, HEK-293 cells co-transfected with miR-331-3p and RAP1A-WT vs HEK-293 cells co-transfected with miR-NC and RAP1A-WT. **C)** Spinal cord segments were collected from SCI + NC agomiR, SCI + miR-331-3p agomiR, and sham groups at day 7 following the treatment of miR-331-3p agomiR. Relative mRNA and protein levels of RAP1A were determined by qRT-PCR (C) and Western blot (D), respectively. ** $p < 0.01$, SCI + NC agomiR vs sham; # $p < 0.05$, ## $p < 0.01$, SCI + miR-331-3p agomiR vs SCI + NC agomiR. **E)** The correlation between miR-331-3p expression and RAP1A mRNA level was analyzed by Spearman's rank correlation test.

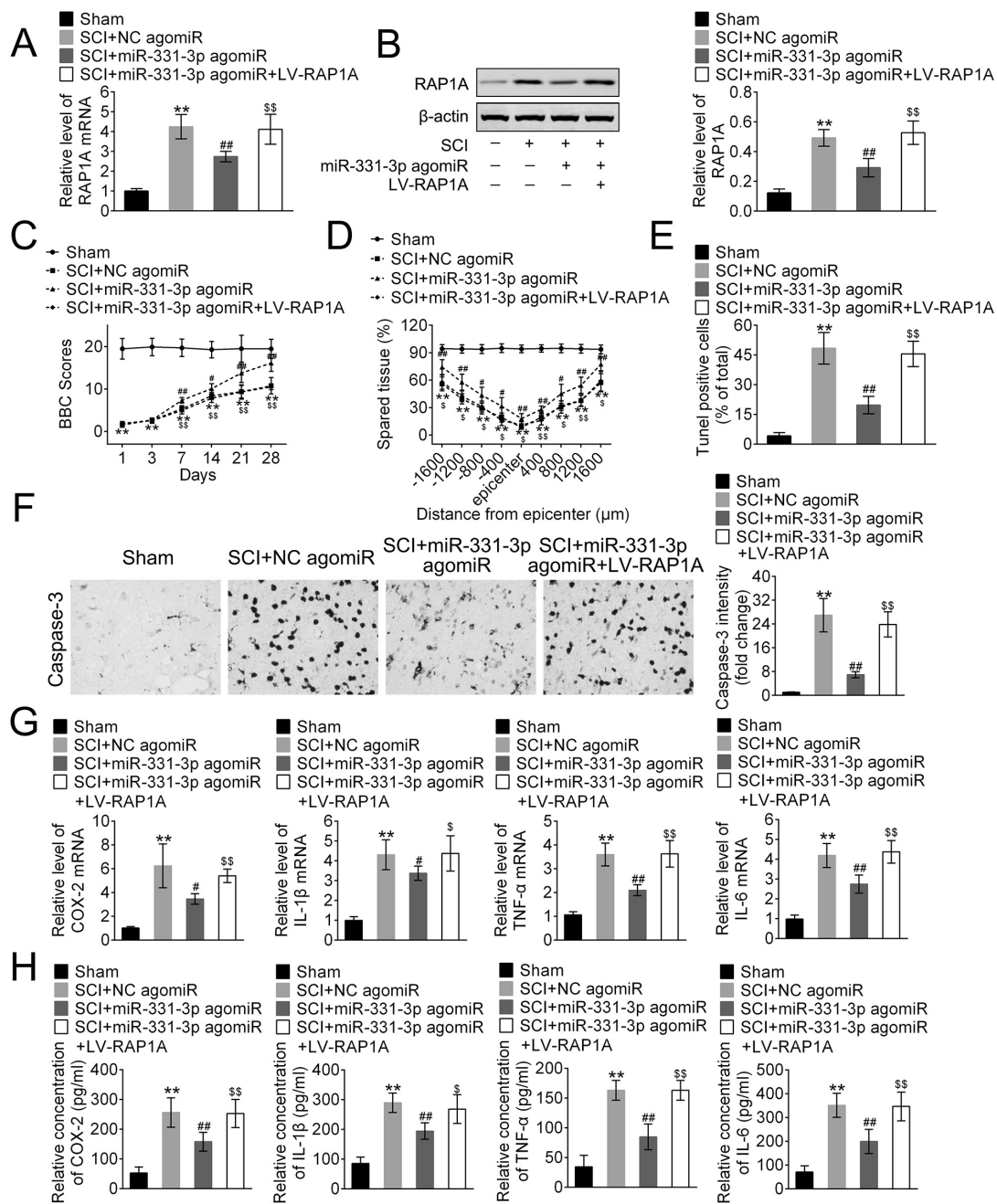


Fig. 6. RAP1A overexpression reverses the inhibitory effect of miR-331-3p on neuropathic pain. Rats were randomly assigned into four groups: SCI + NC agomiR, SCI + miR-331-3p agomiR, SCI + miR-331-3p agomiR + LV-RAP1A and sham group. Recombinant lentiviral vectors expressing RAP1A were constructed and injected in SCI + miR-331-3p agomiR + LV-RAP1A rats followed by the intrathecal treatment of miR-331-3p agomiR. Relative mRNA and protein levels of RAP1A were determined by qRT-PCR (A) and Western blot (B), respectively, in all four groups at day 7 after the treatment. C) The locomotor behavior was evaluated at days 1, 3, 7, 14, 21, and 28 following the treatment. Cresyl violet staining (D), TUNEL staining (E), and immunohistochemistry (F) were performed to detect the spared tissue, residual neurons, and the expression of caspase-3, respectively. Spinal cord segments were collected from rats in each group at day 7 following the treatment. The levels of COX-2, IL-1 β , TNF- α and IL-6 were measured using qRT-PCR (A) and ELISA (B). ** $p < 0.01$, SCI + NC agomiR vs sham; # $p < 0.05$, ## $p < 0.01$, SCI + miR-331-3p agomiR vs SCI + NC agomiR. \$ $p < 0.05$, \$\$ $p < 0.01$, SCI + miR-331-3p agomiR + LV-RAP1A vs SCI + miR-331-3p agomiR.

the lesion after SCI via the inhibition of RAP1A, we constructed recombinant lentiviral vector expressing RAP1A and injected the vectors into the lesion site of SCI (Study 3). The mRNA and protein levels of *rap1a* were downregulated by the overexpression of miR-331-3p in SCI + miR-331-3p agomiR group, whereas the transduction of lentiviral vector expressing RAP1A significantly elevated the level of RAP1A in SCI + miR-331-3p agomiR + LV-RAP1A rats (Fig. 6A, B). The locomotor function and the size of spared tissue were examined in all four groups. The overexpression of RAP1A in SCI + miR-331-3p agomiR + LV-RAP1A group significantly impaired the beneficial effect of miR-331-3p on functional recovery in rats following SCI (Fig. 6C, D). Consistently, suppressed apoptosis by miR-331-3p overexpression was also restored in rats injected with RAP1A-expressing vector as shown in TUNEL and caspase-3 staining (Fig. 6E, F). The assessment of inflammation in spinal cord samples showed that miR-331-3p agomiR treatment partially but significantly inhibited the expressions of COX-2, IL-1 β , TNF- α and IL-6 at both mRNA

and protein levels, whereas the overexpression of RAP1A resulted in significantly higher levels of inflammation in SCI + miR-331-3p agomiR + LV-RAP1A group compared to the ones without lentiviral transduction (Fig. 6G, H). These results suggested that miR-331-3p attenuated the adverse responses during the development of NP following SCI through the inhibition of RAP1A expression.

MiR-331-3p reduces the activation of ERK and p38 signaling via inhibiting RAP1A

ERK and p38 are two downstream genes of Ras family responsible for the regulation of neuronal cell death (24, 25). To further explore whether the regulation of miR-331-3p in SCI is involved in other neuronal apoptotic pathways, we analyzed the expressions of ERK, MRK, and p38 in the spinal cord samples. We found that miR-331-3p treatment significantly suppressed the activation of ERK1/2, MRK1/2, and p38 in SCI model, while the overexpression of RAP1A reversed the inhibitory effect of miR-331-3p, resulting in stimulated activation of all these proteins with statistical

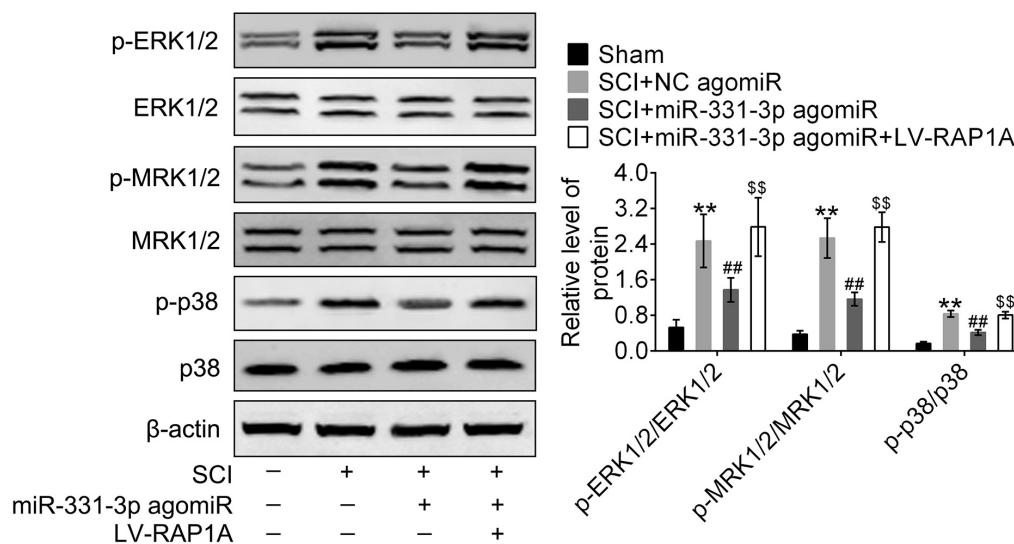


Fig. 7. ERK and p38 expression following SCI was decreased by miR-331-3p but elevated by RAP1A. Spinal cord segments were collected from SCI + NC agomiR, SCI + miR-331-3p agomiR, SCI + miR-331-3p agomiR + LV-RAP1A and sham groups at day 7 following the treatment. The protein expressions of p-ERK1/2, ERK1/2, p-MRK1/2, MRK1/2, p-p38, p38 were measured using Western blot and normalized by β -catenin expression. ** $p < 0.01$, SCI + NC agomiR vs sham; ## $p < 0.01$, SCI + miR-331-3p agomiR vs SCI + NC agomiR. \$\$ $p < 0.01$, SCI + miR-331-3p agomiR + LV-RAP1A vs SCI + miR-331-3p agomiR.

significance (Fig. 7). These findings suggested that miR-331-3p might decreased the activation of ERK and p38 signaling through the inhibition of RAP1A.

DISCUSSION

Neuropathic pain following SCI is a common sequela that leads to compromised physical and cognitive functions, and reduced quality of life for many patients (26). Numerous cellular and molecular interactions take place in the nervous system during the development of NP after SCI, including neuronal inflammatory responses and apoptosis of targeted cells (27). Results from animal studies showed that aberrant expressions of miRNAs post-SCI play vital roles in the pathogenesis of SCI (28). In the current study, we report that the overexpression of miR-331-3p improved the functional recovery, reduced tissues damage, neuronal apoptosis and inflammation in rats following SCI. It was confirmed that *rap1a* was a downstream target for miR-331-3p and RAP1A upregulation impaired the beneficial effect of miR-331-3p on the progress of SCI. Furthermore, miR-331-3p reduced the activation of RAP1A downstream genes, ERK, MRK and p38, via the inhibition of RAP1A expression.

Extensive expression of mature miRNAs has been found in the spinal cord of adult rodents (29). Following SCI, 97 of 269 miRNAs exhibited significantly altered expression in a rat SCI model, which can be categorized into three expression patterns: upregulation, downregulation and bidirectional changes (30). The mature miR-331-3p is derived from pre-miR-331, and has been shown to be a tumor suppressor for colorectal, prostate, and gastric cancer (8, 31, 32). The downregulation of miR-331-3p was also found in mice with amyotrophic lateral sclerosis and glioblastoma multiforme cell lines, suggesting the regulatory effect of miR-331-3p in central nervous system (9, 33). In this study, we showed that the expression of miR-331-3p was significantly decreased in rats following SCI compared to sham-operated counterparts. By treating SCI rats with miR-331-3p-agomiR, the upregulation of miR-331-3p improved the locomotor performance, suppressed tissue damage, neuronal

apoptosis and inflammation. These findings suggest a protective role of miR-331-3p in the development of SCI.

Ras signaling pathway is involved in the promotion of neuronal apoptosis in patients with SCI (34). The inhibition of Ras/Raf/ERK1/2 signaling in neurons harvested from SCI mice significantly restored neuronal adhesion, migration, and dendritic spine development (35). By performing bioinformatic prediction analysis, we found that RAP1A, a member of Rap1 subfamily, was a potential target of miR-331-3p, which was then confirmed by the luciferase reporter assay in HEK 293 cells. The expression of miR-331-3p was inversely correlated with the level of RAP1A in the spinal cord samples. Moreover, the injection of lentivirus containing RAP1A-expressing sequence in SCI rats significantly inhibited the protective effect of miR-331-3p on the pathogenesis of SCI. Compared to rats administered with miR-331-3p agomiR alone, the ones with both treatments of miR-331-3p agomiR and RAP1A-expressing lentivirus showed worse locomotor activity, more severe tissue damage, promoted apoptosis and inflammation. These data indicated that miR-331-3p attenuated the adverse responses following SCI via inhibiting the expression of RAP1A. To further explore whether miR-331-3p mediated the downstream genes of RAP1A, we assessed the protein levels of ERK1/2, MRK1/2, p38, and their phosphorylated forms in SCI rats. Our data show that miR-331-3p treatment significantly suppressed the activation of ERK1/2, MRK1/2, and p38 in rats following SCI, whereas the overexpression of RAP1A restored the phosphorylation level of all three proteins. Taken together, we present that SCI downregulated the expression of miR-331-3p in spinal cord. The upregulation of miR-331-3p improved the locomotor performance, reduced tissue damage, neuronal apoptosis and inflammation in rat SCI model. The expression of miR-331-3p was inversely correlated with the level of its functional target, RAP1A. MiR-331-3p alleviated the adverse responses occurring during the development of NP following SCI via suppressing the expression of RAP1A and the phosphorylation of its downstream targets. Our findings may help to develop novel

therapeutic strategies against SCI-induced complications.

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NOTCH SIGNALING IS INVOLVED IN REGULATION OF LPS-INDUCED MACROPHAGE APOPTOSIS THROUGH JNK/NF- κ B SIGNALING PATHWAY

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Notch signaling plays a critical role in the development and function of macrophages. The aim of the present study was to investigate the relationship between Notch signaling pathway and macrophage apoptosis after LPS stimulation. In RAW 264.7 cells, the mRNA expression of Jagged1, Hes1, Hes 5 and GM-CSF, and protein expression of NICD1 and GM-CSF were increased after LPS stimulation. Inhibition of Notch signaling by γ -secretase inhibitor DAPT and the suppression of Notch1 expression using siRNA both significantly prevented LPS induced activation of JNK and NF- κ B, and simultaneously the expression of GM-CSF was also down regulated significantly. JNK inhibitor SP600125 was used to block the phosphorylation of JNK signaling, Western blot results showed that the activation of NF- κ B was blocked and expression of GM-CSF was down-regulated. Finally, flow cytometry analyses showed that the Notch signaling was involved in the regulation of macrophage apoptosis after LPS stimulation. Our study showed that the Notch signaling pathway was activated and involved in the regulation of macrophage apoptosis after LPS stimulation through JNK/ NF- κ B signaling regulated GM-CSF expression.

Inflammation is a natural defense mechanism of the body against foreign bacteria, viral and fungal infections (1). Macrophages are critical players in inflammatory processes to provide immediate defense against infection or tissue damage. Macrophages can be activated by various signals that stimulate intracellular signaling cascades of cytokines and chemokines (1, 2). Lipopolysaccharide (LPS), a major component from the outer membrane of the cell wall of Gram-negative bacteria, is one of the most powerful activators of macrophages (3). Although LPS induces activation of macrophages through the production of inflammatory cytokines and inflammatory mediators, it is also reported to induce apoptosis in a variety of cell types, including macrophages (4).

Notch signaling is a highly conserved signaling system that plays important roles in cell fate decisions, proliferation, and survival (5). In mammals, there are four transmembrane Notch receptors (Notch-1, Notch-2, Notch-3, and Notch-4), two Jagged-like ligands (Jagged1, Jagged2) and three Delta-like ligands (DLL1, DLL3, DLL4) (6). The interaction of these five ligands with Notch receptors activates the proteolytic cleavage of the Notch intracellular domain (NICD). Then NICD is translocated to the nucleus, where it forms a large transcriptional activator complex with CSL (RBPJk/CBF1) and Mastermind proteins (7). The transcriptional complex then activates the transcription of target genes, such as Hes (Hairy/ Enhancer of split) and

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Hey (Hes-related with YRPW motif).

Notch signaling has been reported to participate in the development and function of macrophages (2). High amounts of Notch1 and Notch2 were expressed in monocytes, and incubation of monocytes with the plastic-immobilized Notch ligand in the presence of macrophage colony-stimulating factor (M-CSF) led to apoptotic cell death (8). Notch signaling amplifies the production of LPS-induced inflammatory cytokines in macrophages (9). Aberrant activation of Notch signals is associated with the dysregulation of macrophage activation and function (10, 11).

Granulocyte macrophage colony stimulating factor (GM-CSF), a multifunctional cytokine and immune-modulator, was originally discovered as a growth factor capable of promoting both macrophage and granulocyte colonies from myeloid precursor cells *in vitro* (12). Recent reports showed that GM-CSF is an important activating and differentiation factor for immune cells, and is involved in a wide range of biological processes related to both innate and adaptive immunity (13, 14).

Previous studies demonstrated significant enhancement in proliferation of RAW 264.7 in the presence of GM-CSF, and GM-CSF can function as a survival factor for macrophage lineage populations (15). In addition, recent studies have found that GM-CSF could inhibit apoptosis of various hematopoietic cell lineages and neural cell lineages (16-18). However, little is known about the role of Notch signaling in the GM-CSF expression after LPS stimulation. And it is not clear whether the Notch signaling was involved in the regulation of macrophage apoptosis through GM-CSF expression after LPS stimulation.

The purpose of this study was to investigate the relationship between Notch signaling pathway and GM-CSF regulated macrophage apoptosis after LPS stimulation. We also further explored the internal mechanism.

MATERIALS AND METHODS

Cell culture and reagents

The RAW 264.7 murine macrophage cell line was purchased from the American type culture collection

(Manassas, VA, USA) and grown in Dulbecco's modified Eagle medium (DMEM, Hyclone, Thermo Fisher, Rockford, IL, USA) supplemented with 10% fetal calf serum (Sigma-Aldrich, St. Louis, MO, USA). Cells were cultured in the presence of LPS (Sigma-Aldrich, St. Louis, MO, USA) with or without the γ -secretase inhibitor DAPT (Santa Cruz, Dallas, CA, USA). The specific MAPK inhibitor SP600125 was purchased from Santa Cruz biotechnology (Santa Cruz, CA, USA).

Real-Time PCR analysis

Total RNA was extracted using a Trizol reagent (Takara Co., Ltd., Dalian, China), and reverse transcription into first-strand cDNA was performed using the First Strand cDNA Synthesis Kit (FSK-100, TOYOBO CO., Ltd., Osaka, Japan) in the presence of the RNase inhibitor diethylpyrocarbonate (DEPC) (Roche Diagnostics GmbH, Mannheim, Germany). Real-time PCR was performed as previously described (19). The following PCR primers were used: Jagged1 forward, 5'- GGCTTGGTCTGTTGCTTGG-3' and reverse, 5'- TGTGGCTGCTGGGCTTCC-3'; Notch1 forward, 5'- GAGGACGAGGTGGACGAGTG-3' and reverse, 5'- AGCCGCCGAGATAGTCAGTG-3'; Hes1 forward, 5'- GAGCACAGAAAGTCATCAAAGCC-3' and reverse, 5'- TTATTCTTGCCCTTCGCCTCTTC-3'; Hes5 forward, 5'- GCGGTGGAGATGCTCAGTC-3' and reverse, 5'- TGCTCTATGCTGCTGTTGATGC-3'; GM-CSF forward, 5'- CCCACCCGCTCACCCATC-3' and reverse, 5'- CGACTTCTACCTCTTCATTCAACG-3'; GAPDH forward, 5'- AATGGTGAAGGTCGGTGTGAAC-3' and reverse, 5'- CGTGAGTGGAGTCATACTGGAAC-3'.

Western blot

Cells were homogenized in cold RIPA buffer (Beyotime, Shanghai, China). Protein was quantified by the Bradford method using the BCA assay reagent (Beyotime, Shanghai, China). Equal amounts of protein were loaded into SDS-polyacrylamide gels and transferred onto PVDF-Plus membranes. After blocking in 5% fat-free milk for 1 h at room temperature, the membranes were incubated overnight at 4°C with the following primary antibodies: NICD2 (ab-52302, Abcam, UK), Hes5 (sc-25395, Santa Cruz, CA, USA), and GAPDH (sc-32233, Santa-Cruz, CA, USA). The primary antibodies were detected with horseradish peroxidase-conjugated

goat anti-rabbit IgG, or goat anti mouse IgG secondary antibody for 1 h at room temperature and detected by the use of a chemiluminescence system (Beyotime, Shanghai, China) and imaging system (Kodak Gel Logic 4000R Imaging System, Carestream, Rochester, NY, USA).

ELISA for GM-CSF

The RAW 264.7 Cells were cultured in the presence of LPS. After LPS stimulation, cells were lysed in cold RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 µg/ml PMSF, 1.0 mM sodium orthovanadate, and 1×mammalian protease inhibitor cocktail; Sigma-Aldrich). The homogenates were centrifuged at 14,000g for 10 min at 4°C. The supernatants were aliquoted and stored at -80°C. GM-CSF in the supernatants was quantified with a commercial ELISA kit (CSB-E04569m, CUSABIO, Wuhan, Hubei, China) according to the manufacturer's instructions. The antibody for GM-CSF was provided by Santa Cruz Biotechnology (sc-398649, Santa-Cruz, CA, USA).

Silencing Notch 1 using siRNA and in-vitro transfection

To inhibit the Notch signaling pathway, an *in vitro* transfection to silence Notch expression was performed. siRNA were chemically synthesized by Ribobio (Guangzhou, China). The sequences of the siRNA targeting Notch1 were as follows: sense 5' CAAAGGGCTGGCTTGTGGTAG dTdT3' and antisense 3' dTdT TGGATGGAGACTGCTGGAATGG'. RAW 264.7 cells were cultured in 6-well plates, transfected with siRNA at a concentration of 50 nmol for each well using the Lipofectamine 2000 reagent (Invitrogen, Shanghai, China) in antibiotic-free and serum-free Opti-MEM medium, according to the manufacturer's instructions.

Detection of apoptosis

In order to detect the apoptosis of RAW 264.7 cells, flow cytometric analysis was applied with Annexin V-FITC Apoptosis Detection Kit (KeyGEN Biotech, Nanjing, China) according to the manufacturer's instructions. IEC-6 cells were cultured as above. Double staining for FITC-Annexin V binding and cellular DNA using propidium iodide (PI) was performed as previously described (20). The acquisition and analysis were performed using MoFlow (Beckman Coulter, Atlanta, GA, USA).

Statistical analysis

Data are presented as the mean ± SD. The Student's *t*-test was used for the comparisons of the mean values between two groups. ANOVA was used for comparisons of more than two groups. All statistical analyses were carried out using SPSS13.0 software. A P value less than 0.05 was considered statistically significant.

RESULTS

LPS induced activation of Notch signaling pathway and increased GM-CSF expression in murine macrophages

To investigate the effect of LPS on Notch signaling activation and GM-CSF expression in murine macrophages, RAW 264.7 cells were treated with different concentrations of LPS (0, 0.5, 1, 2, 4 and 8 µg/ml) for 6 h. As shown in Fig. 1A, the mRNA levels of Jagged1, Hes1, Hes 5 and GM-CSF were increased after LPS stimulation dose-dependently. Western blot analysis also showed that the NICD1 protein expression was increased significantly after LPS stimulation (Fig. 1B).

Then LPS (1 µg/ml) was used to treat RAW 264.7 and the total RNA and protein were harvested at the indicated times (0, 3, 6, 12, and 24 h). As shown in Fig. 1C, the mRNA levels of Jagged1, Hes1, Hes 5 and GM-CSF were increased time-dependently. Jagged1 was remarkably increased 3 h after LPS stimulation. Hes1, Hes 5 and GM-CSF mRNA levels were increased remarkably 6 h after LPS stimulation. Western blot analysis further showed that the NICD1 protein expression was increased remarkably 3 h after LPS stimulation (Fig. 1D).

These results suggested that the Notch signaling pathway was activated and the expression of GM-CSF was increased simultaneously after LPS stimulation in murine macrophages.

DAPT and silencing RNA for Notch1 blocked Notch signaling activation and prevented LPS induced expression of GM-CSF

To investigate whether Notch signaling was required for LPS induced expression of GM-CSF, DAPT, one type of γ-secretase inhibitor, was used to block the activation of Notch signaling pathway.

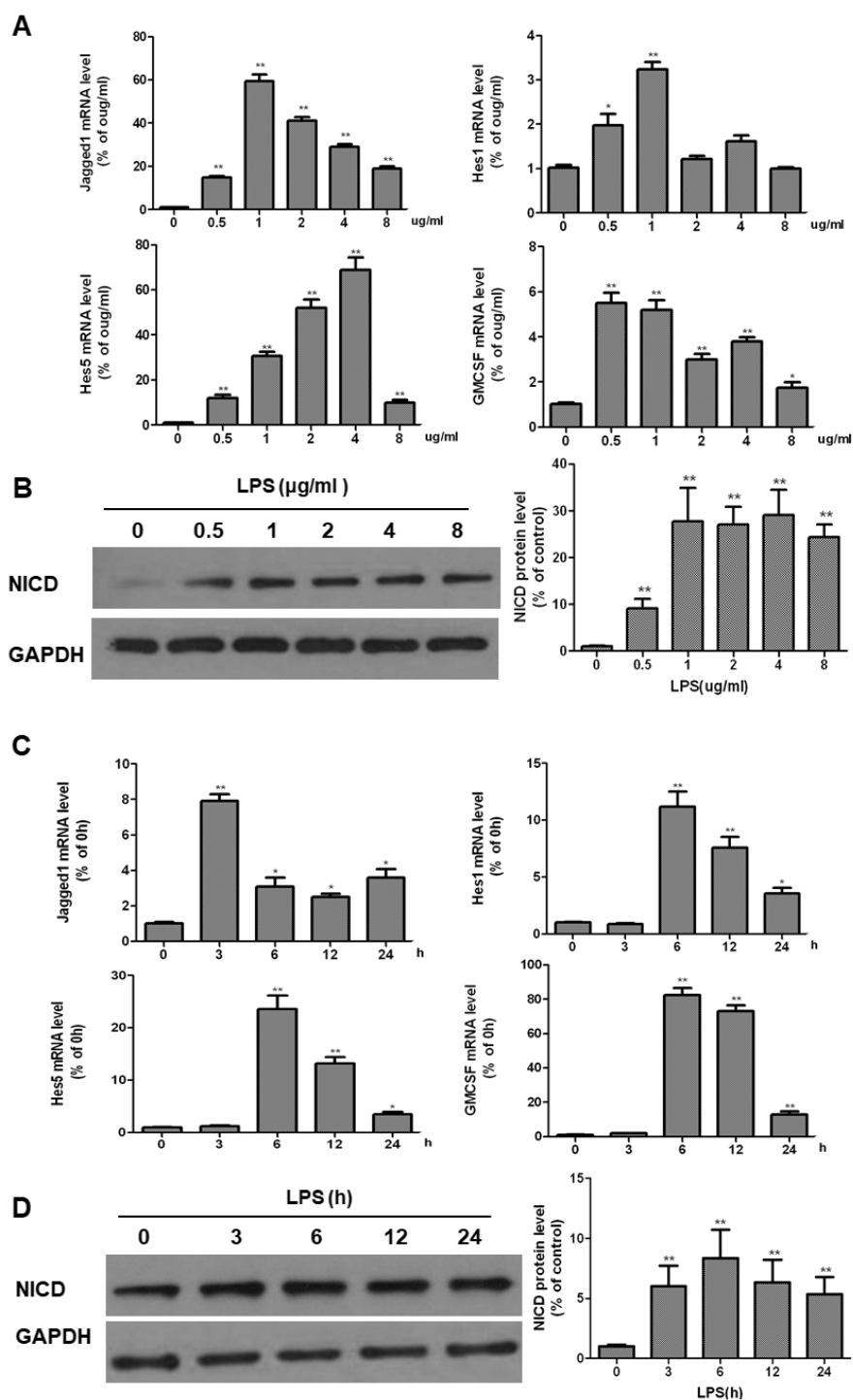


Fig. 1. Expression of Notch signaling components and GM-CSF in murine macrophages. RAW 264.7 cells were treated with different concentrations of LPS (0, 0.5, 1, 2, 4 and 8 µg/ml) for 6 h, mRNA expressions of Jagged1, Hes1, Hes5 and GM-CSF were detected by Real-Time PCR (A) and protein expression of NICD1 was detected by Western blot (B). RAW 264.7 cells were treated with 1 µg/ml LPS for the indicated times (0, 3, 6, 12, and 24 h), mRNA expressions of Jagged1, Hes1, Hes5 and GM-CSF were detected by Real-Time PCR (C) and protein expression of NICD1 was detected by Western blot (D). Data are shown as the means $\pm$ SDs ($n = 3$). ** $p < 0.01$ vs control group; * $p < 0.05$ vs control group. GAPDH was used as the loading control.

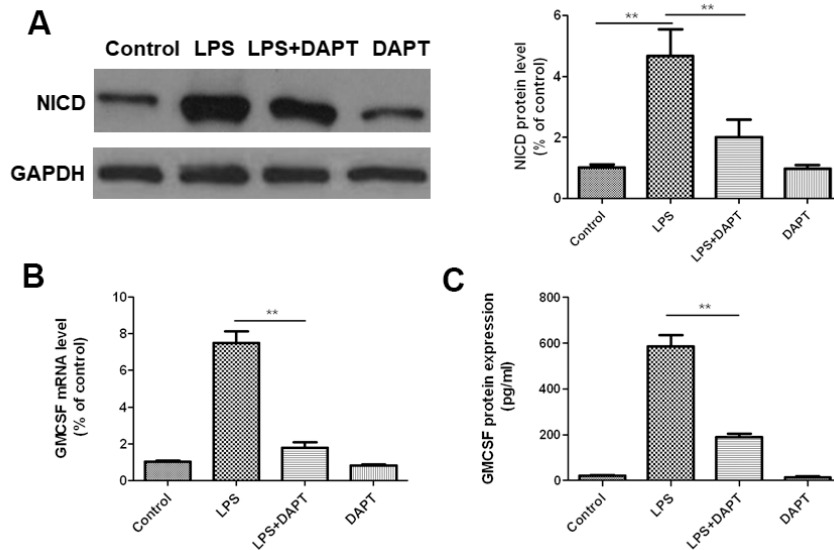


Fig. 2. Inhibition of Notch signaling by DAPT prevented LPS induced expression of GM-CSF. **A** RAW 264.7 cells were treated with 1 g/ml LPS for 6 h, with or without 20 μ M DAPT pre-treated for 12 h. The protein expression of NICD1 was examined by Western blot. GAPDH was used as the loading control. Expression of GM-CSF was detected by Real-Time PCR (**B**) and ELISA (**C**). Data are shown as the means $\pm$ SDs ($n = 3$). ** $p < 0.01$ vs control group; * $p < 0.05$ vs control group.

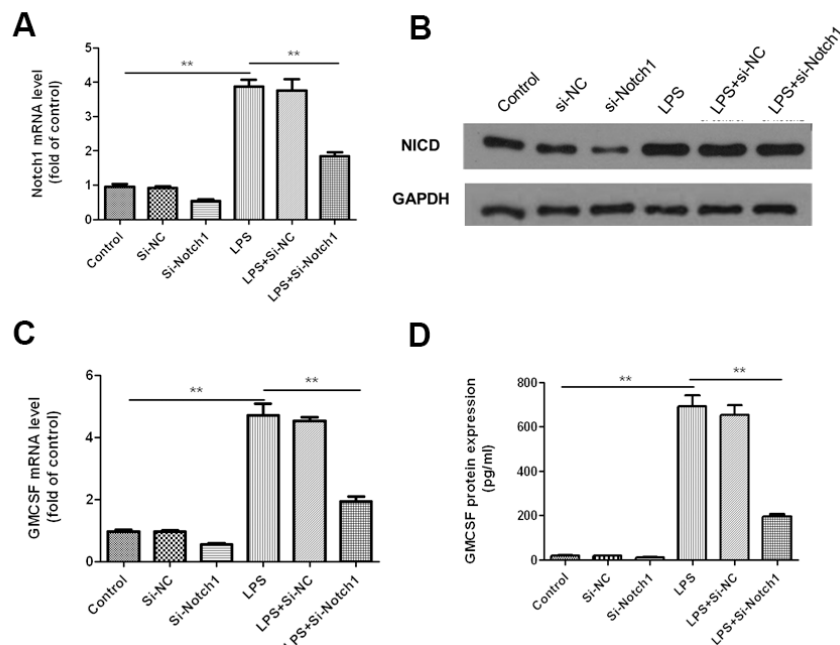


Fig. 3. siRNA for Notch1 prevented LPS-induced expression of GM-CSF. Inhibition of Notch1 with siRNA was carried out as mentioned in materials and methods. After 48 h of culture, LPS was added for 6 h. **A**) Expression of Notch1 mRNA was detected by Real-Time PCR. Data are shown as the means $\pm$ SDs ($n = 3$). ** $p < 0.01$ vs control group; * $p < 0.05$ vs control group. **B**) Expression of NICD1 was detected by Western blot. GAPDH was used as the loading control. **C**) Expression of GM-CSF mRNA was detected by Real-Time PCR. **D**) Protein expression of GM-CSF was detected by ELISA. Data are shown as the means $\pm$ SDs ($n = 3$). ** $p < 0.01$ vs control group; * $p < 0.05$ vs control group.

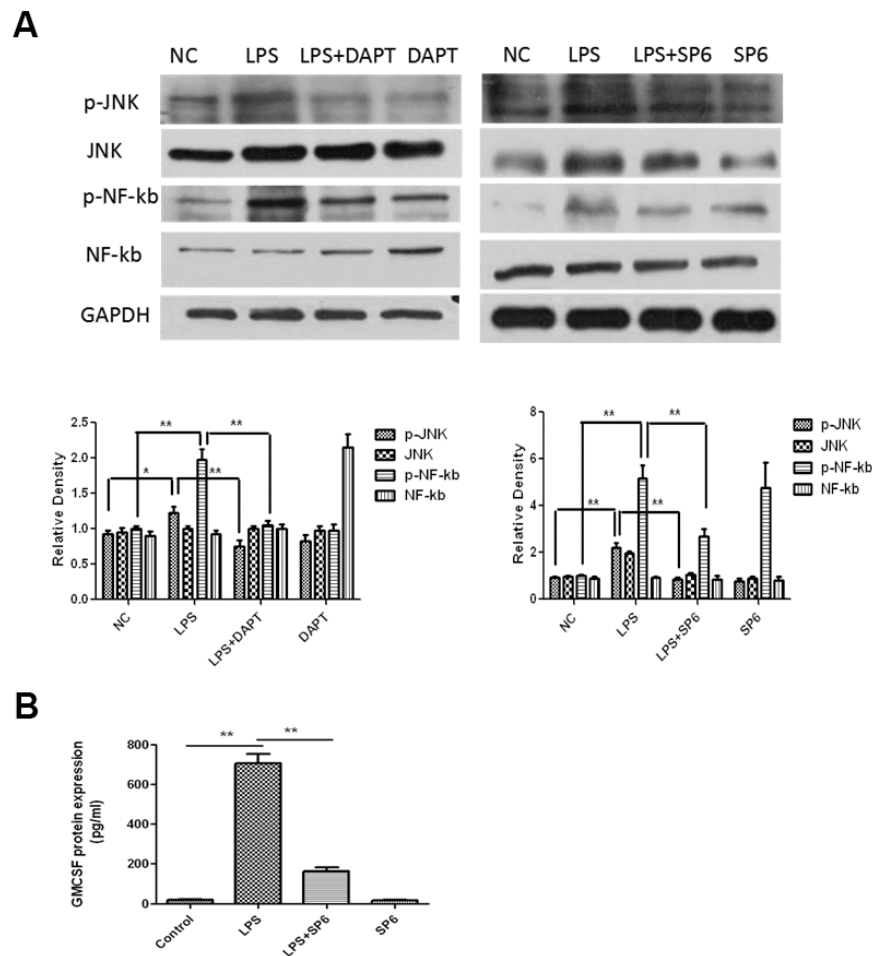


Fig. 4. JNK and NF- κ B activation is required for LPS-induced GM-CSF expression in macrophages. **A)** RAW 264.7 cells were treated with 1 μ g/ml LPS for 6 h, with or without 20 μ M DAPT pre-treated for 12 h. Phosphorylation of JNK and NF- κ B p65 was examined by Western blot. Then RAW 264.7 cells were treated with 1 μ g/ml LPS for 6 h, with or without JNK inhibitor SP600125. Phosphorylation of JNK and NF- κ B p65 was examined by Western blot. **B)** Expression of GM-CSF was detected by ELISA. Data are shown as the means $\pm$ SDs ($n = 3$). ** $p < 0.01$ vs control group; * $p < 0.05$ vs control group.

RAW 264.7 cells were treated with 1 μ g/ml LPS for 6 h, with or without DAPT pre-treated for 12 h. As shown in Fig. 2, DAPT inhibited the LPS-induced NICD1 expression significantly which showed that the Notch signaling pathway was blocked significantly. Furthermore, we examined the effect of DAPT on GM-CSF expression. The Real-Time PCR and ELISA results showed that DAPT inhibited the LPS-induced GM-CSF expression significantly (Fig. 2 B, C).

To further investigate the effect of Notch

signaling activation on GM-CSF expression, RAW 264.7 cells were transfected with siRNA for Notch1. The siRNA for Notch1 significantly down-regulated the expression of NICD1 as shown in Fig. 3A and B. At the same time, Real-Time PCR and ELISA results showed that the GM-CSF expression was significantly down-regulated (Fig. 3C and D). All these results demonstrated that the Notch signaling pathway was activated and involved in the LPS induced GM-CSF expression in macrophages.

JNK/NF-κB was involved in the Notch signaling regulated GM-CSF expression

Previous studies showed that JNK-NF-κB was involved in the LPS induced activation of macrophages (9). However, it remains unclear whether JNK-NF-κB was involved in the Notch signaling regulated GM-CSF expression after LPS stimulation.

DAPT was pretreated to block the Notch signaling activation, and then the phosphorylation of JNK and NF-κB p65 was examined by Western blot after LPS stimulation. The results showed that, compared with control group, LPS induced marked JNK and NF-κB p65 phosphorylation. DAPT pretreatment inhibited JNK and NF-κB p65 phosphorylation induced by

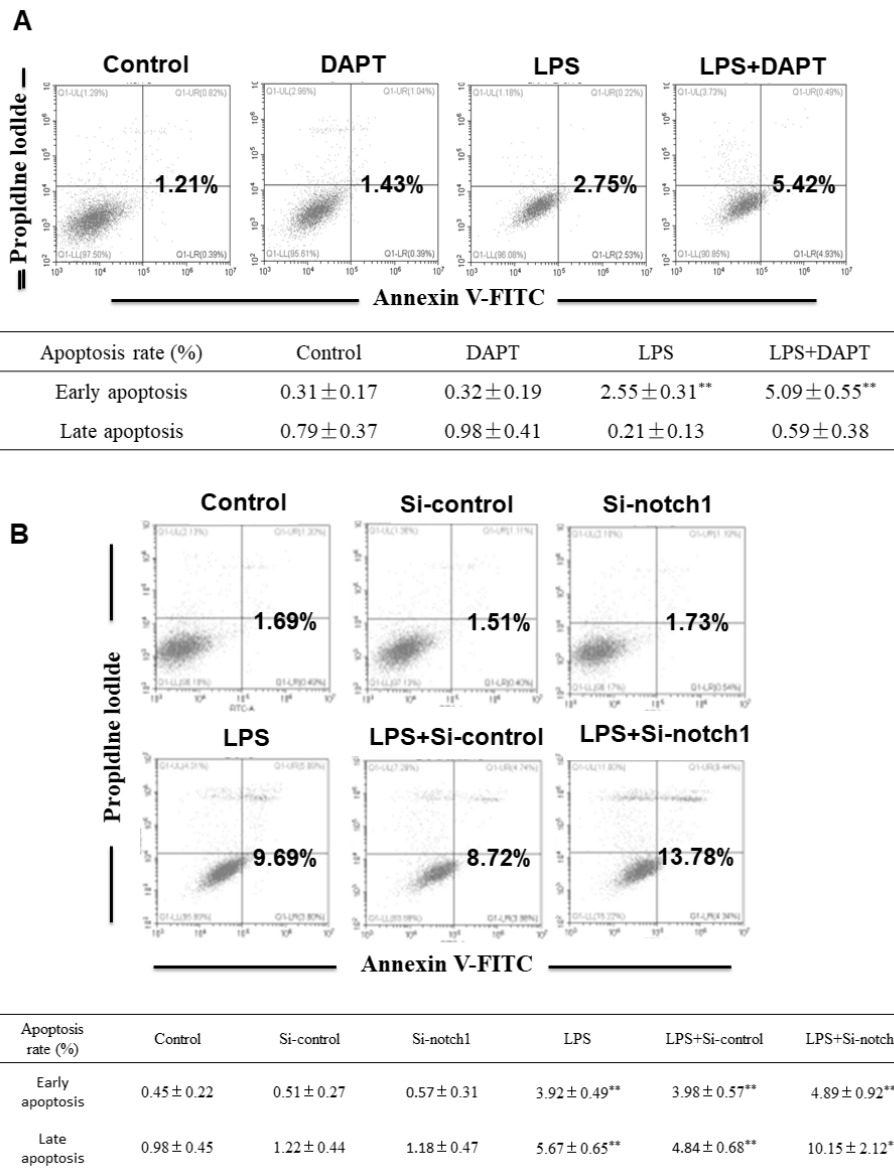


Fig. 5. Notch signaling was involved in the regulation of macrophage apoptosis after LPS stimulation. **A)** RAW 264.7 cells were treated with 1 µg/ml LPS for 6 h, with or without 20 M DAPT pre-treated for 12 h. Flow cytometry was applied to examine the apoptosis of RAW 264.7 cells. **B)** Inhibition of Notch1 with siRNA was carried out as mentioned in materials and methods. After 48 h of culture, LPS was added for 6 h. Flow cytometry was applied to examine the apoptosis of RAW 264.7 cells. In the apoptosis map, FITC-Annexin V+/PI+ indicates late apoptosis, FITC-Annexin V+/PI- indicate early apoptosis, and FITC-Annexin V-/PI- indicate normal live cells. ** $p < 0.01$ vs control group.

LPS significantly (Fig. 4A). Then JNK inhibitor SP600125 was used to block the activation of JNK in RAW 264.7 cells. As shown in Fig. 4A, JNK and NF- κ B p65 phosphorylation was both inhibited by SP600125 after LPS stimulation. These results suggested that Notch signaling was involved in the activation of JNK/ NF- κ B signaling pathway after LPS stimulation.

Interestingly, at the same time LPS-induced GM-CSF expression was also suppressed significantly by SP600125 (Fig. 4B). All these results indicated that JNK/NF- κ B signaling was involved in the Notch signaling induced GM-CSF expression after LPS stimulation in macrophages.

Notch signaling was involved in the regulation of macrophage apoptosis after LPS stimulation through GM-CSF expression

To further investigate the effect of Notch signaling on RAW 264.7 cells apoptosis, we examined the effects of DAPT on the apoptosis of RAW 264.7 cells receiving LPS or not with flow cytometry. The flow cytometry results showed that LPS increased the apoptosis of RAW 264.7 cells compared to control group (Fig. 5A). DAPT had no effect on the apoptosis rate of RAW 264.7 cells statistically. However, after LPS stimulation DAPT increased the apoptosis rate of RAW 264.7 cells significantly (Fig. 5A). Then SiRNA for Notch1 was used to block the Notch signaling after LPS stimulation and flow cytometry was applied to examine the apoptosis rate of RAW 264.7 cells. The results showed that suppression of Notch1 expression through SiRNA further increased the apoptosis of RAW 264.7 cells (Fig. 5B). The results above confirmed that the Notch signaling was activated after LPS stimulation and promoted the survival of RAW 264.7 cells.

DISCUSSION

In this study, we found that the Notch signaling pathway was activated and the expression of GM-CSF was also increased in LPS-treated RAW 264.7 cells. DAPT and Silencing RNA for Notch1 blocked Notch signaling activation and prevented LPS induced GM-CSF expression. Our findings

also demonstrated that JNK and NF- κ B signaling was involved in the Notch signaling regulated macrophage apoptosis after LPS stimulation through GM-CSF expression.

During the immune responses, macrophages must be activated to perform most of the functions. LPS is reported to both modulate macrophage activation and apoptosis (21, 22). Notch signaling has been reported to be activated in LPS-treated RAW 264.7 cells and amplify the production of LPS-induced inflammatory cytokines (8,9,23). Consistent with this, our study also found the activation of Notch signaling in LPS-treated RAW 264.7 cells.

GM-CSF is essential for the production and differentiation of various immune cells including macrophages. GM-CSF can be produced by both haemopoietic and non-haemopoietic cell types upon their stimulation including myeloid cells, dendritic cells, T cells, B cells, epithelial cells, endothelial cells and tumor cells (13, 24, 25). LPS was reported to induce a dose-dependent release of GM-CSF in mouse bladder urothelial cells (13). In this study, we found that GM-CSF was increased after LPS stimulation in RAW 264.7 cells. However, blocking the Notch signaling pathway inhibited the LPS-induced GM-CSF expression. This suggests that Notch signaling was involved in LPS-induced GM-CSF expression.

Recent results demonstrated that the induction of Jagged1 expression is partially dependent on canonical TLR-activated NF- κ B and MAPK signaling pathways (26). Tsao et al. also found that activation of JNK is required for LPS-induced Jagged1 expression in macrophages (9). We then detected whether JNK-NF- κ B was involved in Notch signaling regulated GM-CSF expression after LPS stimulation.

Our results showed that LPS-induced JNK and NF- κ B p65 phosphorylation was inhibited by both DAPT and JNK inhibitor SP600125 in RAW 264.7 cells, and simultaneously GM-CSF protein expression was down-regulated significantly. These results indicated that JNK and NF- κ B signaling was involved in the Notch signaling regulated GM-CSF expression in macrophages.

GM-CSF can function as a survival factor for macrophage lineage populations and inhibit

apoptosis of various hematopoietic cell lineages and neural cell lineages (16-18). GM-CSF is reported to reduce apoptosis of neutrophils through PI3K–ATK and MAPK kinase (MEK) 1–extracellular signal-regulated kinase (ERK) pathways(27). Recent studies showed that GM-CSF has anti-apoptotic activity via the PI3K-NF- κ B-HIF-1 α -Survivin pathway in muse neural progenitor cells (28). It also reported that GM-CSF can deliver Bcl-XL as an extracellular fusion protein to protect human monocyte/macrophage cells from apoptosis (29). In this study, we illuminated whether Notch and JNK-NF- κ B signaling was involved in the anti-apoptotic activity of GM-CSF. RAW 264.7 cells were treated with DAPT or Notch siRNA to block the Notch signaling activation. The flow cytometry results showed that suppression of Notch signaling further increased LPS-induced apoptosis of RAW 264.7 cells. These results suggested that Notch signaling activation inhibited LPS-induced macrophage apoptosis.

In summary, our study demonstrated that the Notch signaling pathway was activated and involved in the regulation of GM-CSF expression and macrophage apoptosis through JNK and NF-B signaling pathway after LPS stimulation. Further studies are needed to gain a more precise understanding of the molecular mechanisms.

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lncRNA CASC2 SUPPRESSES THE GROWTH OF HEMANGIOMA CELLS BY REGULATING miR-18a-5p/FBXL3 AXIS

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Dysregulation of lncRNA cancer susceptibility candidate 2 (CASC2) is involved in the pathogenesis of multiple malignancies. However, the underlying mechanisms by which lncRNA CASC2 regulates the proliferation of hemangiomas (HAs) remain undocumented. Herein, the expression levels of lncRNA CASC2 and VEGF in proliferating or involuting phase HAs were assessed by qRT-PCR analysis, and the effects of lncRNA CASC2 on HAs cell growth were evaluated by MTT, colony formation assays and Western blot analysis. lncRNA CASC2 specific binding with miR-18a-5p was confirmed by luciferase report assay. Consequently, we found that the expression of lncRNA CASC2 was reduced in proliferating phase HAs as compared with the involuting phase HAs or normal tissues, and possessed a negative correlation with VEGF expression in proliferating phase HAs. Restored expression of lncRNA CASC2 repressed cell viability and colony formation and downregulated VEGF expression, while silencing lncRNA CASC2 showed the opposite effects. Moreover, lncRNA CASC2 was confirmed to bind with miR-18a-5p, which could reverse lncRNA CASC2-induced anti-proliferative effects by targeting FBXL3 in HAs cells. Altogether, our findings demonstrated that lncRNA CASC2 suppressed the growth of HAs cells by regulating miR-18a-5p/FBXL3 axis.

Hemangiomas (HAs) as one of the most frequently recurring vascular benign tumors, are linked to the proliferation of vascular endothelial cells, and classified into proliferating phase HAs (PPH) and involuting phase HAs (IPH) (1). The mutations in CCM2 and von Hippel-Lindau contribute to cerebral cavernous malformation and hemangioblasts (2, 3). Our previous studies revealed that VEGF/VEGFR signaling is implicated in the proliferation and apoptosis escape of HAs, and blockage of this signaling produces a therapeutic effect (4, 5).

Long non-coding RNAs (lncRNAs) exceeding 200 nucleotides in length is implicated in cell growth,

invasion and metastasis in a variety of tumors including HAs (6-8). lncRNA cancer susceptibility candidate 2 (CASC2) is downregulated in multiple malignancies, such as thyroid carcinoma (9, 10), osteosarcoma (OS) (11), hepatocellular carcinoma (HCC) (12), and gastric cancer (13), and decreased expression of lncRNA CASC2 is associated with advanced TNM stage and tumor metastasis, predicting a poor prognosis in tumor patients (10-12). lncRNA CASC2 represses cell growth and induces cell apoptosis in thyroid carcinoma by inactivating AKT/ERK1/2 signaling (9), and retards epithelial-mesenchymal transition in HCC by regulating miR-367/FBXW7 axis (12), whereas

Key words: hemangioma; lncRNA CASC2; miR-18a-5p; FBXL3; proliferation

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downregulation of lncRNA CASC2 promotes the tumorigenesis, cell invasion and cisplatin resistance by sponging miR-181a/-19a (10, 11, 13). These studies indicated that lncRNA CASC2 can act as a potential biomarker in cancer.

Dysregulation of lncRNAs is associated with the tumorigenesis of HAs (8). The expression of lncRNA UCA1, Linc00152, SNHG16 and lncRNA CASC9 is increased in PPH samples, and facilitates the proliferation and invasion of HAs cells (14-17). However, the role and underlying mechanisms of lncRNA CASC2 in HAs remain unknown. Herein, we uncovered the mechanisms by which lncRNA CASC2 inhibited HAs cell growth by regulating miR-18a-5p/FBXL3 axis.

MATERIALS AND METHODS

HDEC and CRL-2586 EOMA cell lines used were stored at our laboratory. lncRNA CASC2 plasmids or control vector pEX-3, lncRNA CASC2 siRNA (si-CASC2) or control vector (si-NC), and miR-18a-5p mimic or inhibitor were purchased from GenePharma (Shanghai, China). The primer sequences of CASC2, miR-18a-5p and FBXL3 were synthesized by Sangon Biotech (Shanghai, China).

Frozen HAs tissue samples, consisting of PPH (n=15), IPH (n=15) and normal tissues (n=15), were stored at our laboratory. This study was approved by the Ethics Committee of Xinhua Hospital.

Cell culture

HDEC and CRL-2586 EOMA cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Sigma, Aldrich, USA) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS, Sigma, Aldrich, USA), 100U/ml of penicillin and 100µg/ml of streptomycin (Invitrogen, Carlsbad, CA, USA).

Quantitative real-time PCR (qRT-PCR)

To assess the expression of lncRNA CASC2, miR-18a-5p and FBXL3 in HAs tissues and cells, qRT-PCR analysis was performed according to previous reports (4, 5). PCR primer sequences were listed as follows: lncRNA CASC2 forward: 5'-TACAGGACAGTCAGTGGTGGTA-3' and reverse: 5'-ACATCTAGCTTAGGAATG TGGC-3'; FBXL3 forward: 5'-GCAGCTTG TGATATACTATCGCA-3' and reverse:

5'-TGGTCGAGCAGTTGAAATAAGTC-3'; miR-18a-5p forward: 5'-GTCCGGAC TCAGATCTCGAGCTTG AATCTACTGCAGTGAAGGCACTTG-3', reverse: 5'-TATCTAGATCCGGTGGATCCG-TGCAACTATGCAAACTAACAG-3'; GAPDH forward: 5'-CAACGAATTTGG C TACAGCA-3' and reverse: 5'-AGGGGTCTACAT GGCAACTG-3'. This experiment was repeated three times.

Western blot analysis

To evaluate the protein levels of VEGF in HAs cells, Western blot analysis was performed according to previous reports (4, 5). The primary antibody against VEGF (1:1000, ab46154, Abcam, Cambridge, MA, USA) was diluted and incubated overnight at 4°C. Horseradish peroxidase-linked secondary antibodies (1:1000) were added, and incubated at room temperature for 2 h. The membranes were washed with PBS and the bands were visualized using ECL-PLUS/Kit according to the kit's instruction.

MTT and colony formation assays were conducted as previously reported (4, 5).

Luciferase reporter assay

The fragments of lncRNA CASC2 or FBXL3 3' UTR were amplified by PCR and inserted into a pMIR-Report luciferase reporter vector (Ambion, Austin, TX, USA). After 24-h incubation, they were co-transfected with 60 µM miR-18a-5p mimic or inhibitor. A luciferase reporter assay system was used to analyze the luciferase activities.

Statistical analysis

Statistical analyses were carried out by SPSS 20.0 (IBM, SPSS, Chicago, IL, USA) and GraphPad Prism. Student's *t*-test was used to analyze the statistical significance for comparisons between two groups. Pearson correlation analysis was used to analyze the correlations. *P* < 0.05 was considered statistically significant.

RESULTS

lncRNA CASC2 had a negative correlation with VEGF expression in PPH

Our previous study indicated that VEGF can be used to distinguish PPH and IPH (4, 5). Thus, we assessed the expression of lncRNA CASC2 and VEGF in PPH and IPH by qRT-PCR analysis

and found that lncRNA CASC2 expression was substantially decreased ($P < 0.0001$; $P = 0.0292$), while VEGF expression was increased in PPH ($P = 0.0253$; $P < 0.0001$) as compared with IPH or normal tissues (Fig. 1A). Pearson correlation analysis uncovered that lncRNA CASC2 had a negative correlation with VEGF expression in PPH ($r = -0.6676$, $P = 0.0055$; Fig. 1B) rather than in IPH ($r = -0.3809$, $P = 0.1613$; Fig. 1C) and normal tissues ($r = -0.0577$, $P = 0.8383$).

lncRNA CASC2 reduced the proliferation and colony formation of HAs cells

MTT and colony formation assays were conducted to assess the role of lncRNA CASC2 in HAs cells. After lncRNA CASC2 plasmids had been transfected into HDEC and CRL-2586 EOMA cells for 48 h, their overexpression efficiencies were determined by qRT-PCR analysis ($P < 0.01$; Fig. 2A). Ectopic expression of lncRNA CASC2 repressed the cell viability (Fig. 2B) and colony formation ability (Fig. 2C) in these

two cell lines as compared with the pEX-3 group ($P < 0.01$). Then, Western blot analysis showed that VEGF protein levels were significantly reduced by lncRNA CASC2 as compared with the pEX-3 group in these two cell lines (Fig. 2D).

The knockdown efficiencies of si-CASC2 were defined in HDEC and CRL-2586 EOMA cells ($P < 0.01$; Fig. 3A). Reduced expression of lncRNA CASC2 facilitated cell viability (Fig. 3B) and colony formation (Fig. 3C) in these two cell lines as compared with the si-NC group ($P < 0.05$; $P < 0.01$). Western blot analysis indicated that VEGF protein levels were elevated by silencing lncRNA CASC2 as compared with the si-NC group in these two cell lines (Fig. 3D).

miR-18a-5p was identified to bind with lncRNA CASC2 in HAs cells

Online starBase2.0 (<http://starbase.sysu.edu.cn/starbase2/index.php>) was used to identify lncRNA CASC2-specific binding with miR-18a-5p in tumors. qRT-PCR analysis showed that miR-18a-

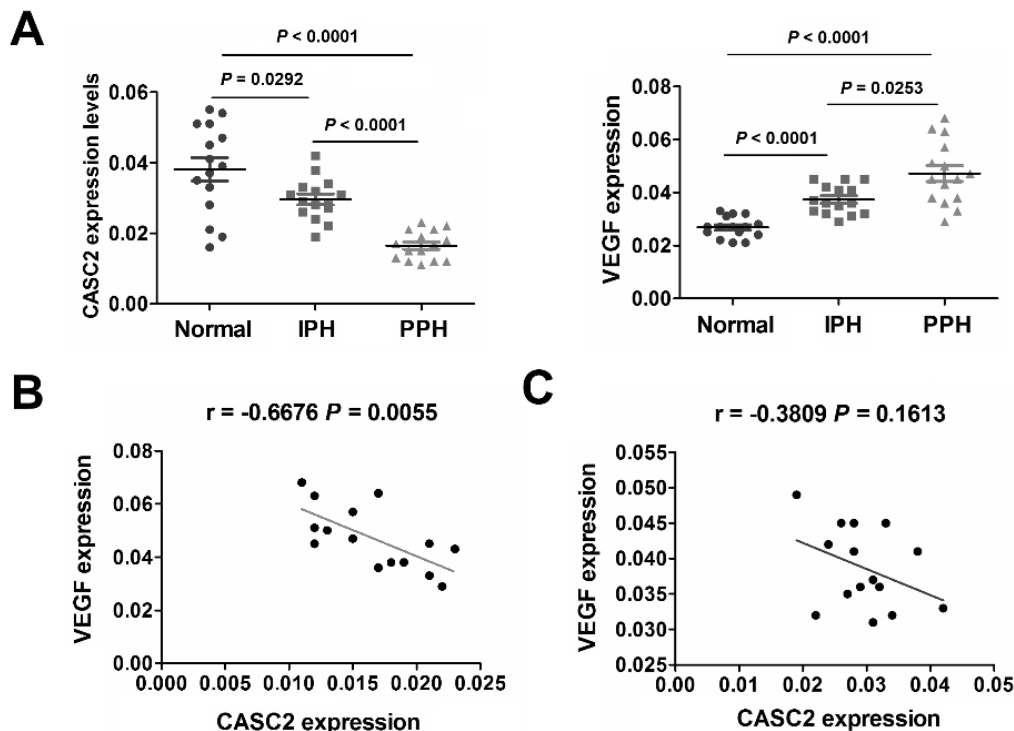


Fig. 1. The expression levels of lncRNA CASC2 and VEGF in HAs tissue samples. **A)** qRT-PCR analysis of the expression levels of CASC2 and VEGF in PPH, IPH and normal tissues. **B)** Pearson correlation analysis of the correlation of CASC2 with VEGF expression in PPH. **C)** Pearson correlation analysis of the correlation of CASC2 with VEGF expression in IPH.

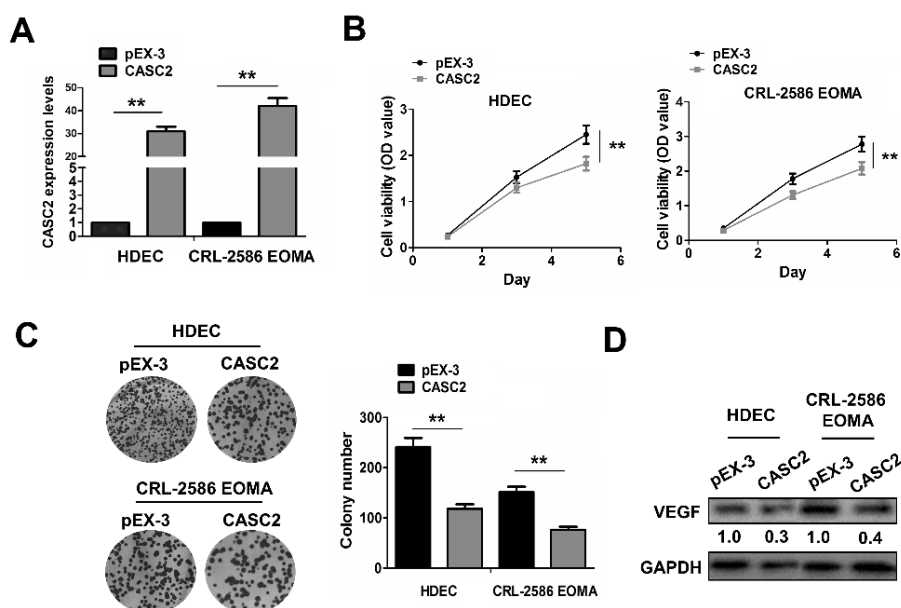


Fig. 2. CASC2 overexpression inhibited HAs cell proliferation and colony formation. **A)** qRT-PCR analysis of the transfection efficiency of CASC2 plasmids in HDEC and CRL-2586 EOMA cells. **B)** MTT analysis of the cell proliferative viability after transfection with CASC2 plasmids in HAs cells. **C)** Colony formation analysis of the cell colony formation number after transfection with CASC2 plasmids in HAs cells. **D)** Western blot analysis of the expression levels of VEGF after transfection with CASC2 plasmids in HAs cells. Data are the means $\pm$ SEM of three experiments. ** $P < 0.01$.

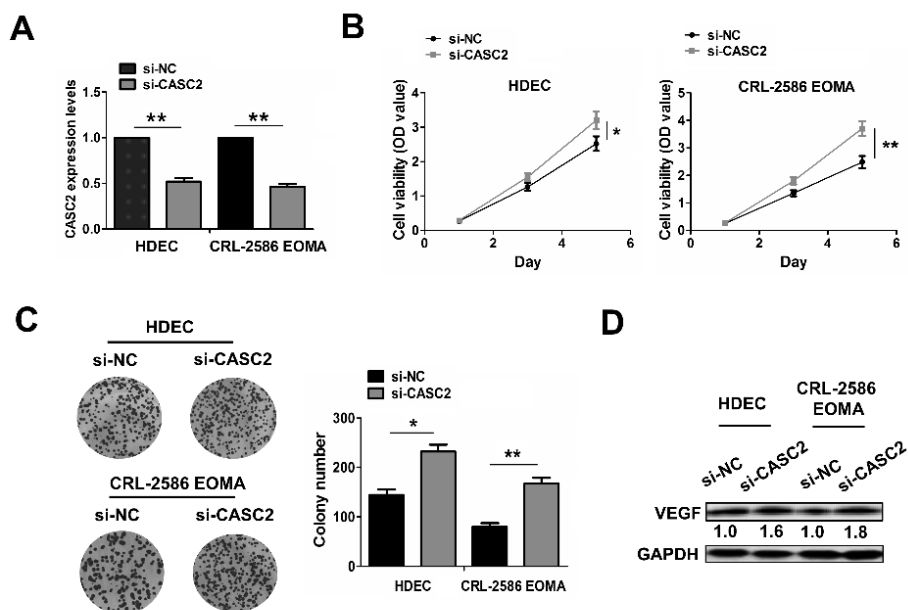


Fig. 3. CASC2 knockdown promoted HAs cell proliferation and colony formation. **A)** qRT-PCR analysis of the transfection efficiency of si-CASC2 plasmids in HDEC and CRL-2586 EOMA cells. **B)** MTT analysis of the cell proliferative viability after transfection with si-CASC2 plasmids in HAs cells. **C)** Colony formation analysis of the colony formation number after transfection with si-CASC2 plasmids in HAs cells. **D)** Western blot analysis of the expression levels of VEGF after transfection with si-CASC2 plasmids in HAs cells. Data are the means $\pm$ SEM of three experiments. * $P < 0.05$; ** $P < 0.01$.

5p expression was markedly increased in PPH as compared with IPH or normal tissues ($P = 0.0079$, $P = 0.0048$; Fig. 4A), and had a negative correlation with VEGF expression in PPH ($r = -0.5931$, $P = 0.0198$; Fig. 4B). The binding sites of miR-18a-5p with wild type (WT) or mutant (Mut) lncRNA CASC2 3'UTR are indicated in Fig. 4C. The transfection efficiency of miR-18a-5p mimic or inhibitor in HDEC and CRL-2586 EOMA cell lines was confirmed by qRT-PCR analysis (Fig. 4D). Then, the luciferase activities of WT lncRNA CASC2 3'UTR were lowered by miR-18a-5p mimic, but raised by its inhibitor, and those of Mut lncRNA CASC2 3'UTR were unaffected by miR-18a-5p in HAs cells (Fig. 4E). Moreover,

miR-18a-5p expression was markedly depressed by lncRNA CASC2, but elevated by silencing lncRNA CASC2 (Fig. 4F), but lncRNA CASC2 expression was unaffected by miR-18a-5p in HAs cells (Fig. 4G).

miR-18a-5p reversed lncRNA CASC2-induced anti-proliferative effects by targeting FBXL3

After si-CASC2 plasmids were co-transfected with miR-18a-5p inhibitor into HDEC and CRL-2586 EOMA cells, we found that miR-18a-5p inhibitor repressed cell viability and reversed si-CASC2-induced cell proliferation in these two cell lines ($P < 0.05$, $P < 0.01$; Fig. 5A). Online TargetScanHuman7.1 (<http://www.targetscan.org/>

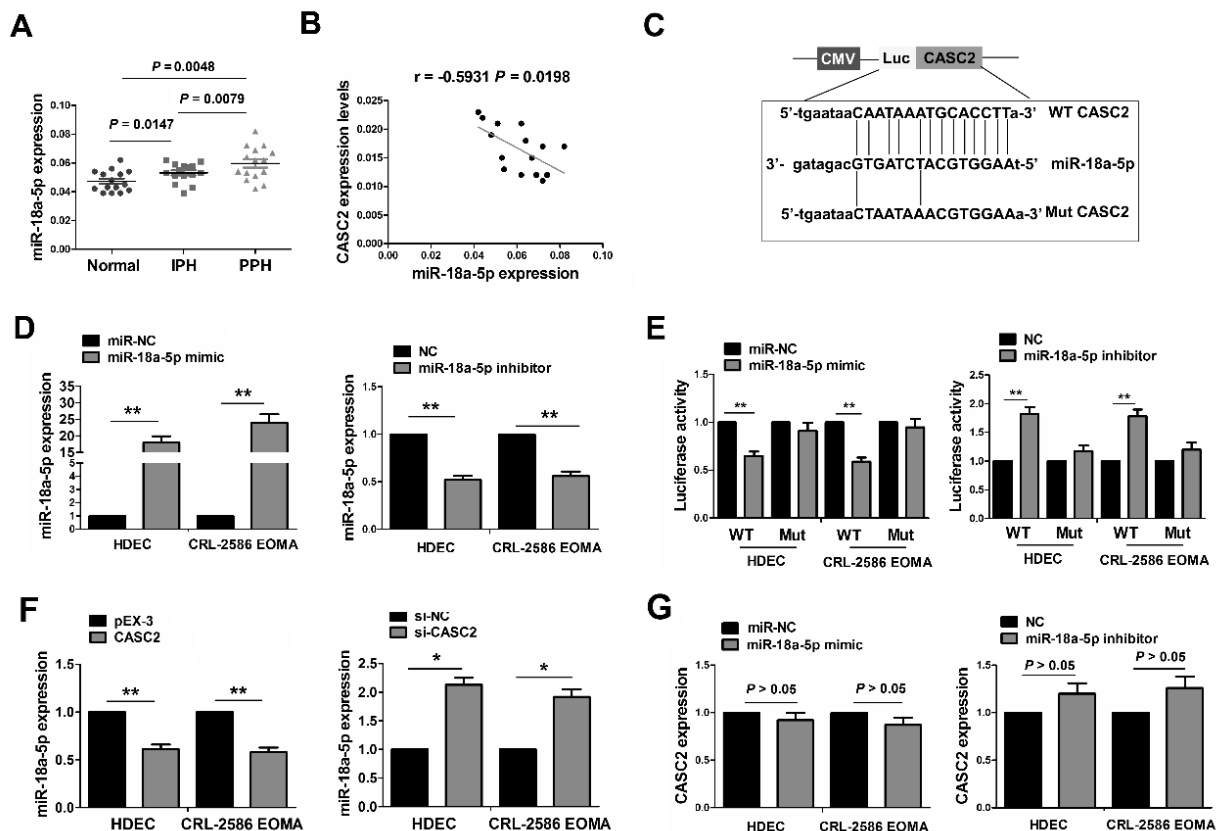


Fig. 4. LncRNA CASC2 could bind with miR-18a-5p in HAs cells. **A)** qRT-PCR analysis of the expression levels of miR-18a-5p in PPH, IPH and normal tissues. **B)** Pearson correlation analysis of the correlation of CASC2 with miR-18a-5p expression in PPH. **C)** Schematic representation of the binding sites of WT or Mut CASC2 with miR-18a-5p. **D)** qRT-PCR analysis of the transfection efficiency of miR-18a-5p mimic or inhibitor in HDEC and CRL-2586 EOMA cells. **E)** Luciferase activities of WT or Mut CASC2 after transfection with miR-18a-5p mimic or inhibitor in HDEC and CRL-2586 EOMA cells. **F)** qRT-PCR analysis of the expression levels of miR-18a-5p after transfection with CASC2 or si-MEG3 plasmids in these two cell lines. **G)** qRT-PCR analysis of the effects of miR-18a-5p mimic or inhibitor on CASC2 expression levels in HDEC and CRL-2586 EOMA cells. Data are the means $\pm$ SEM of three experiments. $*P < 0.05$; $**P < 0.01$.

vert_71/) was used to identify F-box and leucine-rich repeat protein 3 (FBXL3) as a direct target of miR-18a-5p (Cumulative weighted context score = - 0.80). FBXL3 expression levels were substantially decreased in PPH as compared with IPH or normal tissues ($P < 0.0001$, $P < 0.0001$) and displayed a negative correlation with miR-18a-5p expression in PPH ($r = -0.7175$, $P = 0.0026$; Fig. 5B). The binding sites of miR-18a-5p with WT or Mut FBXL3 3'UTR are indicated in Fig. 5C. Then, the luciferase activities of WT FBXL3 3'UTR were lowered by miR-18a-5p mimic, but those of Mut FBXL3 3'UTR were unaffected by miR-18a-5p in HAs cells (Fig. 5D). qRT-PCR and Western blot analysis indicated that miR-18a-5p mimic significantly reduced the expression levels of

FBXL3 as compared with miR-NC group in HDEC and CRL-2586 EOMA cells.

DISCUSSION

Accumulating data have demonstrated that the expression levels of lncRNA CASC2 are reduced in a variety of malignancies, and downregulation of lncRNA CASC2 can be used to predict a poor survival in cancer (9-13), but the expression of lncRNA CASC2 is elevated in HAs and favors the proliferation of HAs cells (17). Our previous study indicated that VEGF can act as a proliferating marker in PPH (4). Herein, lncRNA CASC2 expression levels were diminished as compared with IPH and normal tissues, and displayed a negative correlation

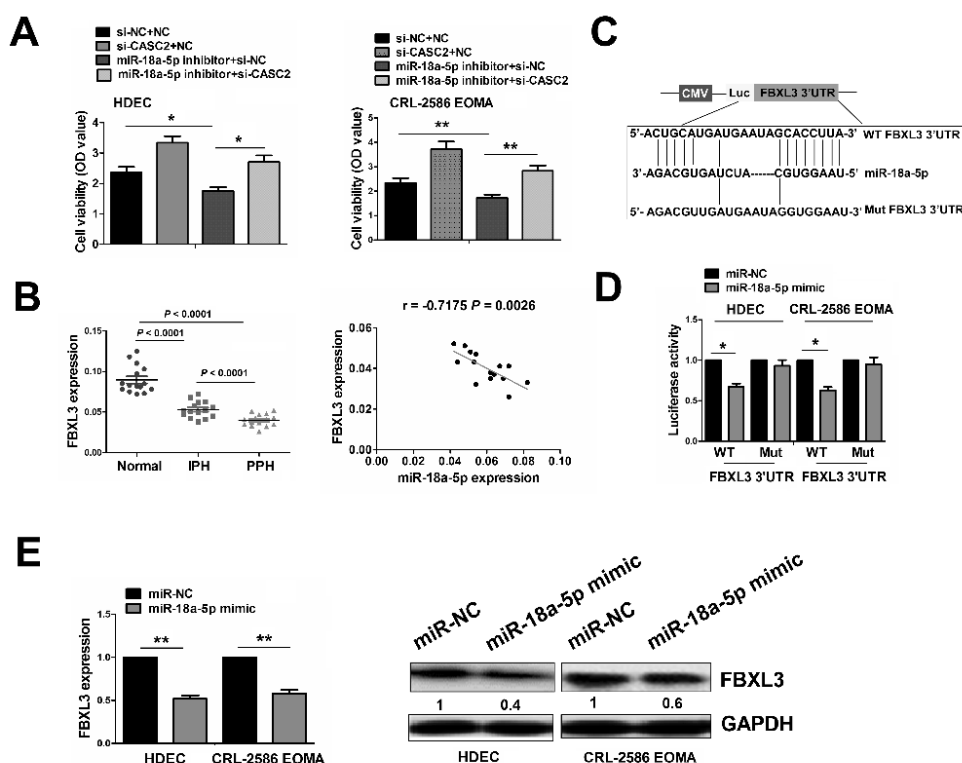


Fig. 5. miR-18a-5p reversed CASC2-induced anti-proliferation effects by targeting FBXL3. **A)** MTT analysis of the cell proliferative viability after transfection with si-CASC2 and (or) miR-18a-5p inhibitor in HDEC and CRL-2586 EOMA cells. **B)** qRT-PCR analysis of the expression levels of FBXL3 in PPH, IPH and normal tissues, and Pearson correlation analysis of the correlation of FBXL3 with miR-18a-5p expression in PPH. **C)** Schematic representation of the binding sites of miR-18a-5p with WT or Mut FBXL 3'UTR. **D)** Luciferase activity of WT or Mut 3'UTR of FBXL3 after transfection with miR-18a-5p mimic in HDEC and CRL-2586 EOMA cells. **E)** qRT-PCR and Western blot analysis of the effects of miR-18a-5p mimic on FBXL3 expression levels in HAs cells. Data are the means $\pm$ SEM of three experiments. * $P < 0.05$; ** $P < 0.01$.

with VEGF expression in PPH rather than IPH, indicating that lncRNA CASC2 might be a negative factor in PPH.

lncRNA CASC2 functions as a tumor suppressive factor in multiple cancers (9-12). We found that lncRNA CASC2 suppressed HAS cell viability and colony formation, while downregulation of lncRNA CASC2 exerted an opposite effect. In terms of the association of VEGF with the proliferation of HAS (4), we found that, lncRNA CASC2 downregulated VEGF expression, thereby leading to the inhibition of HAS cell proliferation.

lncRNA CASC2 can act as the sponge of miRNAs to modulate cancer growth. For example, lncRNA CASC2 sponges miR-18a/-367 to retard tumor proliferation in OS and HCC (11, 12). Herein, we identified that miR-18a-5p had the potential to bind with lncRNA CASC2. It was upregulated in PPH as compared with IPH and normal tissues, and possessed a negative correlation with lncRNA CASC2 in PPH. lncRNA GAS5 also negatively regulates miR-18a-5p to inhibit glioma cell invasion (18). We also found that lncRNA CASC2 negatively regulated miR-18a-5p to inhibit HAS cell growth.

MiR-18a-5p is upregulated in renal cell carcinoma (RCC) and non-small cell lung cancer (NSCLC) and accelerates the carcinogenesis of RCC and NSCLC (19, 20). On the other hand, miR-18a-5p is downregulated in breast cancer and irradiated lung cancer and inhibits the migration and invasion of breast cancer and increases the radio-sensitivity of lung cancer by targeting HIF-1 α (21, 22). Herein, miR-18a-5p was upregulated in PPH, and its inhibitor reduced HAS cell proliferation and reversed si-CASC2-induced cell proliferation. Furthermore, we identified FBXL3 as a direct target of miR-18a-5p in HAS cells and FBXL3 displayed a negative correlation with miR-18a-5p expression in PPH.

In conclusion, our findings demonstrated that lncRNA CASC2 suppressed HAS cell growth by regulating miR-18a-5p/FBXL3 axis and represents a potential therapeutic target for HAS.

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REGULATION OF EPITHELIAL MESENCHYMAL TRANSITION BY THE RENIN-ANGIOTENSIN SYSTEM: A ROLE FOR KLOTHO IN RENAL TUBULAR EPITHELIAL CELLS

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Klotho is a putative aging suppressor gene that is primarily expressed in renal tubular epithelial cells. Its expression has been reported to protect against fibrosis in human chronic kidney disease. However, the roles of klotho in epithelial-mesenchymal transition (EMT) and renal fibrosis are yet to be elucidated. The present study aimed to investigate the putative roles of klotho in angiotensin (Ang) II-induced damage of renal tubular epithelial cells. NRK-52E rat cells were treated with various combinations of Ang II, the Ang-converting enzyme inhibitor fosinopril (Fos) and the Ang II receptor antagonist valsartan (Val). The levels of transforming growth factor (TGF)- β 1, soluble klotho, α -smooth muscle actin (α -SMA) and E-cadherin in NRK-52E culture supernatants were measured using enzyme-linked immunosorbent assays. Furthermore, the mRNA and protein expression of TGF- β 1, klotho, α -SMA and E-cadherin was detected using semiquantitative reverse transcription-polymerase chain reaction, immunocytochemistry and Western blot analysis. The results demonstrated that Ang II inhibited the expression of klotho and E-cadherin, while it upregulated the expression of TGF- β 1 and α -SMA, in NRK52E cells. Fos and/or Val were revealed to enhance klotho and E-cadherin expression, and suppress the expression of TGF- β 1 and α -SMA, compared with the Ang II-only group. Furthermore, a positive linear correlation was detected between the expression of klotho and E-cadherin, while negative linear correlations with klotho expression were detected for TGF- β 1 and α -SMA expression. In conclusion, the expression of klotho was demonstrated to be enhanced following treatment with Fos and Val in Ang II-treated NRK-52E cells. The present results indicate that klotho may be involved in the inhibition of Ang II-induced EMT in renal tubular epithelial cells. Therefore, klotho may serve as a protective factor in renal tubulointerstitial fibrosis and aid the treatment of chronic kidney disease (CKD) patients using precision therapy.

Chronic kidney disease (CKD) is a major health concern worldwide, and its incidence rate is constantly increasing (1). Current treatments effectively slow the progression of CKD to end-stage renal disease (ESRD); however, a large number of patients with CKD still develop ESRD and require dialysis and kidney transplantation (2). CKD is characterized by kidney fibrosis that results from the aberrant deposition of fibrillar matrix components

in the space between renal tubules and peritubular capillaries (3). The fibrillar matrix consists of extracellular matrix proteins, including collagens that are secreted by interstitial fibroblasts. These matrix-producing fibroblasts are generated under pathological conditions from renal tubular epithelial cells that undergo epithelial-mesenchymal transition (EMT) (4). During the phenotypic transition of renal tubular epithelial cells into fibroblasts, the expression

Key words: klotho; angiotensin II; fosinopril; valsartan; epithelial-mesenchymal transition

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of mesenchymal markers is induced, while epithelial markers are downregulated. Therefore, during EMT, the expression of α -smooth muscle actin (α -SMA) and vimentin is upregulated, and the expression of E-cadherin is suppressed (4).

Angiotensin (Ang) II is a vasoconstriction-inducing peptide hormone, and is an effective pharmacological target for the prevention of CKD; the Ang-converting enzyme (ACE) inhibitor fosinopril (Fos) and the Ang II receptor antagonist valsartan (Val) have been reported to protect renal function in CKD (5). Targeting Ang II is an effective therapeutic strategy, as Ang II is actively involved in the development of renal fibrosis, which is partially mediated by transforming growth factor (TGF)- β . TGF- β_1 is reported to be a potent and ubiquitous growth factor that independently induces EMT in multiple cell types (6), while the Ang II receptor type 1 (AT₁) is reported to induce EMT in renal proximal tubular cells (7).

Previous studies have demonstrated that increased endogenous klotho expression protects against renal injury, preserves kidney function and suppresses renal fibrosis during CKD, which indicates that klotho may be a promising early biomarker for renal fibrosis and a potential novel therapeutic agent against CKD (8,9). The klotho gene was originally identified as a potential aging suppressor gene; klotho belongs to the protein family of glycosidases and is an ~130 kD protein with a single transmembrane domain that is primarily expressed in renal tubular epithelial cells (10). The extracellular domain of klotho is cleaved from the cell surface by secretases and released into the circulation where it functions as an endocrine factor. However, the intracellular domain of klotho is short (10 amino acids long) with no established functional domains. Following translocation of klotho from the endosomes to the cell membrane, its extracellular domain (soluble klotho) is cleaved and released into the circulation. Therefore, two types of klotho protein exist: Membrane-bound and secreted. Membrane-bound klotho acts as a co-receptor for fibroblast growth factor (FGF)-23, which is a bone-derived growth factor that

suppresses vitamin D synthesis and promotes the urinary excretion of phosphate, subsequently leading to a negative phosphate balance. Secreted klotho acts as a humoral factor that regulates the activity of several glycoproteins, ion channels and growth factor receptors on the cell surface (11). Soluble klotho levels are reduced in the early stages of CKD (12). It has been hypothesized that interactions between vitamin D, FGF-23 and klotho may form an endocrine axis for Ca²⁺ and phosphate metabolism, and the dysregulation of this axis may contribute to the progression of CKD (13).

Although a protective role has been reported for klotho against renal damage, the exact molecular mechanisms underlying its involvement in EMT and renal fibrosis are yet to be elucidated. In the present study, NRK-52E rat renal tubular epithelial cells were stimulated by Ang II *in vitro*, and pharmacological interventions, including Fos and Val, were applied to investigate the putative physiological roles of klotho during Ang II-induced damage of renal tubular epithelial cells and EMT.

MATERIALS AND METHODS

Rat renal tubular epithelial cell culture

Rat NRK-52E cells were kindly provided by Dr Xue-qing Yu (Department of Nephrology, First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China), and were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). Cells were cultured (37°C, humidity 95%, CO₂ 5%) for 24 h and reached 80% confluence prior to harvesting and allocation into five experimental groups: Control, cells received no drugs only culture medium; Ang II, cells received 100 nM Ang II (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany); Ang II + Fos, cells received 100 nM Ang II and 10 μ M Fos (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China); Ang II + Val, cells received 100 nM Ang II and 10 μ M Val (Novartis International AG, Basel, Switzerland); and Ang II + Fos + Val, cells received 100 nM Ang

II, 10 μ M Fos and 10 μ M Val. Cells in all groups were cultured with treatments for 24 h (37.0°C). The drug doses were selected according to a previous study (14).

TGF- β_1 , soluble klotho, α -SMA and E-cadherin levels in culture supernatants

The concentration of TGF- β_1 , soluble klotho, α -SMA and E-cadherin in cell culture supernatants was measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits. Biologically active TGF- β_1 (cat. no. K4344; BioVision, Inc., Milpitas, CA, USA) was measured in previously acidified samples, following the addition of 0.02 ml HCl (1 M) to 100 ml samples to achieve pH 1-2. Soluble klotho concentration was measured using a solid-phase sandwich ELISA (cat. no. 27601; Immuno-Biological Laboratories Co., Ltd., Gunma, Japan). The intra-assay and inter-assay coefficient of variation, as specified by the manufacturer's protocol, were 2.7-3.5 and 2.9-11.4%, respectively. The levels of α -SMA (cat. no. CSB-E1402789r; Cusabio Biotech Co., Ltd., College Park, MD, USA) and E-cadherin (cat. no. E-EL-R0347; Elabscience Biotechnology Co., Ltd., Wuhan, China) were detected in culture supernatants according to the manufacturers' protocols.

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

RNA was isolated from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA was reverse transcribed (42°C for 60 min then 70°C for 10 min) into cDNA according to manufacturer's protocol for the RevertAid First Strand cDNA Synthesis kit (K1622; Fermentas; Thermo Fisher Scientific, Inc.). The resultant cDNA was stored at -20°C until use. A commercial PCR kit was used for cDNA amplification (B630293-0100 100RXNS, Sangon Biotech Co., Ltd. Shanghai, China) Oligonucleotide primers for TGF- β_1 , klotho, α -SMA, E-cadherin and β -actin were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The sequences of the primers used were as follows:

TGF- β_1 , 5'-GGA CTA CTA CGC CAA AGA AG-3' (sense) and 5'-TCA AAA GAC AGC CAC TCA GG-3' (antisense); klotho, 5'-CAA TGG CTT CCC TCC TTT AC-3' (sense) and 5'-AGC ACA GGT TTG CGT AGT CT-3' (antisense); α -SMA, 5'-GCG TGG CTA TTC CTT CGT TAC-3' (sense) and 5'-CAT AGT GGT GCC CCC TGA TAG-3' (antisense); E-cadherin, 5'-ATT CTG ATT CTG CTG CTC TTG-3' (sense) and 5'-AGT AGT CAT AGT CCT GGT CTT-3' (antisense); and β -actin, 5'-CGT TGA CAT CCG TAA AGA C-3' (sense) and 5'-TGG AAG GTG GAC AGT GAG-3' (antisense). The following thermocycling conditions were employed: Denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, extension at 72°C for 1 min, for a total of 25 cycles, with a final extension step at 72°C for 10 min. A total of 10 μ l final products were subsequently electrophoresed on a 1.5% agarose gel (ethidium bromide 0.5 μ g/ml) and the bands were visualized using a UVP high performance UV transilluminator (UVP, Inc., Upland, CA, USA). The intensity of the TGF- β_1 (292 bp), klotho (512 bp), α -SMA (331 bp) and E-cadherin (420 bp) DNA bands was semiquantified using Bio-Rad Gel Doc 2000 Quantity One software v4.62 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and was expressed as a percentage of the intensity of the β -actin bands (201 bp).

Immunocytochemistry

Cells were cultured on slides (5x10⁵ cells/ml, 37°C) for 24 h for immunocytochemical analysis and fixed with 10% formalin at room temperature for 30 min, PBS wash 5 min twice, 0.5% Txiton X-100 at room temperature for 30 min, PBS wash 5 min twice. A commercial streptavidin-biotin complex (SABC) kit (Wuhan Boster Biological Technology, Ltd., Wuhan, China) was used. Briefly, the cells were incubated in 37°C with 3% H₂O₂ for 15 min to quench endogenous peroxidase activity, followed by incubation with 10% non-immunized serum (Rabbit control serum; Alpha Diagnostics International Inc., San Antonio, TX, USA) for 30 min at 37°C to block non-specific binding. Primary polyclonal antibodies against TGF- β_1 (1:150; cat. no.

sc-130348; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), klotho (1:150; cat. no. sc-22220; Santa Cruz Biotechnology, Inc.), E-cadherin (1:150; cat. no. sc-71008; Santa Cruz Biotechnology, Inc.) and α -SMA (1:150; cat. no. SAB5500002; Sigma-Aldrich; Merck KGaA) were subsequently added overnight at 4°C. Following primary antibody incubation, slides were incubated with biotinylated secondary antibodies (1:150; cat. no. BA1003; Wuhan Boster Biological Technology, Ltd.) for 90 min at 37°C, followed by SABC (1:100; SABC-POD kit; cat. no. BA0120; Wuhan Boster Biological Technology, Ltd.) reagent solution for 30 min at 37°C. Subsequently, slides were incubated with streptavidin peroxidase until the desired stain intensity was developed at 37°C, hematoxylin staining was performed at room temperature for 3 min and the slides were washed with flowing water, dehydrated through graded alcohols and mounted with coverslips. All the solutions were diluted in PBS and the slides were washed with PBS three times (each for 3 min) following each step. Brown and yellow coloring in the cytoplasm indicated positive results for TGF- β_1 , klotho, α -SMA and E-cadherin expression. A total of three randomly selected fields of view/slide under a polarizing microscope (magnification, x400; Olympus BX51, Olympus corporation, Tokyo, Japan) were assessed to detect protein expression; a positive signal was automatically semiquantified using the absorbance value representing the intensity of positive staining with a pathology image analysis system (PIPS-2000; Chongqing Tianhai Medical Equipment Co., Ltd., Chongqing Shi, China).

Western blot analysis

Cells were collected into lysis buffer ($1/10^8$ cells/ml, 2XSDS buffer; SDS lysis buffer P0013G; Beyotime Biotechnology, China.) in an EP tube on ice (4°C) for 30 min, subsequently centrifuged for 20 min (4°C, $16099.2 \times g$). Protein yield was measured by Bradford Protein Assay kit (Hangzhou MultiSciences Lianke Biotech Co., Ltd., Hangzhou, China). Extracted protein samples were boiled for 5 min and 50 μ g protein was separated by

12.5% SDS-PAGE and electrotransferred onto nitrocellulose membranes. Membranes were blocked in 5% non-fat milk in PBS for 2 h at room temperature, followed by incubation with primary antibodies against TGF- β_1 (1:300; sc-130348; Santa Cruz Biotechnology, Inc.), klotho (1:300; cat. no. sc-22220; Santa Cruz Biotechnology, Inc.), α -SMA (1:300; cat. no. SAB5500002; Sigma-Aldrich; Merck KGaA) and E-cadherin (1:300; cat. no. sc-71008; Santa Cruz Biotechnology, Inc.) overnight at 4°C. The amount of protein expression was detected by Western blotting with GAPDH (1:300; cat. no. sc-47724; Santa Cruz Biotechnology, Inc.) as a control. Membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (1:300; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence Western blotting detection system (The ECL Western Blotting Substrate kit; cat. no. ab65623; Abcam, Cambridge, UK; Image Quant 300/Image Master Total Lab; GE Healthcare Life Sciences, Little Chalfont, UK). IPP 6.0 was used for densitometry (Image Pro Plus 6.0, Medin Cybernetics Inc).

Statistical analysis

Data are presented as the mean $\pm$ standard deviation of three independent experiments. The statistical significance of the differences between groups was assessed using one-way analysis of variance followed by a post-hoc Student-Newman-Keuls test for multiple comparisons. Correlations between the expression of klotho and various proteins of interest were analyzed using Pearson's correlation coefficient. Statistical analysis was performed using SPSS software version 22.0 (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Effects of Fos and Val on the secreted levels of TGF- β_1 , soluble klotho, α -SMA and E-cadherin

The concentrations of TGF- β_1 , soluble klotho,

α -SMA and E-cadherin in culture supernatants of NRK-52E cells were measured using ELISA. The present results demonstrated that levels of soluble klotho and E-cadherin were decreased following Ang II stimulation, compared with control cells (Fig. 1). However, soluble klotho and E-cadherin concentrations in the Ang II + Fos, Ang II + Val and Ang II + Fos + Val groups were significantly increased compared with the Ang II group (Fig. 1). Conversely, the concentrations of TGF- β_1 and α -SMA were increased in conditioned media from Ang II-treated NRK-52E cells, compared with control cells, while levels were downregulated following treatment with Fos and/or Val compared with the Ang II group (Fig. 1). No significant differences were detected in the levels of TGF- β_1 , soluble klotho, α -SMA and E-cadherin between the control and the Ang II + Fos, Ang II + Val and Ang II + Fos + Val groups (Fig. 1).

TGF- β_1 , klotho, α -SMA and E-cadherin mRNA expression in NRK-52E cells

Semiquantitative RT-PCR was performed to assess the mRNA expression of TGF- β_1 , klotho, α -SMA and E-cadherin in NRK-52E cells from the

various treatment groups. The results demonstrated that the mRNA expression of klotho and E-cadherin was significantly downregulated in Ang II-treated cells, compared with the control group (Fig. 2); however, Fos and/or Val treatment prevented the Ang II-induced decrease in klotho and E-cadherin mRNA expression. The expression of TGF- β_1 and α -SMA mRNA demonstrated the opposite trend: Expression was significantly upregulated by Ang II stimulation, while Fos and/or Val treatment suppressed the Ang II-induced increase in the mRNA expression levels (Fig. 2). No significant differences in TGF- β_1 , klotho, α -SMA and E-cadherin mRNA expression were detected between cells treated with Fos or Val alone and those that received combination treatment (Fig. 2).

Immunocytochemical detection of TGF- β_1 , klotho, α -SMA and E-cadherin in NRK-52E cells

Immunocytochemical analysis demonstrated that the protein expression of klotho and E-cadherin in Ang II-treated cells was significantly suppressed compared with control cells; however, following treatment with Fos and/or Val, the expression of klotho and E-cadherin was significantly

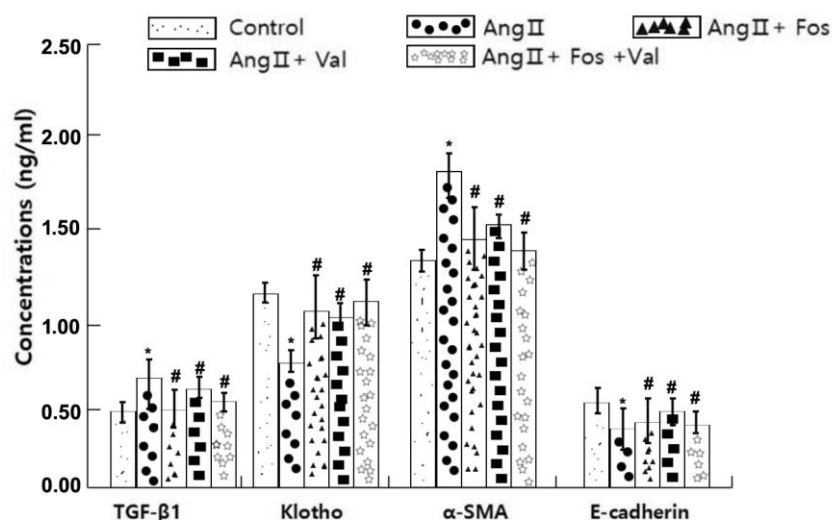


Fig.1. Concentration of TGF- β_1 , soluble klotho, α -SMA and E-cadherin in NRK-52E cell culture supernatants. NRK-52E cells received treatment with Ang II, with or without treatment with Fos and/or Val, and ELISA was performed to determine the levels of TGF- β_1 , soluble klotho, α -SMA and E-cadherin in NRK-52E cell culture supernatants. Data are presented as the mean $\pm$ standard deviation, $n=7$. * $P<0.05$ vs control group; # $P<0.05$ vs Ang II group. TGF: transforming growth factor; SMA: smooth muscle actin; Ang: angiotensin; Fos: fosinopril; Val: valsartan.

upregulated in Ang II-treated cells (Fig. 3A and B). Compared with the control group, the protein expression of TGF- β_1 and α -SMA in the Ang II group was significantly enhanced, while Fos and/or Val treatment attenuated the Ang II-induced increase in TGF- β_1 and α -SMA expression. No significant differences were detected between the control, Ang II + Fos, Ang II + Val and Ang II + Fos + Val groups (Fig. 3A and B).

Western blot analysis of TGF- β_1 , klotho, α -SMA and E-cadherin in NRK-52E cells

In order to confirm the results of immunocytochemical analysis, Western blot analysis was performed. The results demonstrated that cells in the Ang II group exhibited decreased klotho and E-cadherin protein expression levels, compared with the control group, while treatment with Fos and/or Val significantly counteracted the Ang II-induced decrease in protein expression (Fig. 3C and D). Conversely, the protein expression of TGF- β_1 and α -SMA was significantly enhanced in Ang II-treated

NRK-52E cells, compared with the control group; however, Fos and/or Val treatment suppressed the Ang II-induced increase in protein expression (Fig. 3C and D). There were no significant differences in protein expression between the control and the Fos- and/or Val-treated cells (Fig. 3C and D).

Correlation analysis of klotho and TGF- β_1 , α -SMA and E-cadherin protein expression

Correlation analysis indicated that the protein expression of klotho exhibited a negative linear correlation with TGF- β_1 and α -SMA expression ($r=-0.685$ and $r=-0.641$, respectively; $P<0.05$; Fig. 4A and B). Conversely, a positive linear correlation was identified between the expression of klotho and E-cadherin in NRK-52E cells ($r=0.478$; $P<0.05$; Fig. 4C).

DISCUSSION

The aim of the present study was to investigate the potential inhibitory roles of klotho against Ang II-induced EMT, and to investigate the protective

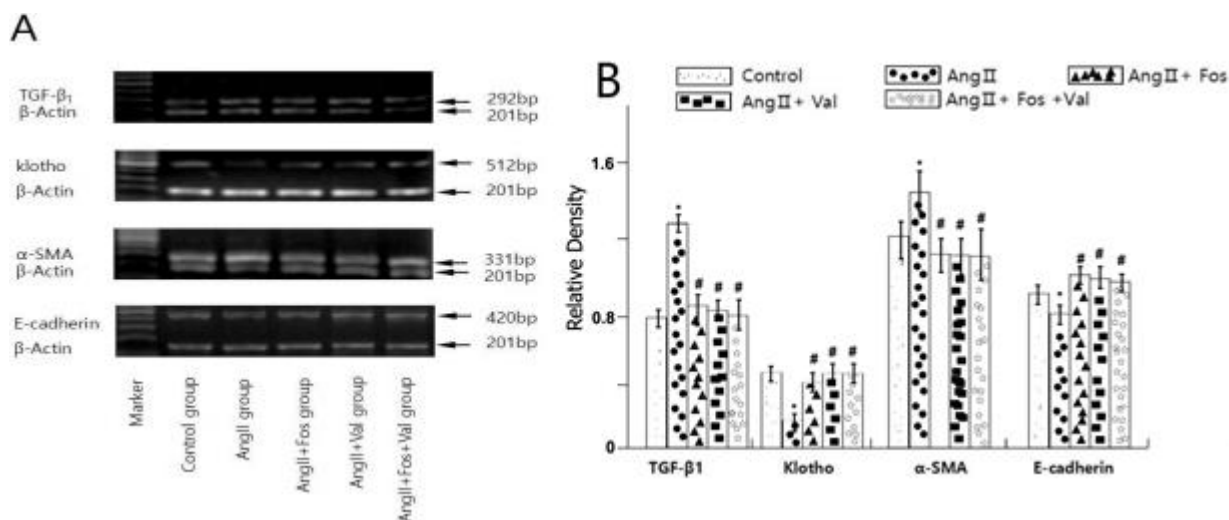


Fig. 2. mRNA expression levels of TGF- β_1 , klotho, α -SMA and E-cadherin in NRK-52E cells were determined by RT-PCR. **A)** Representative gel demonstrating the mRNA expression of TGF- β_1 , klotho, α -SMA and E-cadherin in NRK-52E cell from various treatment groups. **B)** RT-PCR results were semiquantified by densitometry. Data are presented as the mean $\pm$ standard error, $n=3$. * $P<0.05$ vs control group; # $P<0.05$ vs Ang II group. TGF: transforming growth factor; SMA: smooth muscle actin; RT-PCR: reverse transcription-polymerase chain reaction; Ang: angiotensin; Fos: fosinopril; Val: valsartan.

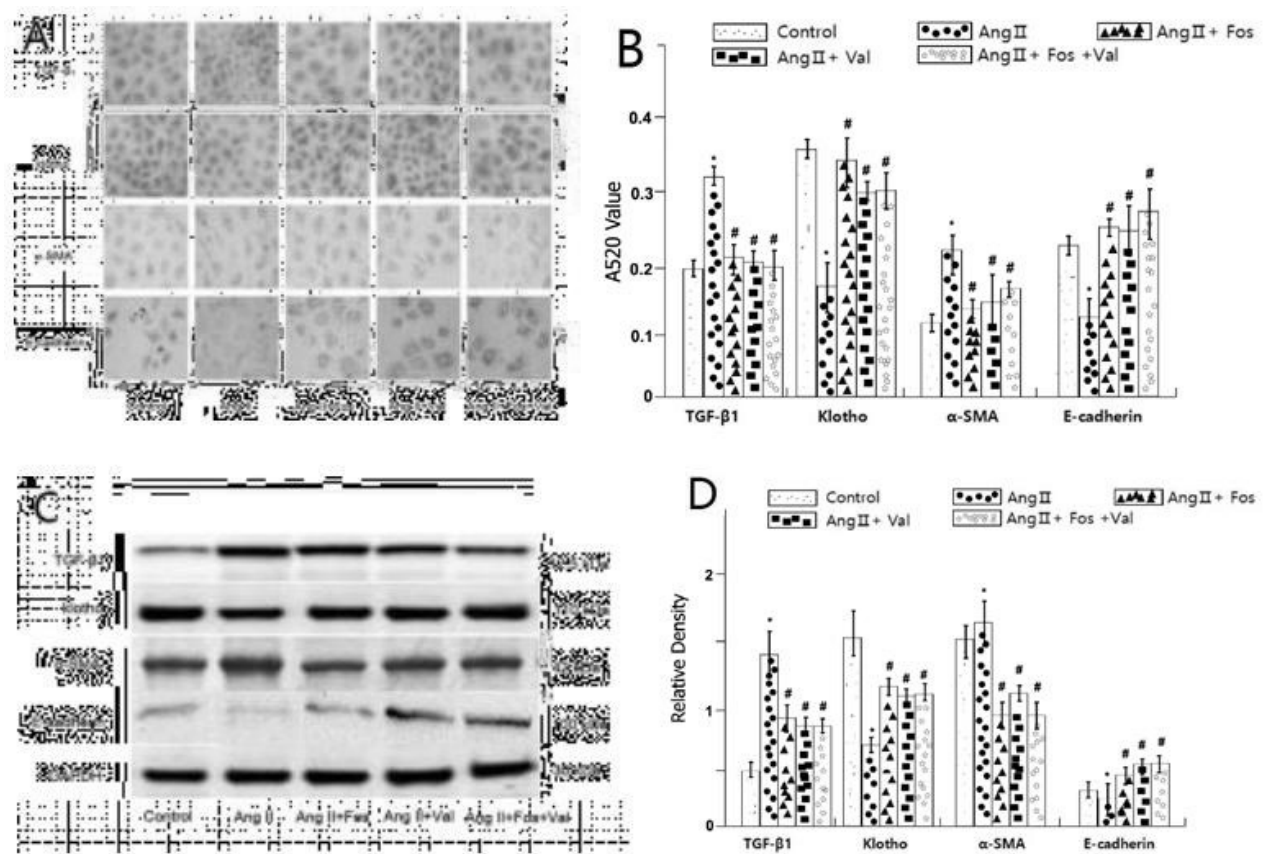


Fig. 3. Protein expression levels of TGF- β_1 , klotho, α -SMA and E-cadherin in NRK-52E cells were determined by ICC and Western blotting. **A)** Representative ICC staining images demonstrating the expression of TGF- β_1 , klotho, α -SMA and E-cadherin under $\times 400$ magnification. **B)** Semiquantitative analysis of ICC staining results. **C)** Western blot analysis was performed to detect the protein expression of TGF- β_1 , klotho, α -SMA and E-cadherin. Representative Western blot bands are presented. **D)** Blots were semiquantified by densitometry. Data are presented as the mean $\pm$ standard error, $n=3$. * $P<0.05$ vs control group; # $P<0.05$ vs Ang II group. TGF: transforming growth factor; SMA: smooth muscle actin; ICC: immunocytochemistry; Ang: angiotensin; Fos: fosinopril; Val: valsartan.

effects of Fos and Val in Ang II-induced renal damage in renal tubular epithelial cells *in vitro*. The results of the current study indicated that altered klotho expression may be implicated in the Ang II-induced EMT of NRK-52E cells. In addition, previous studies performed in our lab reported that Ang II suppressed klotho expression in NRK-52E renal epithelial cells, while Fos and/or Val reversed the Ang II-induced downregulation of klotho expression (14, 15). The present study demonstrated that Ang II stimulation increased the expression of TGF- β_1 and α -SMA in NRK-52E cells, and inhibited the expression of

klotho and E-cadherin. In addition, to the best of our knowledge, the present study demonstrated for the first time that klotho expression exhibited a negative linear correlation with the expression of TGF- β_1 and α -SMA, while it was positively correlated with the expression of E-cadherin. Furthermore, in the current study, Fos and/or Val treatment counteracted the Ang II-induced decreases in the expression of klotho and E-cadherin, and downregulated the Ang II-induced increases in TGF- β_1 and α -SMA expression. Therefore, the present results indicate that Ang II may stimulate the expression of TGF- β_1

and α -SMA to promote EMT, which may be attenuated following the upregulation of klotho and E-cadherin expression through treatment with Fos and/or Val. In combination with the results from our previous studies, it may be hypothesized that klotho is a pivotal component in the regulation of TGF- β_1 -induced α -SMA expression in renal epithelial cells during EMT, and Fos and Val may exert a protective role against Ang II-induced EMT by enhancing the expression of klotho and E-cadherin, and suppressing

the expression of TGF- β_1 and α -SMA. Further studies are required to fully elucidate the roles of klotho in renal interstitial transdifferentiation during EMT processes and to investigate the molecular mechanisms that may be involved (6, 16).

A previous study (17) demonstrated that the chronic activation of the renin-Ang system served a deleterious role in hypertension and progressive renal injury, and the renal proximal tubule has an important role in tubulointerstitial fibrosis. Ang II

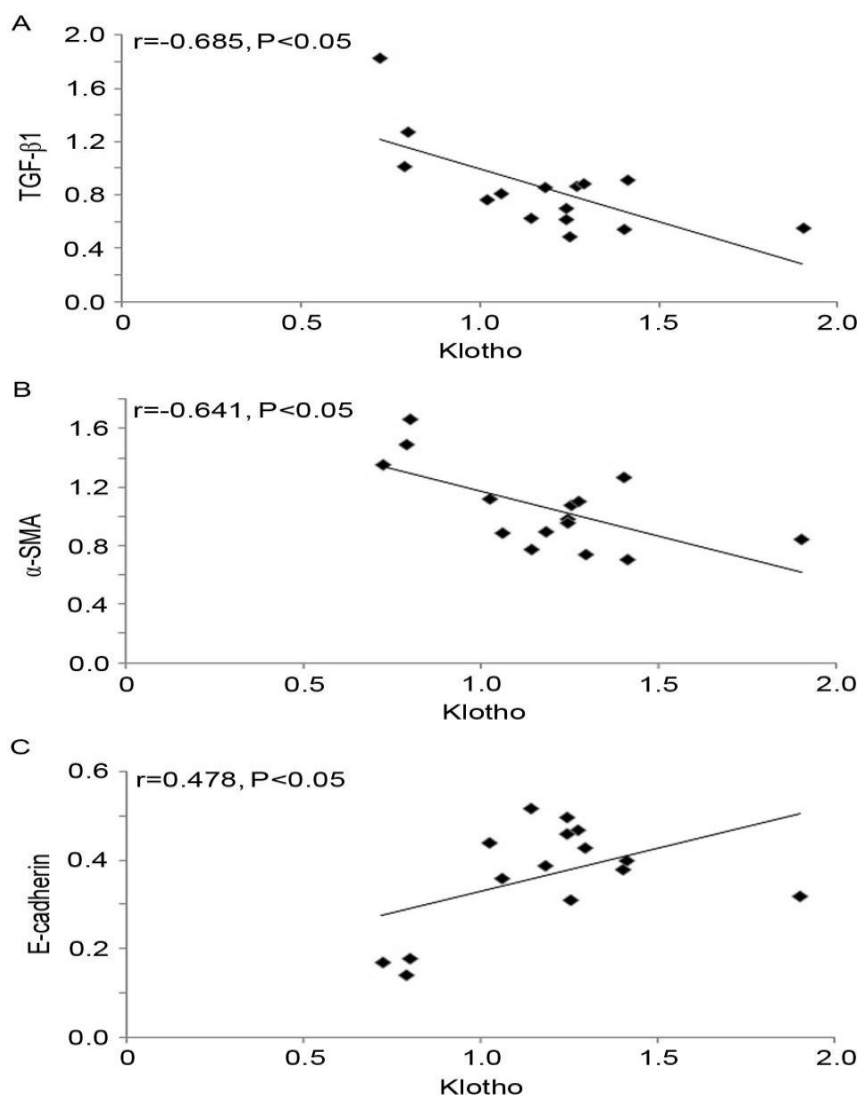


Fig. 4. Pearson's correlation analysis was performed to investigate the correlation between the protein expression levels of klotho and TGF- β_1 , α -SMA and E-cadherin in Ang II-stimulated NRK-52E cells. **A)** TGF- β_1 and klotho expression demonstrated a negative linear correlation ($r = -0.685$, $P < 0.05$). **B)** α -SMA and klotho expression exhibited a negative linear correlation ($r = -0.641$, $P < 0.05$). **C)** A positive linear correlation was detected between the expression of E-cadherin and klotho ($r = 0.478$, $P < 0.05$). TGF: transforming growth factor; SMA: smooth muscle actin.

is one of the major components of the renin-Ang system, and activates mesangial cells, tubular cells and interstitial fibroblasts, increases the expression and synthesis of extracellular matrix proteins, and serves an important role in the progression of renal disease. Ang II has been reported to alter epithelial cell morphology and induce the expression of mesenchymal markers; these effects may be partially mediated by the upregulation of TGF- β_1 (18), which has a central role in EMT. In the present study, Ang II was demonstrated to induce upregulation of TGF- β_1 expression, which may in turn enhance α -SMA expression and, consequently, suppress the expression of klotho and E-cadherin, thus causing NRK-52E cells to lose their epithelial features. Notably, these effects were revealed to be counteracted through the inhibition of the renin-Ang system by the administration of Fos and Val. The results of the present study indicated that Ang II-induced EMT may be prevented through the upregulation of klotho expression by inhibitors of the renin-Ang system.

In the kidneys, during EMT, renal tubular epithelial cells lose their epithelial and adhesive properties, and undergo a phenotypic transformation into myofibroblasts. This transformation is associated with the loss or downregulation of epithelial markers, such as E-cadherin, and increased expression of mesenchymal markers, including vimentin and α -SMA (19). During EMT, the loss of epithelial markers, particularly E-cadherin, which is essential for the structural integrity of the renal epithelium, is a critical process that is regulated by various transcriptional repressors, including klotho and p53 (20). Renal EMT may be triggered by various growth factors, including TGF- β_1 , which is a pivotal stimulus that is regulated by Ang II. Numerous studies have demonstrated that increased TGF- β_1 expression and secretion may be used as a biomarker for renal damage (21-24), and it was reported to be implicated in the dedifferentiation of epithelial cells in response to chronic Ang II treatment (6). These findings indicated that TGF- β_1 may be among the most potent inducers of EMT.

EMT in the kidneys is associated with tubulointerstitial fibrosis and renal dysfunction; Li et

al. (25) demonstrated that EMT contributed to the early development and progression of renal interstitial fibrosis. The renin-Ang system has been reported to contribute to ESRD, while ACE inhibitors and AT₁ blockers are often employed to treat patients with CKD and are effective in delaying the progression of renal fibrosis. However, ACE inhibitors and AT₁ blockers are incapable of halting or reversing the fibrotic progress (2, 5). The results of the present study demonstrated that Ang II stimulation resulted in EMT in NRK-52E cells; cells were revealed to lose their epithelial marker E-cadherin, and were characterized by decreased klotho expression, and increased expression of the fibrosis inducer TGF- β_1 and the myofibroblast marker α -SMA. In addition, the current study also demonstrated that Fos and Val treatment attenuated the downregulation of klotho expression and may partially reverse the Ang II-induced EMT of NRK-52E cells, as indicated by decreases in TGF- β_1 and α -SMA expression, and increased E-cadherin expression, following treatment with Fos and/or Val in Ang II-exposed cells.

In order to further investigate the association between expression of klotho and EMT in renal epithelial cells, correlations between the expression of klotho and TGF- β_1 , α -SMA and E-cadherin were analyzed in NRK-52E cells following exposure to Ang II. The results demonstrated that a significant negative correlation with klotho was detected for TGF- β_1 and α -SMA expression, while the expression of klotho exhibited a significant positive correlation with E-cadherin expression. Therefore, the results suggest that the increased klotho expression may decrease the Ang II-induced EMT and serve as a protective factor in renal tubulointerstitial fibrosis.

Evidence has indicated that klotho may exert renoprotective and antifibrotic properties. Klotho was first identified as a single-pass transmembrane protein with potential anti-aging properties (26). Membrane-anchored klotho functions as a co-receptor for FGF-23 to promote renal phosphate excretion and inhibit active vitamin D synthesis, while soluble klotho is cleaved from the cell membrane (6,27) and acts as a hormone to suppress fibrosis (28). Previous studies have reported that

klotho deficiency in animals and humans resulted in severe kidney damage and renal fibrosis, and directly promoted EMT in renal epithelial cells (9, 29). Klotho has also been identified as an endogenous inhibitor of several growth factors, including TGF- β_1 (6,28); TGF- β_1 may suppress the development of renal fibrosis through the inhibition of TGF- β_1 signaling (15), and has been implicated in the pathogenesis of renal fibrosis in renal diseases (30). TGF- β_1 has been reported to inhibit the expression of genes that are crucial for the maintenance of an epithelial phenotype, including E-cadherin, and to promote the expression of factors that are required for a mesenchymal phenotype, including α -SMA (18). The antifibrotic properties of klotho may be attributed to its ability to suppress TGF- β_1 signaling. Klotho is thought to bind with high affinity to the TGF- β receptor II and inhibit the binding of TGF- β_1 , thus blocking TGF- β_1 signaling. Therefore, klotho may suppress the EMT process and the development of fibrosis by preventing the interaction between TGF- β_1 and the TGF- β receptor II (6).

In conclusion, the results of the present study indicate that the activation of klotho expression may attenuate Ang II-induced EMT in NRK-52E cells, which may occur, at least partially, through inhibition of the TGF- β_1 signaling pathway. Future study may confirm that klotho inhibits the renal tubular EMT process by TGF- β_1 signaling pathway, which will provide novel mechanistic insights into the role of Klotho in renal tubulointerstitial fibrosis and contribute to treatment for CKD patients by precision therapy.

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BAICALIN ATTENUATES LIPOPOLYSACCHARIDE-INDUCED RENAL TUBULAR EPITHELIAL CELL INJURY BY INHIBITING THE TXNIP/NLRP3 SIGNALLING PATHWAY VIA INCREASING miR-223-3p EXPRESSION

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Baicalin has been used in China to treat inflammation-related diseases, such as inflammation-induced acute kidney injury (AKI). However, the specific mechanism of baicalin remains unclear. To observe the protective effects of baicalin on lipopolysaccharide (LPS)-induced inflammatory injury of renal tubular epithelial cells (HK-2 cells) and to explore its protective mechanism. LPS (1 mg/L) was used to induce an HK-2 cell inflammatory injury model *in vitro*. The cells were divided into seven groups: the normal control group, LPS-induced group, LPS plus 5 µmol/L baicalin treatment group, LPS plus 15 µmol/L baicalin treatment group, LPS plus 25 µmol/L baicalin treatment group, LPS plus 50 µmol/L baicalin treatment group, and LPS plus 75 µmol/L baicalin treatment group. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay was employed for detecting the relative survival rate of HK-2 cells. Enzyme-linked immunosorbent assay was used for detecting the levels of inflammatory factors, including interleukin-6 (IL-6), IL-1β, and tumor necrosis factor-α (TNF-α). Moreover, the expression of inducible nitric oxide synthase (iNOS); cyclooxygenase-2 (COX-2); nuclear factor κB65 (NF-κB65); phosphorylated NF-κB inhibitory protein-α (p-IκB-α); NF-κB inhibitory protein (IκB); human thioredoxin interacting protein (TXNIP); and human NACHT, LRR, and PYD domain-containing protein 3 (NLRP3) were determined by Western blot analysis. The expression levels of NLRP3 and TXNIP mRNA and miR-223-3p were determined by RT-PCR. Results found that the relative survival rate of HK-2 cells treated with different baicalin concentrations was significantly increased ($P<0.05$) and the levels of the inflammatory factors IL-6, IL-1β, and TNF-α were significantly decreased ($P<0.05$) compared with those of the LPS-induced group. The expression levels of the inflammatory proteins inducible nitric oxide synthase and cyclooxygenase-2 and the genes expressions of TXNIP and NLRP3 were significantly decreased in the cells ($P<0.05$), while the expression level of miR-223-3p was significantly increased ($P<0.05$). These changes were induced in a dose-dependent manner. The results suggest that baicalin significantly inhibited the expression of inflammation-related proteins and alleviated LPS-induced inflammatory injury in HK-2 cells. The mechanism may be associated with the inhibition of activation of the TXNIP/NLRP3 inflammatory pathway, which might be mediated by increased expression of miR-223-3p. Thus, NLRP3 is a regulatory target of miR-223-3p.

Key words: renal tubular epithelial cells, inflammatory response, mir-223-3p, NLRP3 inflammasome

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The kidney is one of the most vulnerable target organs of inflammation in endotoxin-induced sepsis (1). More than 50% of patients in the intensive care unit have acute kidney injury (AKI) (2), which has a mortality rate of 30–60% and seems to be increasing (3, 4). The mortality from sepsis-induced AKI is significantly higher than that of normal AKI. When sepsis occurs, a systemic inflammatory reaction is induced, a large number of inflammatory mediators are released, and kidney inflammation is exacerbated by a cascade of reactions, which then triggers the inflammatory immune response, inducing effects such as cell apoptosis, cell infiltration, and renal tubular necrosis. Moreover, thromboplastin and fibrinolytic cascade imbalance causes microthrombus formation in the glomeruli and renal tubules. All of these factors are the direct and/or indirect causes of kidney damage. To date, in addition to the timely use of antibiotics and fluid replacement, clinical treatments for sepsis and its complications, such as AKI (5), include surgical drainage of fluid, haemodialysis to protect against renal failure, mechanical ventilation to improve lung function, and providing appropriate support for organ dysfunction (5). However, these treatments cannot reduce the high mortality rate. Therefore, effective prevention and treatment methods for kidney damage are an important part of the therapeutic treatment of sepsis.

NACHT, LRR, and PYD domain-containing protein 3 (NLRP3) is a pattern recognition receptor that senses tissue damage and risk signals in the cytoplasm (6). It forms a multi-protein complex (NLRP3 inflammasome) with apoptosis-associated Speck-like protein containing a CARD and effector protein caspase-1 (7). This multi-protein complex is involved in innate immune responses derived from viral or bacterial pathogen molecules or from aseptic risk-signalling molecules. Nuclear factor kappa B (NF- κ B) is induced by activating Toll-like receptors, thereby activating expression of NLRP3 and interleukin (IL)-1 β proprotein (8). Thioredoxin-interacting protein (TXNIP) activates the NLRP3 inflammatory pathway under various stimuli (9, 10). Overactivation of the NLRP3 inflammasome induces the activation of caspase-1 and caspase-1 cleaves the proproteins IL-1 β and IL-18 into mature IL-1 β and

IL-18, which in turn activate the downstream pro-inflammatory signal transduction pathway, causing various inflammatory lesions (11). The TXNIP/NLRP3 signalling pathway is also highly activated in the AKI model (10).

Scutellaria baicalensis is a traditional Chinese medicine with a long history. It is associated with cooling the blood, detoxification, haemostasis and miscarriage prevention. Baicalein is one of the main active ingredients of *S. baicalensis*. It has various biological activities, such as anti-inflammatory, anti-oxidative, and anti-apoptotic effects. Recent studies have found that baicalein can alleviate inflammatory responses by inhibiting the toll-like receptor 4 (TLR4)-mediated NF- κ B and mitogen-activated protein kinase (MAPK) signalling pathways in a mouse model of lipopolysaccharide (LPS)-induced mastitis (12). Baicalein can reduce oxidized low-density lipoprotein (oxLDL)-induced oxidative stress and the inflammatory response by regulating adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK)- α expression (13). Baicalein combined with 12/15-lipoxygenase can alleviate myocardial ischaemia/reperfusion injury through multiple signalling pathways (14). It can also reduce heart and kidney damage caused by myocardial ischaemia/reperfusion injury (15, 16). Some studies also found that baicalein improves the viability of hypoxia-reoxygenation-treated HK-2 cells (17), prevents cisplatin-induced AKI by downregulating the activities of the MAPK and NF- κ B pathways and increasing antioxidant production (18).

MicroRNAs (miRNAs) are considered an important class of gene expression regulators. They consist of approximately 18–25 nucleotides and are a class of small non-coding RNAs that inhibit RNA translation. miRNAs also promote mRNA degradation by binding to the 3'-end untranslated region of an mRNA, playing a regulatory role in gene expression (19). A growing number of studies have focused on miRNAs for disease detection or as therapeutic targets (20, 21). Different miRNAs play various roles in the inflammatory response and promote or inhibit inflammation (22–24). miR-223 regulates IKK α together with miR-15a and miR-16 during the differentiation of monocytes into

macrophages; when the expression levels of these three miRNAs are decreased, IKK α phosphorylation is increased, which in turn activates NF- κ B (25).

miRNA-223 is known as a myeloid-rich anti-inflammatory miRNA. In miR-223-deficient zebrafish, activation of the NF- κ B pathway is elevated, and neutrophilic inflammation (wound response) is increased (26). miR-223 inhibits lipid deposition and inflammation by inhibiting the TLR4 signalling pathway in macrophages (27). However, the underlying mechanism remains unclear. The present study predicted that NLRP3 is a miRNA-223 regulatory target by using TargetScan software. Therefore, we hypothesized that miR-223 plays an important role in the occurrence and development of endotoxin-induced acute renal tubular injury and further explored whether baicalin can reduce the activity of the TXNIP/NLRP3 signalling pathway and improve endotoxin-induced acute renal tubular injury by increasing the expression of miR-223.

MATERIALS AND METHODS

HK-2 cell culture and groupings

HK-2 cells obtained from the US ATCC cell bank (Shanghai Sixin Biotechnology Co., Ltd., Shanghai, China) were cultured in RPMI 1640 medium containing 10% foetal bovine serum. When the cells reached 70% confluence, they were seeded in a Petri dish at a density of 1×10^4 /mL per well and cultured in an incubator with 5% CO₂ at 37°C. The cells were digested with 0.25% trypsin, split 1:2 and cultured for 7 days. Then, the cells were seeded in a 25 cm² culture flask at a density of 1×10^5 cells/mL, and were synchronized for 24 h and then grouped after reaching 60% confluence. To observe the effects of LPS on HK-2 cell activity and survival rate, as previously described (28), different concentrations of LPS (0.25, 0.5, 1.0, 1.5, and 2.0 mg/L) were added to the cell cultures for 24 h. To observe the effects of baicalin on endotoxin-induced HK-2 cell injury, the cells were divided into the following groups: normal control group, LPS-induced group, LPS plus 5 μ mol/L baicalin treatment group, LPS plus 15 μ mol/L baicalin treatment group, LPS plus 25 μ mol/L baicalin treatment group, LPS plus 50 μ mol/L baicalin treatment group, and LPS plus 75 μ mol/L baicalin treatment group. Based on previous research

(29), different concentrations of baicalin (5–75 μ M) were added to the cell cultures firstly for 2 h and then LPS solution (1mg/L) was added and cultured for another 24 h. Subsequently, various related indicators were measured.

Analysis of cell viability

The proliferation activity of cells in the different treatment groups was determined using a CCK-8 kit (Beijing Think-Far Technology Co., Ltd., Beijing, China). HK-2 cells were inoculated into a 96-well plate. After the cells reached 70% confluence, the medium was replaced with serum-free medium. Approximately 10 μ L of CCK-8 solution was added to each well after treatment at different conditions, and the samples were incubated for 2–4 h in the dark. A microplate reader was then used to detect the absorbance value (A450 nm) at a wavelength of 450 nm.

Cell transfection

Approximately 2×10^4 /mL HK-2 cells in the logarithmic growth phase were inoculated into a six-well plate. When the cells reached 60% confluence, the miR-223-3p mimic, miR-223-3p inhibitor, mimic control, and inhibitor control were transfected into the HK-2 cells following the instructions for the Lipofectamine 2000 transfection reagent; the transfection concentration was 50 nmol/L. The medium was replaced with fresh medium after 6 h of transfection and cultured for 24 h. Subsequently, the cells were collected for RT-PCR and Western blot analysis.

Determination of the survival rate and cell proliferation inhibition rate of HK-2 cells

The liquid from the 96-well plate was discarded. Approximately 270 μ L of DMEM and 30 μ L of 5 g/L MTT working solution were added, mixed well, and incubated at 37°C for 4 h. The culture solution in the well was discarded, and 270 μ L of dimethyl sulfoxide was added into each well and fully dissolved after 10 min of shaking. The OD value of each well was measured with a microplate reader at a wavelength of 490 nm. The OD value of the normal group was used as a control to calculate the survival rate (%) in each well: survival rate (%) = the OD value of each well/the mean OD value of the normal group $\times$ 100 (6). The cell proliferation inhibition rate was calculated using the following formula: cell proliferation inhibition rate (%) = (OD value in control group – OD

value in experimental group)/OD value in control group $\times 100\%$. ED50 and IC50 was calculated with the Bliss method, as previously described (30, 31).

Target gene prediction

NLRP3 was predicted to be the target gene of miR-223-3p by using target gene prediction software, including miR-Base (<http://www.mirbase.org/>), TargetScan (<http://www.targetscan.org/>), and PicTar (<http://pictar.mdc-berlin.de/>). The plasmids of wild-type (wt)-NLRP3- and mutant (mut)-NLRP3-fused luciferase genes were constructed using conventional methods. The two plasmids were mixed with the miR-223-3p mimic, miR-223-3p inhibitor, mimic control, and inhibitor control and then co-transfected into HK-2 cells and labelled as Wt+miR-223-3p mimics, Mut+miR-223-3p, Wt+negative control, and Mut+negative control. After incubation at 37°C for 48 h in an incubator with 5% CO₂, a dual luciferase reporter gene kit was used to detect dual luciferase activity. The levels of NLRP3 in miR-223-3p-overexpressing HK-2 cells were detected by RT-PCR and Western blot analysis.

RT-PCR

HK-2 cells were collected from each treatment group, and cellular RNA was extracted with a total RNA extraction kit (Dalian Bao Biotech, Dalian, China). Total RNA was used to generate cDNA in accordance with the PrimeScript™ RT reagent kit (Shanghai

Roche Pharmaceutical Co., Ltd., Shanghai, China). The expression levels of TXNIP and NLRP3 mRNA and miR-223-3p were detected via RT-PCR. The reaction system (20 μ L total volume) was as follows: 10 μ L of SYBR Premix Ex Taq II, 2 μ L of PCR primer, 2 μ L of DNA template, and 6 μ L of DEPC water. Three parallel replicate wells were prepared for each sample, and the reaction was performed on a PCR instrument. The TaqMan microRNA assay kit (Applied Biosystems, Foster City, CA, USA) was used to determine the miR-223-3p expression levels using the 2^{- $\Delta\Delta$ Ct} method. The reaction conditions were as follows: pre-denaturation at 95°C for 30 s, reaction at 95°C for 5 s, and annealing at 60°C for 30 s for a total of 40 cycles. The primer series is shown in Table I. U6 was used as a miR-223-3p internal reference, and β -actin was used as an internal reference for detecting TXNIP and NLRP3 gene expression. After the reaction, the cycle threshold (Ct) of each gene was calculated and compared with that of the internal reference gene U6 to determine the expression levels.

Western blot analysis

The cell lysis buffer was prepared by mixing with protease inhibitors, and phosphatase inhibitors at an 8:1:1 ratio. Approximately 150 μ L of RIPA cell lysis buffer was added to each well. The samples were lysed in an ice bath for 30 min and centrifuged at 4°C for 15 min to extract the total proteins from each group. The purity of the proteins

Table I. Primer sequences for RT-PCR analysis

Gene	Primer	Product(bp)
miR-223-3p	F-5'-AGCTGGTGTGTGAATCAGGCCG-3' R-5'-TGGTGTCGTGGAGTCG-3'	198bp
U6	F-5'-CTCGCTTCGGCAGCAC-3' R-5'-AACGCTTCACGAATTTGCGT-3'	144bp
TXNIPmRNA	F-5'-AGTGATTGGCAGCAGGTC-3' R-5'-GGTGTCTGGGATGTTTAGG-3'	171bp
NLRP3mRNA	F-5'-ATTACCCGCCCCGACAATAGG-3' R-5'-CATGAGTCAGCTAGGCTAGAA-3'	187bp
β -actin	F-5'-ATGGTGAAGGTCGGTGTG-3' R-5'-AACTTGCCGTGGGTAGAG-3'	154bp

was determined using a BCA protein quantification kit. Approximately 50 µg of protein extract was separated by 10% SDS-PAGE and blocked by incubation in 5% skim milk for 1.5 h. The following primary antibodies were added and incubated overnight at 4°C: NF-κB65 (1:1,000; Cat. no. ab152104), p-IκB-α (1:1,000; Cat. no. ab105217), IκB-α (1:1000; Cat. no. ab141539), β-actin (1:1000; Cat. no. ab4793), IκB (1:1,000; Cat. no. ab139247), TXNIP (1:1,000; Cat. no. ab1520107), and NLRP3 (1:1,000; Cat.

no. ab1663570) (Abcam, UK). The membrane was washed three times with TBST buffer and HRP-labelled goat anti-rabbit IgG secondary antibody (Wuhan Biotechnology Co., Ltd., Wuhan, China) was added. The samples were labelled with goat anti-rabbit IgG antibody (1:3000 volume dilution ratio) and incubated at room temperature for 1 h. The membrane was then washed three times for 10 min each with TBST buffer, and enhanced chemiluminescence solution was added for colour development. Image Lab

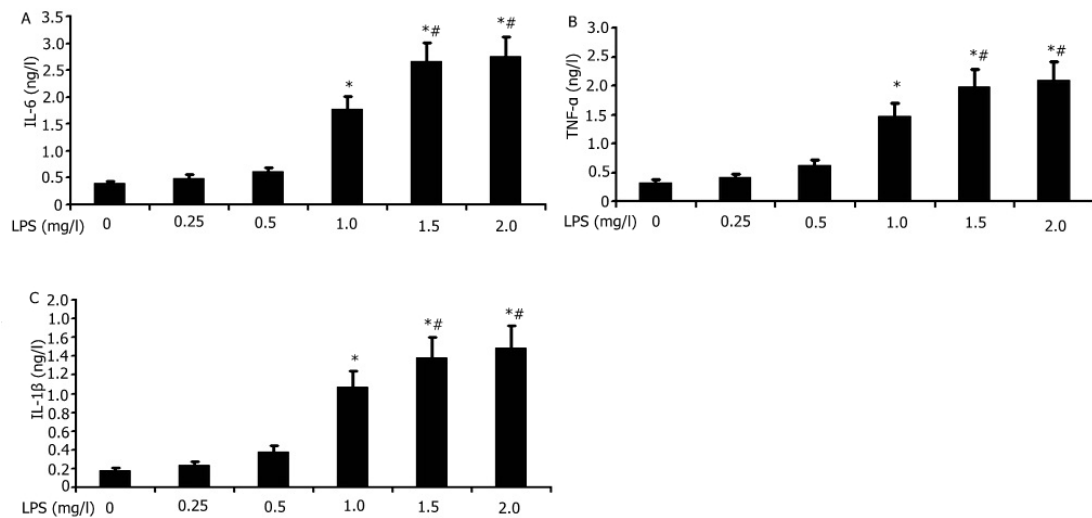


Fig. 1. Effect of LPS on HK-2 cell inflammation. HK-2 cells were incubated with various concentrations of LPS (0.25, 0.5, 1.0, 1.5, and 2.0 mg/L) for 24 h. IL-6, IL-1β, and TNF-α produced by HK-2 cells were measured using ELISA. Data from three independent experiments are presented as the mean ± SEM. * $P < 0.05$ versus the control group. # $P < 0.05$ versus the LPS (0.25 mg/L and 0.5 mg/L) group. $P > 0.05$ in the LPS (1.5 mg/L) group versus the LPS (2.0 mg/L) group.

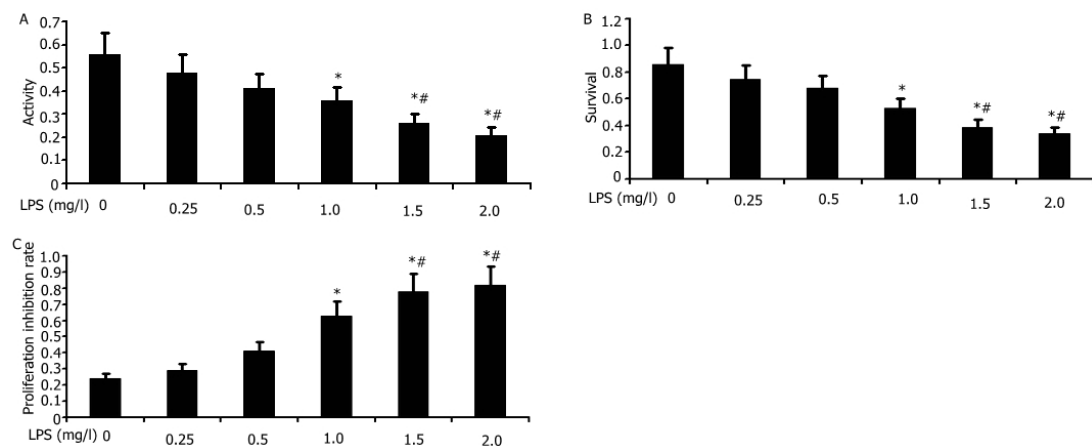


Fig. 2. Effect of LPS on HK-2 cell viability and survival rate. After HK-2 cells were incubated with various concentrations of LPS (0.25–2.0 mg/L) for 24 h, cell viability was measured using a CCK-8 assay, and the survival rate was determined using the MTT assay. Data from three independent experiments are presented as the mean ± SEM. * $P < 0.05$ versus the control group. # $P < 0.05$ versus the LPS (0.25 mg/L and 0.5 mg/L) group. $P > 0.05$ in the LPS (1.5 mg/L) group versus the LPS (2.0 mg/L) group.

software was used to analyse the greyscale values of the bands, and β -actin was used as an internal control.

Determination of inflammatory factors in HK-2 cells

Cells in the logarithmic growth phase were resuspended and inoculated into a six-well plate. The old medium was discarded after the cells attached to the wells. The cells and cell supernatant in each treatment group were collected after 24 h of cell culture, and the cell supernatant was used to assay the levels of inflammatory factors IL-6, IL-1 β , and TNF- α by using an ELISA kit (Cusabio Biotech Co., Ltd., Wuhan, China; Cat. nos. WK15204, WK25540, and WK36640, respectively). The procedure was carried out in strict accordance with the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using GraphPad

Prism 6. The experimental data are expressed as the mean $\pm$ standard deviation. One-way ANOVA was used for comparisons between groups, and the LSD method was used for the comparison of pairwise significance between groups. $P < 0.05$ was considered statistically significant.

RESULTS

LPS increased HK-2 cell inflammation and attenuated HK-2 cell viability and survival rate

To observe the effect of LPS on HK-2 cell inflammation, viability and survival rate, HK-2 cells were incubated with various concentrations of LPS (0.25, 0.5, 1.0, 1.5, and 2.0 mg/L) for 24 h. HK-2 cell production of IL-6, IL-1 β , and TNF- α were measured by ELISA, HK-2 cell viability was measured using a CCK-8 assay, and the survival rate was determined using the MTT assay. As

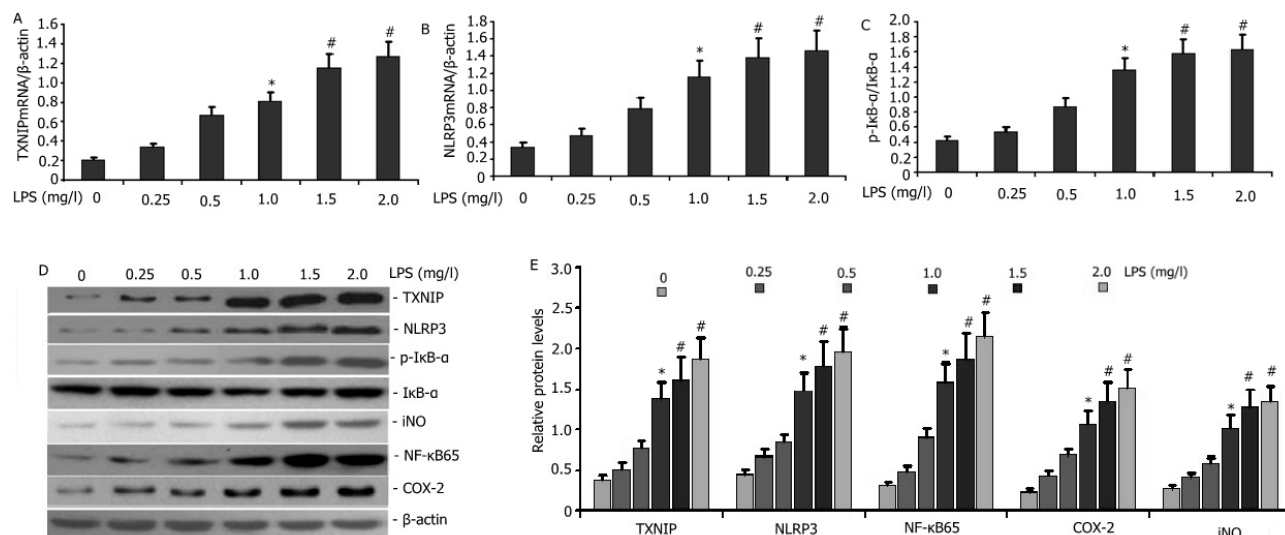


Fig. 3. Effect of LPS on the activity of the TXNIP/NLRP3 and NF- κ B/I κ B signalling pathways in HK-2 cells. HK-2 cells were incubated with various concentrations of LPS (0.25, 0.5, 1.0, 1.5, and 2.0 mg/L) for 24 h. TXNIP and NLRP3 gene expression levels in HK-2 cells were measured using RT-PCR. TXNIP, NLRP3, NF- κ B65, I κ B- α , p-I κ B- α , iNOS, and COX-2 protein expression levels were measured by Western blot analysis. Data from three independent experiments are presented as the mean $\pm$ SEM. * $P < 0.05$ versus the control group. # $P < 0.05$ versus the LPS (0.25 mg/L and 0.5 mg/L) group. $P > 0.05$ in the LPS (1.5 mg/L) group versus the LPS (2.0 mg/L) group.

shown in Figs. 1-2, LPS increased the levels of IL-6, IL-1 β , and TNF- α produced by HK-2 cells in a concentration-dependent manner (Fig. 1 A–C), and decreased HK-2 cell viability and survival rate in a concentration-dependent manner (Fig. 2 A and B).

Approximately 1.0 mg/L LPS effectively increased HK-2 cell inflammation (Fig. 1 A–C), decreased HK-2 cell viability and survival rate (Fig. 2 A and B). The concentration of the LPS was 0.64 mg/L for IC₅₀ (Fig. 2C).

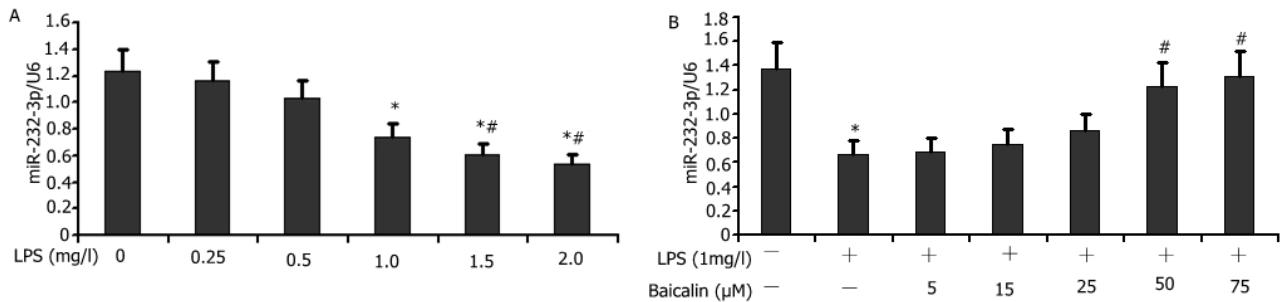


Fig. 4. Effect of baicalin on miR-223-3p expression in LPS-induced HK-2 cells. HK-2 cells were incubated with various concentrations of LPS alone (0.25, 0.5, 1.0, 1.5, and 2.0 mg/L) or LPS (1.0 mg/L) and various concentrations of baicalin (25, 50, and 75 μ M) for 24 h. miR-223-3p expression levels in HK-2 cells were measured using RT-PCR. Data from three independent experiments are presented as the mean $\pm$ SEM. * P <0.05 versus the control group. # P <0.05 versus the LPS (1mg/L) group. P >0.05 in the baicalin (50 μ M) group versus the baicalin (75 μ M) group.

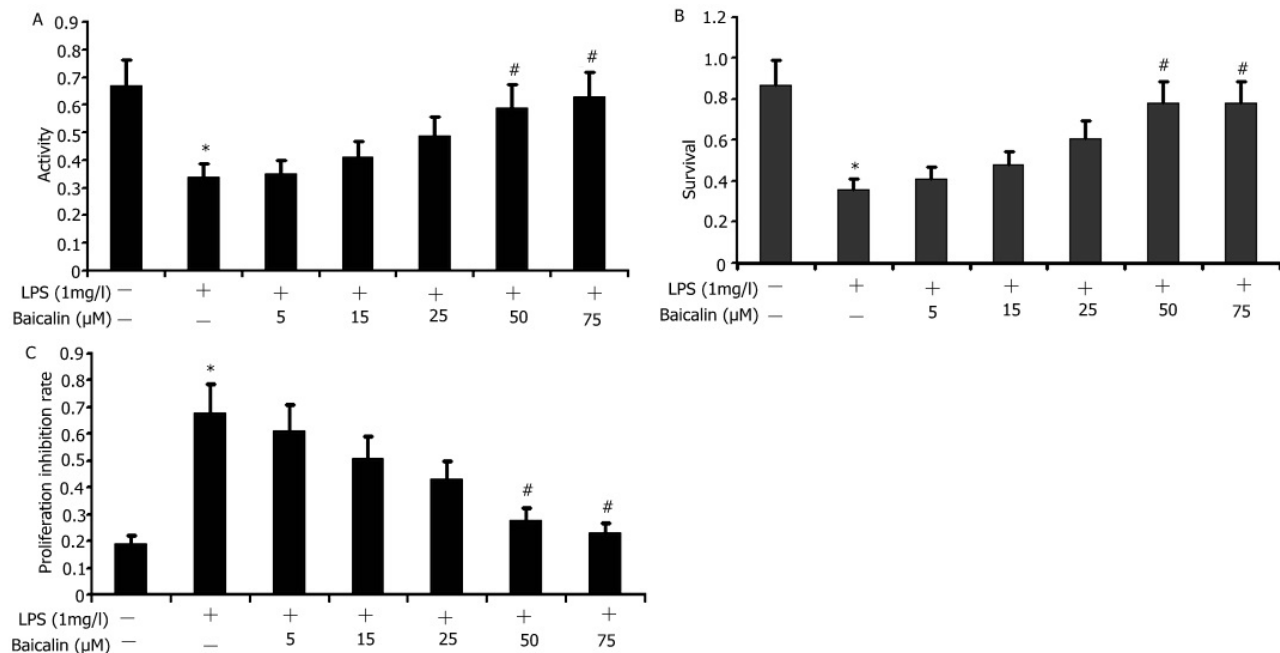


Fig. 5. Effect of baicalin on cell viability, and survival rate. HK-2 cells were incubated with LPS (1.0 mg/L) and various concentrations of baicalin (5, 15, 25, 50, and 75 μ M) for 24 h. HK-2 cell viability was measured using a CCK-8 assay, and the survival rate was determined using the MTT assay. Data from three independent experiments are presented as the mean $\pm$ SEM. * P <0.05 versus the control group. # P <0.05 versus the LPS (1mg/L) group. P >0.05 in the baicalin (50 μ M) group versus the baicalin (75 μ M) group.

LPS increased the activity of the TXNIP/NLRP3 and NF- κ B/I κ B signalling pathways in HK-2 cells

To evaluate the effect of LPS on the TXNIP/NLRP3 and NF- κ B/I κ B signalling pathways in HK-2 cells, HK-2 cells were incubated with various concentrations of LPS (0.25, 0.5, 1.0, 1.5, and 2.0 mg/L) for 24 h. TXNIP and NLRP3 gene expression levels were measured using RT-PCR. The protein expression levels of TXNIP, NLRP3, NF- κ B65, I κ B- α , p-I κ B- α , inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) were measured by Western blot analysis. As shown in Fig. 3, LPS increased TXNIP and NLRP3 gene expression levels (Fig. 3 A and B) and TXNIP, NLRP3, NF- κ B65, I κ B- α , p-I κ B- α , iNOS, and COX-2 protein expression levels (Fig. 3 C–E) in HK-2 cells in a concentration-dependent manner. Approximately 1.0 μ M LPS effectively increased the activity of the TXNIP/NLRP3 and NF- κ B/I κ B signalling pathways in HK-2 cells (Fig. 3 A–E).

Baicalin increased miR-223-3p expression via LPS induction in HK-2 cells

To analyse the effect of LPS on miR-223-3p expression in HK-2 cells, HK-2 cells were incubated with various concentrations of LPS (0.25, 0.5, 1.0, 1.5, and 2.0 mg/L) for 24 h. miR-223-3p expression was measured using RT-PCR. As shown in Fig. 4, LPS decreased miR-223-3p expression in HK-2 cells in a concentration-dependent manner (Fig. 4A). Approximately 1.0 μ M LPS effectively decreased miR-223-3p expression in HK-2 cells (Fig. 4A). To further analyse the effect of baicalin on the LPS-induced decrease in miR-223-3p expression, HK-2 cells were incubated with LPS (1.0 mg/l) and various concentrations of baicalin (5, 15, 25, 50, and 75 μ M) for 24 h. We measured miR-223-3p expression using RT-PCR and found that baicalin rescued miR-223-3p expression following LPS induction in HK-2 cells in a concentration-dependent manner (Fig. 4B). Approximately 50 μ M baicalin effectively rescued miR-223-3p expression in LPS-induced HK-2 cells (Fig. 4B).

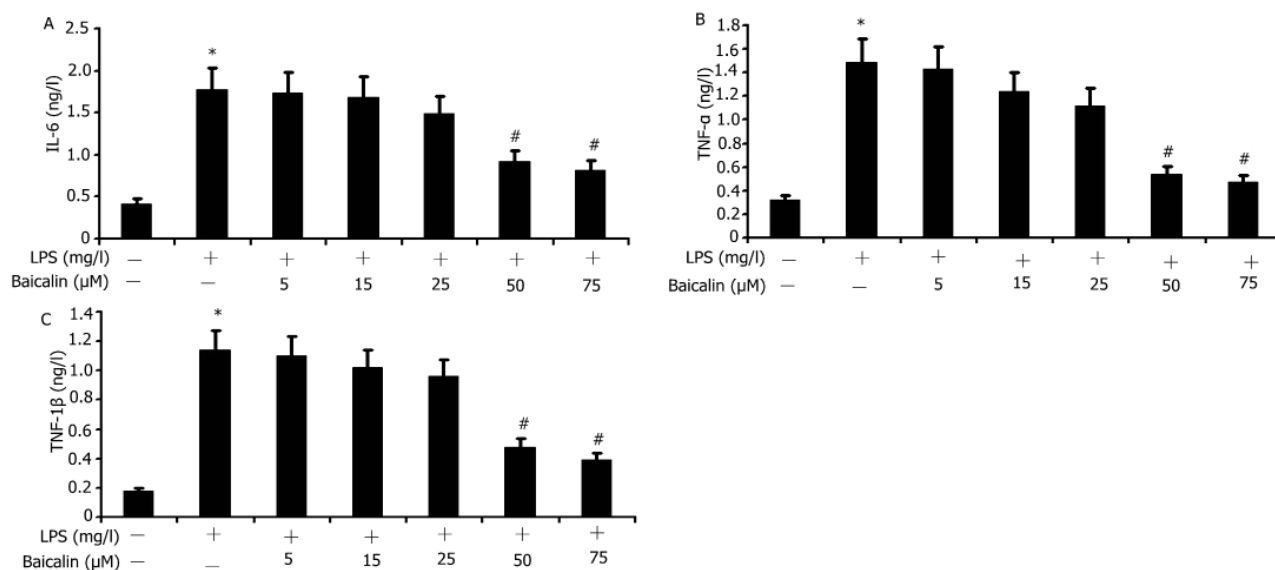


Fig. 6. Effect of baicalin on HK-2 cell inflammation. HK-2 cells were incubated with LPS (1.0 mg/L) and various concentrations of baicalin (5, 15, 25, 50, and 75 μ M) for 24 h. IL-6, IL-1 β , and TNF- α produced by HK-2 cells were measured using ELISA. Data from three independent experiments are presented as the mean $\pm$ SEM. * P < 0.05 versus the control group. # P < 0.05 versus the LPS (1 mg/L) group. P > 0.05 in the baicalin (50 μ M) group versus the baicalin (75 μ M) group.

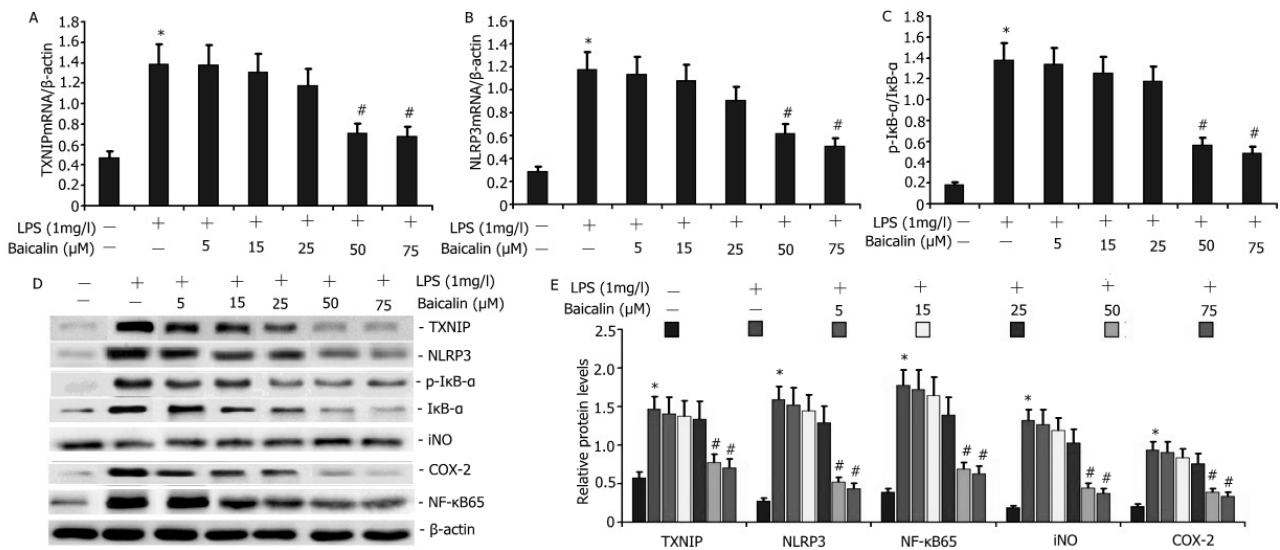


Fig. 7. Effect of baicalin on the activity of the TXNIP/NLRP3 and NF- κ B/I κ B signalling pathways in LPS-induced HK-2 cells. HK-2 cells were incubated with LPS (1.0 mg/L) and various concentrations of baicalin (5, 15, 25, 50, and 75 μ M) for 24 h. TXNIP and NLRP3 gene expression levels in HK-2 cells were measured using RT-PCR. TXNIP, NLRP3, NF- κ B65, I κ B- α , p-I κ B- α , iNOS, and COX-2 protein expression levels were measured by Western blot analysis. Data from three independent experiments are presented as the mean $\pm$ SEM. * P <0.05 versus the control group. # P <0.05 versus the LPS (1mg/L) group. P >0.05 in the baicalin (50 μ M) group versus the LPS (75 μ M) group.

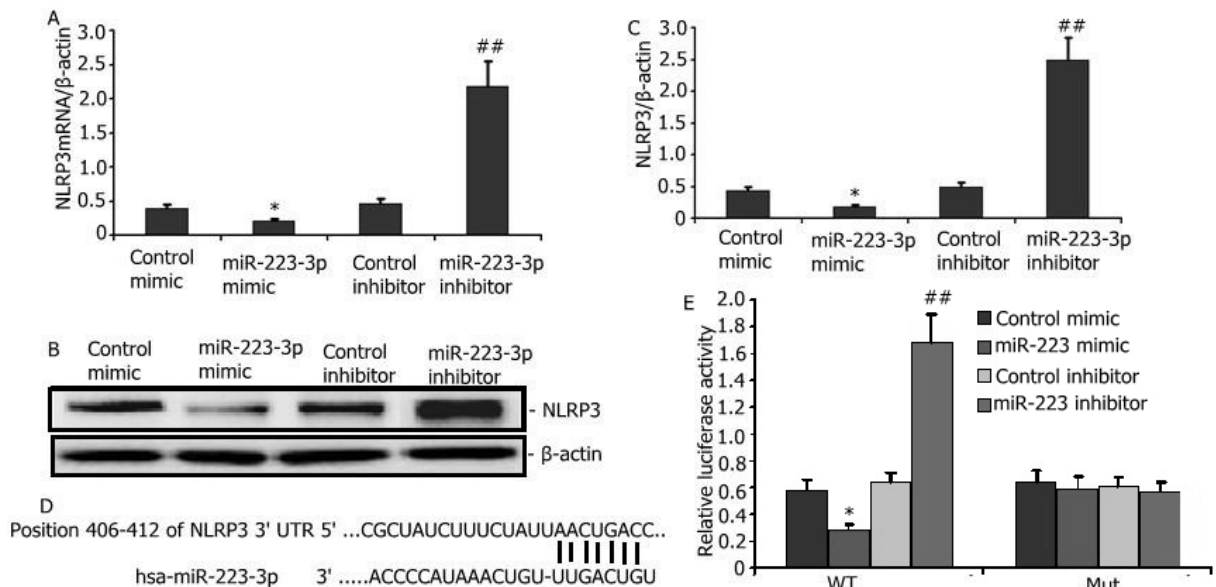


Fig. 8. NLRP3 is a downstream target of miR-223-3p. **A)** Decreased mRNA levels of NLRP3 in LPS-induced HK-2 cells after miR-223-3p overexpression (P <0.05). Levels of NLRP3 mRNA were enhanced after miR-223-3p downregulation (P <0.05). **B and C)** Levels of NLRP3 protein were decreased in LPS-induced HK-2 cells with miR-223-3p overexpression, relative to that of the empty vector control (P <0.05). **D)** The levels of NLRP3 protein were significantly increased in cells with miR-223-3p downregulation. **E)** miR-223-3p was bound to the 3'-UTR regions of NLRP3. Binding was disrupted in mutant NLRP3. **F)** Dual luciferase reporter assay indicated that the miR-223-3p mimic was bound to the 3'-UTR region of the wild-type NLRP3, rather than to that of NLRP3 mutants (P <0.05).

Baicalin increased HK-2 cell viability and survival rate

To observe the effect of baicalin on HK-2 cell inflammation, viability, and survival rate, HK-2 cells were incubated with LPS (1.0 mg/L) and various concentrations of baicalin (5, 10, 25, 50, and 75 μ M) for 24 h. HK-2 cell viability was measured using a CCK-8 assay, and the survival rate was determined using an MTT assay. As shown in Fig. 5, baicalin increased the cell viability and survival rate of LPS-induced HK-2 cells in a concentration-dependent manner (Fig. 5A–C). Approximately 50 μ M baicalin effectively increased cell viability and survival rate of LPS-induced HK-2 cells (Fig. 5A–C). The concentration of the baicalin was 38.6 mg/L for ED50.

Baicalin decreased HK-2 cell inflammation

To observe the effect of baicalin on HK-2 cell inflammation, viability, and survival rate, HK-2 cells were incubated with LPS (1.0 mg/L) and various concentrations of baicalin (5, 10, 25, 50, and 75 μ M) for 24 h. IL-6, IL-1 β , and TNF- α production by HK-2 cells was measured using ELISA. As shown in Fig. 5, baicalin decreased the expression levels of IL-6, IL-1 β , and TNF- α of LPS-induced HK-2 cells in a concentration-dependent manner (Fig. 5A–E). Approximately 50 μ M baicalin effectively decreased IL-6, IL-1 β , and TNF- α levels of LPS-induced HK-2 cells (Fig. 6A–C).

Baicalin decreased the activity of the TXNIP/NLRP3 and NF- κ B/I κ B signalling pathways

To observe the effect of baicalin on the activity of the TXNIP/NLRP3 and NF- κ B/I κ B signalling pathways, HK-2 cells were incubated with LPS (1.0 mg/L) and various concentrations of baicalin (5, 15, 25, 50, and 75 μ M) for 24 h. TXNIP and NLRP3 gene expression levels were measured using RT-PCR. The protein expression levels of TXNIP, NLRP3, NF- κ B65, I κ B- α , p-I κ B- α , iNOS, and COX-2 were measured by Western blot analysis. As shown in Fig. 6, baicalin decreased the gene expression levels of TXNIP and NLRP3 (Fig. 7A and B) and protein expression levels of TXNIP, NLRP3, NF- κ B65, I κ B- α , p-I κ B- α , iNOS, and COX-2 (Fig. 6C–E) in LPS-induced HK-2 cells in a concentration-dependent manner. Approximately 50 μ M baicalin

effectively decreased the activity of the TXNIP/NLRP3 and NF- κ B/I κ B signalling pathways in LPS-induced HK-2 cells (Fig. 7A–E).

NLRP3 is a regulatory target of miR-223-3p

To determine how miR-223-3p mediates LPS-induced HK-2 cell inflammation, we used a well-known database to predict likely targets of miR-223-3p. We found that NLRP3 is an important regulator of LPS-induced cell inflammation. Analysis revealed that NLRP3 mRNA and protein levels were dramatically decreased after miR-223-3p overexpression (Fig. 8A–C). To validate the interaction between miR-223-3p and NLRP3, we constructed wild-type and mutant NLRP3 for a dual luciferase reporter assay. As hypothesized, miR-223-3p bound to the wild-type NLRP3, rather than to the mutants (Fig. 8D and E).

DISCUSSION

Severe sepsis and septic shock are the most common causes of AKI, and AKI occurs in approximately 30–50% of patients with sepsis (32). Sepsis-induced AKI accounts for approximately 50% of all cases of AKI (32). Moreover, the incidence of AKI is directly proportional to the severity of infection. The prevalence rates in patients with systemic infection, severe infection, and septic shock are 19%, 23%, and 53%, respectively (33). AKI promotes and exacerbates organ damage, leading to multiple organ dysfunction and increasing the mortality rate. In this study, we found that the cell activity and survival rate of HK-2 cells were significantly decreased after 24 h of culture with different concentrations of LPS (Fig. 1A and B). The decreases in cell activity and survival rate were significant when the LPS concentration was high (Fig. 1A and B). At 1.0 mg/L LPS, the cell viability and survival rate of HK-2 cells decreased significantly compared with those of the control group (Fig. 1A and B). At 1.5–2.0 mg/L LPS, the cell viability and survival rate of HK-2 cells decreased further compared with those of the control group (Fig. 1A and B). However, the cell viability and survival rate of HK-2 cells treated with 2.0 mg/L LPS were not

significantly decreased compared with those treated with 1.5 mg/L LPS (Fig. 1 A and B). Therefore, infectious sepsis may lead to the occurrence and development of AKI.

In recent years, with continuous improvements in early diagnosis and renal replacement therapy, patient prognosis has improved; however, the mortality rate of patients with sepsis-induced AKI has increased significantly and remained at approximately 50–70% compared with those of patients without AKI and with simple sepsis (32–34). Therefore, the mortality rate is still high, and finding a reasonable and effective treatment can help reduce the occurrence and development of sepsis-induced AKI. In the present study, LPS significantly reduced the cell viability and survival rate of HK-2 cells in a dose-dependent manner (Fig. 1 A and B). To further investigate the effects of baicalein on LPS-induced AKI cells, we administered LPS to HK-2 cells and cultured the cells with different concentrations of baicalein. Medium and high doses of baicalein (50 and 75 μ M, respectively) significantly rescued the LPS-induced decrease in cell activity and survival rate of HK-2 cells (Fig. 5 A–C). A high dose of baicalein (75 μ M) further rescued the LPS-induced decrease in the cell viability and survival rate of HK-2 cells (Fig. 5 A–C); however, the difference between the medium dose (50 μ M) and the high dose (75 μ M) of baicalein was not significant (Fig. 5 A–C). Our experiments also confirmed that baicalein is effective in treating sepsis-induced AKI.

AKI caused by sepsis may be affected by many factors. In addition to haemodynamic changes, renal cell apoptosis, endotoxin or endotoxin-like substance-induced complex inflammation and immune network response, endothelial dysfunction, glomerular embolism, and necrotic cell-induced renal tubular obstruction are also involved in the pathophysiological changes of AKI. Inflammatory damage of renal tubular epithelial cells may play a key role in sepsis-induced AKI (35). In this study, we treated HK-2 cells with different concentrations of LPS and found that HK-2 cells significantly increased secretion of the inflammatory mediators IL-6, IL-1 β , and TNF- α (Fig. 2 A–C). With increasing LPS doses, HK-2 cell secretion of these

inflammatory mediators was further increased (Fig. 2 A–C). Administration of baicalein to LPS-induced HK-2 cells significantly reduced the secretion of the inflammatory mediators IL-6, IL-1 β , and TNF- α (Fig. 6 A–C). In addition, an increased baicalein concentration evidently decreased the secretion of the inflammatory mediators IL-6, IL-1 β , and TNF- α by LPS-induced HK-2 cells (Fig. 6 A–C). Therefore, baicalein can significantly reduce the inflammatory response of renal tubular cells induced by sepsis and has a protective effect on renal tubular damage caused by infection.

We found that LPS secreted by bacteria could activate the NLRP3 signalling pathway (Fig. 3 A–E), which directly and indirectly activates the NF- κ B signalling pathway, increases NF- κ B phosphorylation (Fig. 3 C–E), promotes the phosphorylation of I κ B- α (Fig. 3 C–E), and increases the secretion of inflammatory mediators by renal tubular HK-2 cells (Fig. 2 A–C). Consequently, the activities of renal tubular cells were decreased (Fig. 1A), and the survival rate was also significantly decreased (Fig. 1B). Previous studies have shown that the interaction of TXNIP with NLRP3 induces the activation of the NLRP3/caspase-1/IL-1 β signalling pathway in an inflammatory state (36). NLRP3 plays a key role in diabetic nephropathy and kidney ischaemic reperfusion injury; thus, reducing the activation of the NLRP3 inflammasome can inhibit the inflammatory response and further protect against kidney damage (37). Therefore, inhibiting the activity of the TXNIP/NLRP3 signalling pathway may help to alleviate inflammation in HK-2 cells. Baicalein was found to inhibit the activity of the TXNIP/NLRP3 signalling pathway and reduce the expression of TXNIP and NLRP3 (Fig. 7 A–E), inhibit the activity of the NF- κ B/I κ B pathway (Fig. 6C–E), and reduce the protein expression of NF- κ B65, p-I κ B- α , iNOS, and COX-2 (Fig. 7 C–E), decreasing HK-2 cell inflammation (Fig. 6 C–E). Therefore, our findings suggest that baicalein can improve infection-induced renal tubular injury by inhibiting the activity of the TXNIP/NLRP3 signalling pathway.

Studies on AKI have found that the expression profile of miRNAs changes significantly after acute renal ischaemia/reperfusion injury (38). Zager et

al. established a uraemia model of renal ischaemia/reperfusion injury and found that 69 miRNAs were abnormally expressed and may participate in post-injury inflammation by affecting the expression of anti-inflammatory factor-10 (39). After specifically knocking out the Dicer gene in the proximal renal tubular cells of mice, Wei et al. found that 139 miRNAs in the renal cortex of these mice showed decreased expression, and the expression of 28 miRNAs was increased. In addition, the resistance of these mice to renal ischaemia/reperfusion injury was also significantly increased, and tissue inflammatory injury, cell apoptosis, and renal function were significantly improved (40). In the present study, we found that the expression level of miR-223-3p was decreased after HK-2 cells were treated with different concentrations of LPS (Fig. 4A). The higher the LPS concentration, the lower the expression of miR-223-3p (Fig. 4A) and levels of the inflammatory mediators IL-6, IL-1 β , and TNF- α secreted by HK-2 cells (Fig. 2A–C). As such, the survival rate and activity of HK-2 cells were significantly reduced.

miR-223-3p and NLRP3 play an important role in acute tubular inflammatory injury. To further investigate the relationship between miR-223-3p and NLRP3 in renal tubular injury, we predicted that NLRP3 is a miR-223-3p regulatory target by using TargetScan, and miR-223-3p has a common binding site in the 3'-UTR region of NLRP3 (Fig. 8D). We transfected LPS-induced HK-2 cells with the miR-223-3p mimic and found that increasing the expression of miR-223-3p significantly decreased the expression of the NLRP3 gene and protein in LPS-induced HK-2 cells (Fig. 8A–C). A dual luciferase reporter assay confirmed that NLRP3 is a regulatory target of miR-223-3p (Fig. 8E). This result suggests that miR-223-3p affects the development and progression of AKI by regulating NLRP3 expression.

Therefore, miR-223 may regulate the occurrence and development of endotoxin-induced acute tubular injury by regulating the TXNIP/NLRP3 signalling pathway. Moreover, baicalein can inhibit the activity of the TXNIP/NLRP3 signalling pathway by increasing miR-223 expression, thereby reducing the expression levels of pro-inflammatory genes and proteins and the inflammation of renal tubular cells,

increasing the activity and survival rate of renal tubular cells, and improving endotoxin-induced acute tubular injury.

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MicroRNA-142-3p SUPPRESSES CELL PROLIFERATION AND MIGRATION IN BLADDER CANCER VIA Rac1

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Expression of microRNA(miR)-142-3p has been implicated to be associated with several cancers, whereas its function in bladder cancer (BC) remains unknown. The present study aimed to explore the correlation between the expression of miR-142-3p and the proliferation, migration and invasion of bladder cancer cells by activating Rac1. qRT-PCR was used to measure the expression of miR-142-3p in bladder cancer tissues and cell lines. RNA transfection was used to silence and accelerate the expression of miR-142-3p in bladder cancer cells. CCK-8 and trans-well assays were used to detect the proliferation, migration and invasion of cells before and after RNA transfection. The direct interaction between Rac1 and miR-142-3p was demonstrated by a dual luciferase reporter assay. qRT-PCR and Western blot assays were used to detect the expression changes in Rac1 before and after transfection. The results showed that miR-142-3p in bladder cancer tissues was significantly lower than that in adjacent tissues and lower than that in HT1376 and T-24 cells but higher than that in T5637 and BIU-87 cells. Additionally, upregulating miR-142-3p expression not only inhibits the proliferation of SV-HUC-1 and BIU-87 cells but also inhibits migration and invasion, and downregulating miR-142-3p expression showed the opposite results. The expression of Rac1 was promoted after stimulating miR-142-3p expression, but was inhibited after silencing miR-142-3p expression. In conclusion, miR-142-3p affects the proliferation, migration and invasion of bladder cancer cells by regulating Rac1.

Bladder cancer (BC) is one of the most life-threatening urogenital malignant tumours worldwide, with increasing morbidity and mortality rates (1). Based on their pathological features, BCs are classified into two types: non-muscle-invasive (NMIBC) and muscle-invasive (MIBC). Although combined treatments for NMIBC (surgical intervention and adjuvant chemoradiotherapy) have been well developed, there are still many patients who experience recurrence and progress

to MIBC. MIBC accounts for approximately 30% of cases and is associated with a poor prognosis and a higher rate of metastasis (2, 3). Therefore, a better understanding of the molecular mechanism underlying the malignant progression of BC and the identification of novel therapeutic targets is urgent.

MicroRNAs(miRNAs), a class of short non-coding sequences with approximately 22 nucleotides, regulate gene expression post-transcriptionally through interference with special binding sites in

Key words: microRNA-142-3p; bladder cancer; Rac1; proliferation; migration; invasion

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the 3' untranslated region (3'-UTR) of the target mRNAs (4, 5). Recently, accumulating evidence has demonstrated that some miRNAs might serve as oncogenes or tumour suppressors in the process of BC development and progression (6-9). MiR-142-3p, which is encoded by human chromosome 17q22, is a new tumour suppressor observed in a variety of tumours, such as breast cancer, hepatocellular carcinoma, and ovarian cancer (10-14). The downregulation of miR-142-3p is associated with tumourigenicity through regulation, cell migration, cell apoptosis, and invasion via different signalling pathways. However, the functions of miR-142-3p in BCs have not yet been fully understood.

In this study, we determined that miR-142-3p expression was downregulated in BC tissues and cell lines. Transfection with miR-142-3p mimics significantly reduced the proliferation, migration and invasion of BC cells. We also demonstrated that Rac1 is a direct target of miR-142-3p. Collectively, these data illustrate that miR-142-3p inhibits BC progression via Rac1 suppression, suggesting that miR-142-3p might be a promising therapeutic target for BC treatment.

MATERIALS AND METHODS

Tissue samples

A total of 48 tumour tissues and matched adjacent normal tissues were obtained from 48 patients diagnosed with BC at the Taizhou People's Hospital (Jiangsu, China) between April 2014 and April 2019. Individuals who were treated with radiotherapy or chemotherapy before surgery were excluded. Patient information, including age, sex, tumor size, pTNM category and differentiation, are summarized in Table I. Samples were maintained in liquid nitrogen at -80°C for further use. Patient follow-up and survival data were obtained by telephone follow-ups and home visits as well as patient reviews. All participants provided informed consent. This study was approved by the Ethics Committee of Taizhou People's Hospital (Jiangsu, China).

Cell culture and transfection

BIU-87, HT1376, T24 and T5637 cell lines as well as the normal human uroepithelial cell line

SV-HUV-1 were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). All cancer cells were cultured in Dulbecco's modified eagle medium (DMEM, Gibco, Rockville, MD, USA) supplemented with 10% foetal bovine serum (FBS, Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin (Solarbio, Beijing, China) and maintained at 37°C under a 5% CO₂ atmosphere. MiR-142-3p mimics, negative control (NC) mimics, shcontrol (NC), and shmiR-142-3p were obtained from GenePharma (Shanghai, China). The miR-142-3p and NC control mimics were transfected into HT1376 and T-24 cells, while shcontrol and shmiR-142-3p were transfected into T5637 and BIU-87 cells; all transfections were performed with Lipofectamine 2000™ reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Cell counting kit (CCK)-8 assay

The effect of miR-142-3p on cell proliferation was measured by CCK-8 assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Cells were seeded into 96-well plates at a density of 2×10^3 cells per well and maintained at 37°C under a 5% CO₂ atmosphere. After transfection with miR-142-3p mimics, 10 µl CCK-8 reagent was added and incubated for 1 h at 37°C. Finally, the optical density was detected at a wavelength of 450 nm using a microplate reader (Berthold Technologies GmbH, Bad Wildbad, Germany).

Migration and invasion assays

Cell migration and invasion abilities were determined using a Transwell chamber (BD Biosciences, Franklin Lakes, NJ, USA) coated with Matrigel (BD Biosciences, San Jose, CA, USA). After being suspended in serum-free medium, cells (5×10^4) were seeded into the upper chamber. The lower chamber was filled with complete medium containing 10% FBS. Following incubation at 37°C for 24 h, the migrated/invaded cells on the lower side of the membrane were fixed with 100% ethanol and stained with 0.5% crystal violet (Beyotime Biotechnology, Shanghai, China) at room temperature for 30 min. Invaded cells were captured under an optical microscope.

Western blot

Total proteins were extracted from cells and tissues using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) and quantified with a bicinchoninic acid (BCA) Protein Assay Kit (Thermo Scientific, USA) and a bicinchoninic acid assay. The cell lysates were separated by 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corporation, USA). After blocking with 5% non-fat milk for 2 h at room temperature, the membranes were incubated with primary antibodies against Rac1 and GAPDH (1:500, Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:2000; Abcam, Cambridge, MA, USA) for 1 h at room temperature. The protein bands were detected with Electrochemiluminescence (ECL) Plus Western Blotting Substrate (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's protocol. GAPDH served as the internal control.

Real-time quantitative polymerase chain reaction

To determine the expression of miRNAs and mRNAs, total RNA was extracted from BC cells and tissues using TRIzol reagent (Invitrogen, USA). Subsequently, reverse transcription was performed with the MicroRNA Real-time quantitative polymerase chain reaction (qRT-qPCR) SYBR Green Detection Kit for miRNA and the PrimeScript™ RT Reagent Kit for mRNA (Takara Biotechnology Co, Ltd, Dalian, China) following the manufacturer's instructions. qRT-PCR was carried out using SYBR Green I Supermix (Takara Biotechnology Co., Ltd.). The sequences used in the study are listed in Table II. The miRNA and mRNA levels were normalized to that of the endogenous control (GAPDH) using the $2^{-\Delta\Delta C_q}$ method (15).

Dual-luciferase reporter assay

The target binding site for miR-142-3p in the 3'-UTR of Rac1 was predicted by the TargetScan Human 7.2 tool (http://www.targetscan.org/vert_72/). Plasmids containing the wild-type (WT) or mutated (MUT) fragment of Rac1 3'-UTR mRNA, which contains a putative miR-142-3p complementary site,

were synthesized by RiboBio Co, Ltd. (Shanghai, China). Plasmid DNA (WT-Rac1 or MUT-Rac1) and miR-142-3p or NC mimics were co-transfected into BC cells according to the manufacturer's instructions. After 24 h of transfection, luciferase activity was determined by the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Statistical analysis

All data were analysed using SPSS 20.0 (IBM Corp, Armonk, NY, USA). The comparisons were conducted using Student's t-test between two groups or one-way analysis of variance (ANOVA) among multiple groups. Kaplan-Meier analysis was performed to confirm the overall survival of BC patients with different miR-142-3p expression levels. Spearman's correlation was used to analyse the relationship between Rac1 and miR-142-3p expression. The in vitro experiments were repeated at least three times, and the data are presented as the means $\pm$ standard deviations (SDs) from at least three independent experiments. P values ≤ 0.05 were considered significant for all analyses.

RESULTS

Expression of miR-142-3p is reduced in BC tissues and cell lines

To understand the relationship between the miR-142-3p level and BC, we first examined its expression using qRT-PCR in tumour tissues and adjacent healthy tissues from 46 BC patients. As shown in Fig. 1A, miR-142-3p was significantly downregulated in BC tissues compared with paired adjacent normal tissues. To evaluate the clinical significance of miR-142-3p in BC, we divided BC patients into two groups according to the median miR-142-3p expression value: a high miR-142-3p level group (n=22) and a low miR-142-3p level group (n=26). The survival analysis revealed that lower miR-142-3p levels in BC tissues were correlated with shorter patient survival ($p < 0.05$, Fig. 1B). Moreover, the expression of miR-142-3p was also reduced in human BC cell lines (BIU-87, HT1376, T24 and T5637) compared with the normal

cell line SV-HUC-1 (Fig. 1C). Taken together, these results indicate that the downregulation of miR-142-3p might be involved in the progression of BC and may be a novel prognostic biomarker in the clinic.

MiR-142-3p overexpression inhibits BC cell proliferation, migration and invasion

Because HT1376 and T24 cell lines express low levels of miR-142, we used these two cell lines in the following *in vitro* experiments. To explore the function of miR-142-3p in BC, miR-142-3p mimics were transfected into HT1376 and T24 cells. RT-qPCR analysis confirmed that miR-142-3p levels were markedly upregulated in HT1376 and T24 cells after transfection with miR-142-3p mimics compared with those transfected with NC mimics (Fig. 2A and B). CCK-8 assays were conducted to evaluate the role of miR-142-3p in BC cell proliferation. The results showed that the proliferation ability of HT1376 and T24 cells was suppressed by miR-142-3p overexpression (Fig. 2C and D). In

addition, the results of migration and invasion assays indicated that transfection with miR-142-3p mimics significantly inhibited the migration and invasion of HT1376 and T24 cells (Fig. 2E and F). These results reveal that miR-142-3p significantly suppresses BC cell proliferation, migration and invasion.

MiR-142-3p knockdown promotes BC cell proliferation, migration and invasion

RT-qPCR confirmed that shmiR-142-3p effectively silenced the expression of miR-142-3p compared with shNC in T5637 and BIU-87 cells ($p < 0.01$, Fig. 3A and B). The CCK-8 results suggested that the proliferation of T5637 and BIU-87 cells in the shmiR-142-3p group was significantly promoted compared with cells in the shNC group ($p < 0.01$, Fig. 3C-D). Similarly, as shown in Fig. 3E-F, the number of migrated and invaded cells in the shmiR-142-3p group was higher than that in the shNC group ($p < 0.01$). In summary, the proliferation, migration and invasion of T5637 and BIU-87 cells

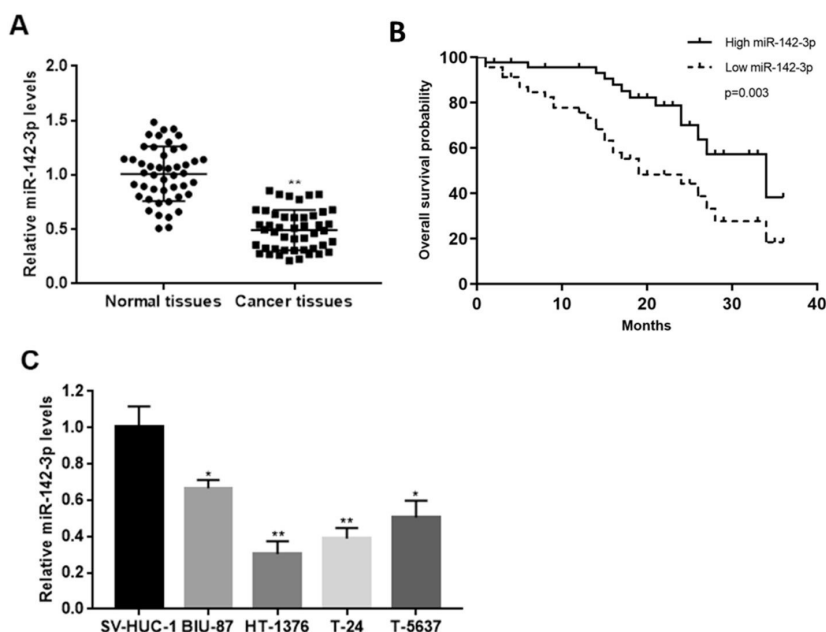


Fig. 1. miR-142-3p expression is reduced in BC tissues and cell lines. **A** and **C**) MiR-142-3p expression in BC tissues and cells. qRT-PCR indicated that miR-142-3p was downregulated in BC tissues and cells. **B**) Kaplan-Meier analysis indicated that low miR-142-3p expression was related to a short survival time of BC patients. Data are expressed as the mean $\pm$ SEM. $n = 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

are closely related to miR-142-3p. In conclusion, miR-142-3p promotes not only the proliferation of BC cells but also migration and invasion.

Rac1 is a target of miR-142-3p in BC cells

Previous studies have determined that Rac1 is a

downstream target of miR-142-3p in several cancer cells, and Rac1 was proven to be associated with BC progression (16-19). Thus, we selected Rac1 for further investigation. We first determined the complementary sequence of miR-142-3p in the 3'-UTR of Rac1 mRNA using TargetScan software

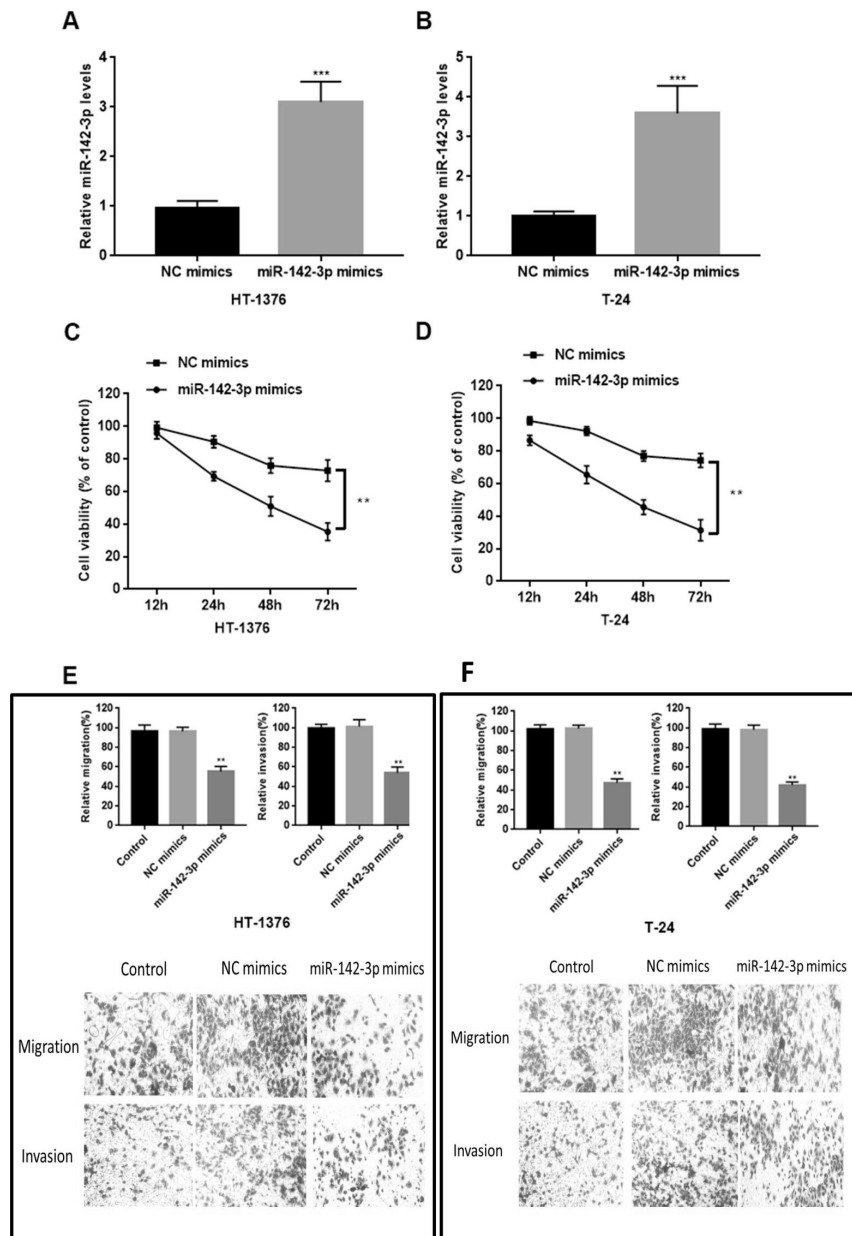


Fig. 2. Effects of miR-142-3p overexpression in HT1376 and T24 cells. After transfection with miR-142-3p mimics, miR-142-3p expression was effectively enhanced in comparison with transfection with NC mimics in HT1376 (A) and T-24 (B) cells, as determined by reverse transcription-quantitative polymerase chain reaction. The effect of miR-142-3p overexpression on the proliferation of HT1376 and T-24 cells was determined by CCK-8 assays (C and D). E and F) Migration and invasion assays were performed to examine the migration and invasion capabilities of BC cells. Data are expressed as the mean $\pm$ SEM. $n=3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

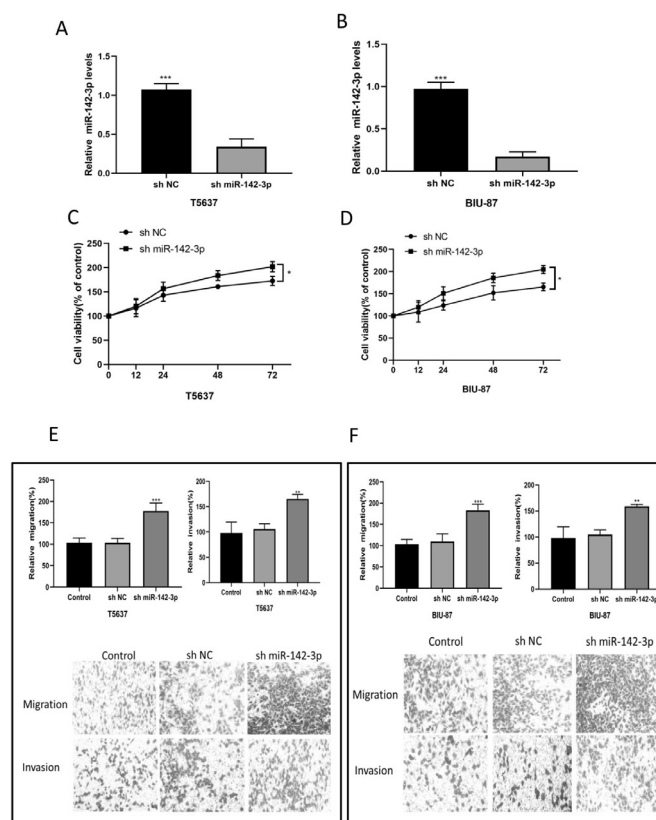


Fig. 3. Effects of miR-142-3p knockdown in T5637 and BIU-87 cells. After transfection with shmiR-142-3p, miR-142-3p expression was effectively enhanced compared with that after transfection with shNC in T5637 (A) and BIU-87 (B) cells, as determined by qRT-PCR. The effect of miR-142-3p knockdown on the proliferation of T5637 and BIU-87 cells was determined by CCK-8 assays (C and D). E and F) Migration and invasion assays were performed to examine the migration and invasion capabilities of T5637 and BIU-87 cells. Data are expressed as the mean $\pm$ SEM. $n=3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

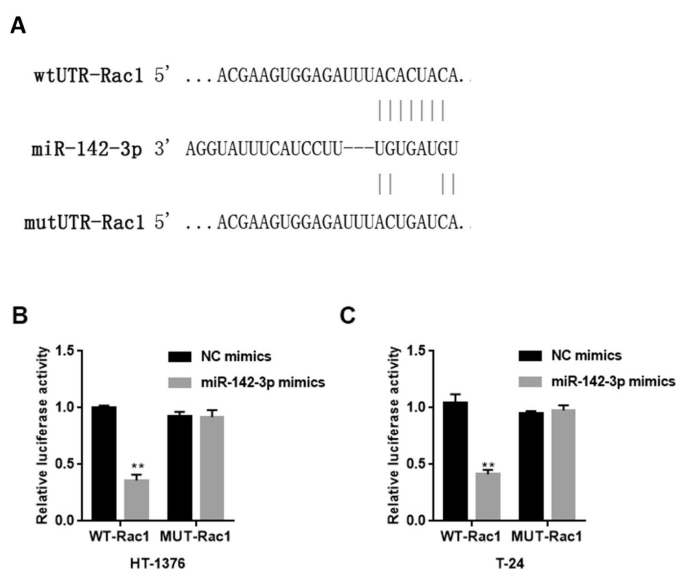


Fig. 4. Rac1 is a direct target of miR-142-3p. A) TargetScan software indicated that Rac1 is a target gene of miR-142-3p. A luciferase reporter assay was conducted on HT1376 (B) and T-24 (C) cells to confirm the relative luciferase activity of Rac1 after treatment with miR-142-3p mimics. Data are expressed as the mean $\pm$ SEM. $n=3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

(Fig. 4A). Next, we tested the direct binding interaction between miR-142-3p and Rac1 using a luciferase reporter assay in BC cells. The luciferase intensity of WT-Rac1 cells was significantly reduced following transfection with miR-142-3p mimics compared with NC mimics, while MUT-Rac1 abolished this influence (Fig. 4 B and C). In addition, the Western blot and qRT-PCR results showed that miR-142-3p mimics markedly inhibited the expression of Rac1 in HT1376 (Fig. 5 A and B) and T24 (Fig. 5C and 5D) cells. However, shmiR-142-3p significantly promoted the expression of Rac1 in T5637 (Fig. 5 E and F) and BIU-87 (Fig. 5G and 5H) cells. Taken together, these data indicate that Rac1 is a target of miR-142-3p.

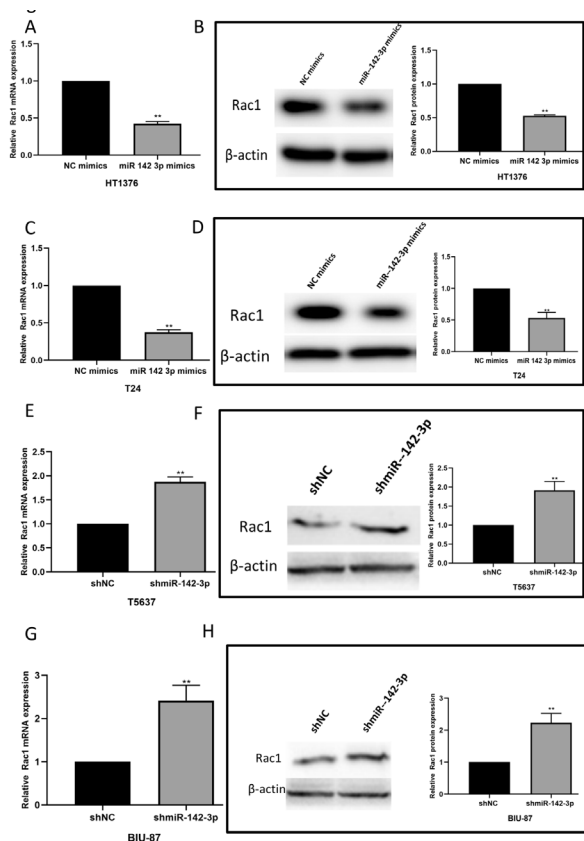


Fig. 5. miR-142-3p regulates Rac1 expression. Following transfection with miR-142-3p mimics or NC mimics, RT-qPCR and Western blot were performed to examine the expression of Rac1 in HT1376 (A and B) and T-24 (C and D) cells. Data are expressed as the mean $\pm$ SEM. $n=3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

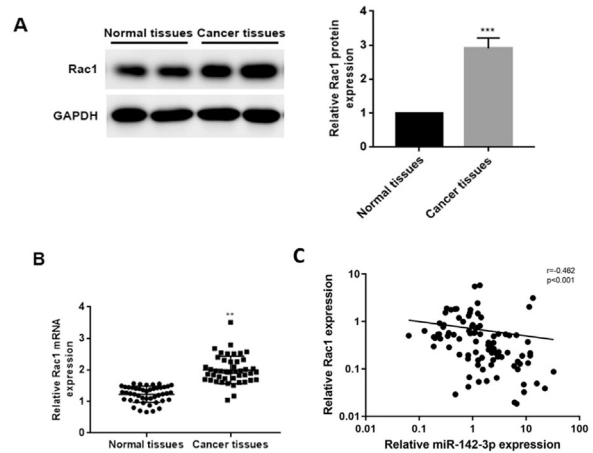


Fig. 6. MiR-142-3p inhibits BC cell proliferation, migration and invasion by targeting Rac1. A) Western blot analysis was used to assess the protein levels of Rac1 in BC tissues. B) qRT-PCR results of Rac1 mRNA in BC tissues and matched normal tissues. Rac1 expression was normalized to that of GAPDH. C) Rac1 mRNA expression was negatively correlated with miR-142-3p levels in BC tissues. Data are expressed as the mean $\pm$ SEM. $n=3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Expression of Rac1 in tumour tissues

As Rac1 was identified as one of the miR-142-3p target genes, Western blot (Fig. 6A) and qRT-PCR (Fig. 6B) were performed to detect Rac1 levels in BC. The expression of Rac1 was increased in BC tissues compared with that in adjacent normal tissues. Moreover, the mRNA levels of Rac1 in BC tumour tissue showed an inverse relationship with the expression of miR-142-3p (Fig. 6C). These findings suggest that miR-142-3p might inhibit BC cell proliferation, migration and invasion by directly targeting Rac1 mRNA.

DISCUSSION

Accumulating studies have determined the essential role of miR-142-3p in the progression of cancers by modulating cell proliferation, apoptosis and invasion. For example, the levels of miR-142-3p in hepatocellular carcinoma tissues are reduced, and the hypermethylation of miR-142-3p contributes to development by targeting transforming growth

Table I. Clinicopathological features of the patients with bladder cancer

Characteristics	Number of cases (n=48)
Age, years	
≤60	34
>60	14
Sex	
Male	31
Female	27
Tumor size, cm	
≤4	38
>4	10
pTNM category	
I/II	40
III	8
Differentiation	
Well/moderate	35
Poor	13

factor β (TGF- β) (20). Macrophage differentiation, a distinctive feature during the process of cancer genesis resulting in an increase in immunosuppressive cells, was found to be inhibited by enforced miR-142-3p expression by regulating gp-130 (21). In another study, miR-142-3p expression was reported to be reduced in colon cancer tissues, and the overexpression of miR-142-3p induced G1 phase cell cycle arrest by regulating CD133, ABCG2, and Lgr5 (22). These studies provide evidence of miR-142-3p as a tumour suppressor. However, the function of miR-142-3p in BC remains elusive. In our study, we determined that miR-142-3p expression was significantly decreased in BC tissues and cell lines compared with that in adjacent normal tissues and normal bladder urothelial cells, respectively. miR-142-3p overexpression significantly inhibited bladder cancer cell proliferation, migration and invasion, and miR-142-3p knockdown inhibited bladder cancer cells, showing the opposite results.

Rac1, a member of the GTPase family, plays an important role in controlling numerous cell events. Increasing studies have determined that Rac1 signalling pathways are involved in the development

Table II. Primer sequences used in qRT-PCR

Primers	Primer sequences
Rac1	
Forward:	5'-GGCTAAGGAGATTGGTGCTGTA-3'
Reverse:	5'-ACGAGGGGCTG-AGACATTAC-3'
GAPDH	
Forward:	5'-GCTGGCGCTGAGTACGTC-GTGGAGT-3'
Reverse:	5'-CACAGTCTTCTGGGTGGCAGTGATGG-3'
miR-142-3p	
Forward:	5'-TGTAGTGTTTCCTACTTTAT-3'
Reverse:	5'-GTCGTATCCAGTGC-AGGG-3'
Sh miR-142-3p	
Forward:	5'-GCTTCGGCAGCACATATACTAAAAT-3'
Reverse:	5'-CGCTTCACGAATTTGCGTGTCAT-3'

and progression of human tumours. As an upstream regulator of many essential signalling pathways in tumours, the expression level of Rac1 has been proven to be overexpressed in many cancers, including gastric cancer (23), lung cancer (24), colorectal cancer (25) and bladder cancer (19). A high level of Rac1 is associated with cell proliferation, apoptosis, migration and invasion in cancers (26). A study showed that miR-142 suppressed the migration and invasion of glioma through Rac1 (16). In accordance with previous studies, we found that Rac1 is a target of miR-142-3p in BC cells. Further investigation indicated that the expression of Rac1 was negatively controlled by miR-142-3p, which indicates that the close association between miR-142-3p and Rac1 affects the malignant biological behaviour of bladder cancer.

In conclusion, our study indicates that miR-142-3p plays a very important positive role in bladder cancer cell proliferation, migration and invasion. Silencing miR-142-3p by shRNA interference technology can effectively promote malignant biological behaviours, such as bladder cancer cell proliferation and invasion, by controlling the expression of Rac1. Our findings offer a new therapeutic target for the treatment of human bladder cancer.

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KALLIKREIN-RELATED PEPTIDASE 4 PROMOTES MIGRATION AND INVASION OF ENDOMETRIAL STROMAL CELLS IN ENDOMETRIOSIS BY INDUCING EPITHELIAL-MESENCHYMAL TRANSITION

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Endometriosis is a gynecological health problem for women of reproductive stage. Kallikrein 4 is a proliferative factor and has important roles in cancer development and progression. To explore the role of Kallikrein 4 in endometriosis, we detected the expression of Kallikrein 4 in ectopic and normal control endometriosis tissues. Moreover, the underlying influence of Kallikrein 4 on migration and invasion of endometrial stromal cells was investigated. Furthermore, the correlations between this gene and E-cadherin and N-cadherin were also evaluated. The results demonstrated that the expression level of Kallikrein 4 in endometrial tissues was significantly increased compared to normal endometrial tissues, and over-expression of Kallikrein 4 up-regulated expression of N-cadherin but down-regulated E-cadherin in endometrial stromal cells. The ability of migration and invasion of endometrial stromal cells *in vitro* was increased by up-regulating Kallikrein 4 expression, suggesting that Kallikrein 4 might be involved in the development of ovarian endometriosis.

Endometriosis is a chronic gynecological disease and one of its significant manifestations is that endometrial cells grow outside the uterus (1). The epidemiological evidence suggests that the incidence rate of endometriosis reaches around 10% in women of reproductive age, representing a serious public health issue for women worldwide (2). Previous studies indicated that endometriosis acted as a precursor for many invasive carcinomas, such as ovarian epithelial carcinoma, by the migration and invasion of stromal cells to distant tissues (3, 4). Although endometriosis is a benign disorder, it shares many similar features with cancers, such

as strong migration and invasive behaviors (5). Currently, apart from several traditional treatments including drug therapy and surgery, investigators have concentrated on exploring the molecular mechanisms of endometriosis to develop more effective therapeutic strategies.

Kallikrein-related peptidases (*KLKs*) belong to a serine protease family containing 15 members and are implicated with tumor genesis (6, 7). Lei et al. found that *KLK5-8* members were up-regulated in those patients with advanced stage and high-grade endometrial carcinoma by analyzing the mRNA sequencing data of endometrial cancer. Furthermore,

Key words: Kallikrein 4; endometriosis; migration; invasion

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they were strongly correlated with aggressive pathological phenotypes (8). Extensive investigations also suggested that *KLK4* might play essential roles in the pathogenesis of endometrial cancers (9, 10). Notably, the researchers highlighted that strong migration capability of endometriosis cells was possibly a key factor in the pathological mechanisms of endometriosis (11). The epithelial-mesenchymal transition (EMT) was characterized by cell migration and invasiveness, and this physiological process tended to give rise to endometriosis lesions (12). Moreover, existing evidence has shown that EMT exerts roles in decreasing cell adhesion and enhancing migration or invasion by regulating expression levels of cadherin proteins family, especially for E-cadherin and N-cadherin (13). Whitbread et al. pointed out that *KLK4* dramatically decreased the expression level of E-cadherin to trigger EMT, which was associated with the development and progression of prostate cancer (14). However, the impact of *KLK4* on the development of ovarian endometriosis has not been elaborated.

The aim of this study is to examine the expression of *KLK4* in ovarian endometriosis and explore the effect of *KLK4* on migration and invasion of endometrial stoma cells, which would provide deeper insight into the role of *KLK4* in the pathogenesis of ovarian endometriosis.

MATERIALS AND METHODS

Sample collection

The inclusion criteria for patient selection in the study were as follows: i) the patients did not take hormone drugs or have an intrauterine device; ii) the lesions were confirmed to be endometrial by the histology method; iii) the endometrial was in proliferative stage by the determination of the last menstrual period and reproductive hormone; iv) the patients did not have medical or surgery complications; v) the patients had not received preoperative chemo/radiotherapy; vi) the patients were non-pregnant women; and vii) all patients could receive follow-up care. Subsequently, the tissue specimens, including ectopic endometrial tissue and the corresponding matched normal endometrial tissue near to ectopic endometrial tissue, were collected from patients

who were diagnosed with ovarian endometriosis. Twenty endometrial samples were used in this study. Written informed consent was obtained from each participant and the study was approved by the ethics committee of the Affiliated Hospital of Changchun University of Chinese Medicine (Changchun, China).

Immunohistochemistry

The tissue samples were fixed in 10% formaldehyde at 20°C for 24 h, embedded in paraffin and then sliced into sections of 5- μ m thickness. The sections were dehydrated in ethanol in a graded concentration series, immersed in xylene at 100°C and alcohol, and then incubated with 1% bovine serum albumin at 20°C for 1 h and in hydrogen peroxide at 20°C for 30 min. Thereafter, they were probed with primary antibodies against *KLK4*, N-cadherin and E-cadherin at 4°C overnight followed by three washes with PBS (phosphate buffer saline) solution. The horseradish peroxidase-conjugated secondary antibodies were added for 1 h at 20°C. The sections were then treated with 3'-diaminobenzidine at 20°C for 30 min and counterstained with hematoxylin for 5 min. Finally, the immunoassaying was visualized by inverted microscope.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from ectopic endometrium, and normal endometrial tissues with Trizol reagent, respectively. Reverse transcription was performed according to standard protocol. qPCR was performed using SYBR Green PCR kit with a Real-Time PCR system. The expression levels of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were used as an internal control and fold changes were calculated by the $2^{-\Delta\Delta t}$ relative quantification method. The relative expression of mRNAs was detected in triplicate for each sample. The primer sequences used in this study are shown in Table I.

Western blot assay

The 100 g test tissues were lysed with 500 μ L lysis buffer and then centrifuged. The supernatant was obtained, and protein concentration was determined by BCA Protein Assay Kit. Each sample (30 μ g) was separated by 10% SDS-polyacrylamide gel, and then transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 10% bovine serum albumin at 20°C for 1 h and then

incubated with 1:1000 dilution of specific primary antibodies at 4°C overnight. After being washed with 0.1% Tween in Tris-buffered saline (TBST), the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Immunoreactive bands were detected by the enhanced chemiluminescence kit. Proteins were analyzed with ImageQuantTL7.0 software.

Endometrial stromal cells (ESCs) isolation and culture

The endometrial tissue was sliced and stored a container at 4°C for 60 min during operation. If the tissue specimen was diagnosed as ovary endometriosis cyst, the fresh capsule wall was cut and endometriosis tissues were scraped. If the tissue specimens were ectopic or ectopic endometrial tissues, they were minced into about the size of a grain of rice. Then, the obtained tissues were digested with 3 mL collagens at 37°C for 1 h. The cells were dispersed with DNase I for 60 m at 37°C by shaking and pipetting. The cells were plated, washed and suspended in High-glucose Dulbecco's modified Eagle's medium (DMEM)/F12 containing 15% fetal bovine serum (FBS) after passing through a 40-mm cell strainer. Finally, the cells were plated into a 6-well plate and cultured in an incubator containing 5% CO₂ at 37°C.

Cell transfection

The Silencer Select Predesigned siRNA targeting *KLK4* (siRNA-*KLK4*) was synthesized (target sequence 5'-AATCCGTGTCCGAGTCTGAC-3'). The endometrial stromal cells were transfected with siRNA-*KLK4*. Meanwhile, the *KLK4* overexpression vector was established and transected into endometrial stromal cells using Lipofectamine 2000 according to the manufacturer's protocols.

Cell migration

Transwell assay was performed to measure cell migration ability. Briefly, a cell suspension was prepared using 50,000 cells in DMEM supplemented with mitomycin C. The cells were then inoculated on the upper chamber. In the lower chamber, DMEM containing 15% fetal bovine serum was added. Following 24 h of culture at 37°C, cells that failed to pass through the membrane between the upper and lower chambers were discarded while those in the lower chamber were fixed with 100% methanol at 20°C for 15 min and then stained with 1%

crystal violet at 20°C for 30 min. Cells that passed through the membrane were counted in six microscopic fields that were selected randomly.

Cell invasion

For the invasion assay, a Transwell chamber coated with Matrigel was used. Following transfection, cells (1.0×10⁵ cells/chamber) were transferred to the upper chamber for incubation while in the lower chamber, DMEM with 20% FBS was added at 37°C. Following 24 h, cells that did not pass through the membrane between the upper and lower chambers were removed, and those in the lower chamber were fixed with 100% methanol at 20°C for 15 min and then stained with 1% crystal violet at 20°C for 30 min. Cells that passed through the membrane were counted in six microscopic fields that were selected randomly.

RT-qPCR and Western blot assays

Total RNA was extracted from four types of cells, including ectopic ESCs, ESCs with siRNA-*KLK4* transfection, ESCs transfected with *KLK4* overexpression plasmid and ESCs with blank plasmid. The qPCR was performed using SYBR Green PCR kit by a real-time PCR system. The relative expression of mRNAs was detected in triplicate for each sample. All primer sequences used are shown in Table I. Similarly, the total proteins were extracted from these four kinds of cells according to the method described above. The cells were incubated with primary antibody *KLK4* (ab71234, dilution ratio of 1:500), N-cadherin (ab98952, dilution ratio of 1:1000), E-cadherin (ab1416, dilution ratio of 1:50) and GAPDH (ab181602, dilution ratio of 1:10,000) at 4°C overnight.

Statistical analysis

All statistical analyses were performed by Prism 6 software. All the data were presented as the mean ± SD. All data have at least three replicates. The difference was analyzed with Student's *t*-test. When *P* < 0.05, the differences between groups were considered significant.

RESULTS

KLK4 is up-regulated in ectopic endometrial tissues

As shown in Fig. 1, the immunohistochemistry analysis revealed that *KLK4* was expressed in ectopic,

ectopic endometrial, and normal endometrial tissues. Moreover, there was a higher level of *KLK4* mRNA and protein in ectopic endometrium tissues than in the other two types of tissues (Fig. 2). We observed that E-cadherin and N-cadherin were also expressed in eutopic, ectopic and normal endometrium tissues. The RT-qPCR analysis showed that N-cadherin was increased in ectopic endometrium tissues compared with others, while E-cadherin was down-regulated in ectopic endometrium tissues (Fig. 2A). Similarly, there was an elevated level of N-cadherin protein and a decreased E-cadherin level in ectopic endometrium tissues compared to other forms of tissues (Fig. 2B). These results indicated that *KLK4* level had a tendency of positive association with N-cadherin expression while *KLK4* was negatively correlated with E-cadherin.

KLK4 overexpression increases the migration and invasion of ESCs

To further explore the underlying roles of *KLK4* on endometriosis, we investigated the influence of *KLK4* on the migration and invasion of ESCs. The results of migration assay demonstrated that *KLK4* overexpression significantly increased cell migration (Fig. 3A). Meanwhile, the invasion assay revealed that the number of ESCs with *KLK4* overexpression transfection that successfully passed through the Transwell membrane exceeded that of other groups

(Fig. 3B), indicating that *KLK4* overexpression might be involved in the molecular mechanisms of ESC migration and invasion.

KLK4 overexpression increases N-cadherin level but decreases E-cadherin level

Our findings indicated that, in endometriosis tissues *KLK4* and N-cadherin were up-regulated while E-cadherin level was decreased. Next, we investigated the interactions between *KLK4* and N-cadherin and E-cadherin. The qPCR and Western blot assays showed that *KLK4* overexpression markedly up-regulated N-cadherin expression but down-regulated E-cadherin expression (Fig. 4). These results demonstrated that *KLK4* increased the migration and invasion of ESCs, probably via enhancing N-cadherin level and reducing E-cadherin expression.

DISCUSSION

Endometriosis is characterized by the growth of stromal tissue outside of the uterus cavity (15). Convincing evidence has suggested that endometriosis is associated with ovarian epithelial tumors and this correlation might serve as an intermediate stage that caused a malignant transformation from endometriosis to other aggressive cancers (16). Although a large body of research has identified several underlying biomarkers in initiation and

Table I. Primers used for RT-qPCR.

Gene	sequence
KLK4	Forward: 5'-ATGGCCACAGCAGGAAATC-3' Reverse: 5'-CAGGATGTATTTGGGGGTCAAT-3'
E-cadherin	Forward: 5'-GGCGGGTAGTGTAAGTGTCA-3' Reverse: 5'-CTCAAATGGATGTTGGGTCA-3'
N-cadherin	Forward: 5'-AAAGAGTGAGCGACAAGGAA-3' Reverse: 5'-AAAGTAGGCAGAACAAAGGTG-3'
GAPDH	Forward: 5'-ATGGAAATCCCATCACCATCTT-3' Reverse: 5'-CGCCCCACTTGATTTTGG-3'

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; *RT-qPCR*: Reverse transcription-quantitative polymerase chain reaction.

development of endometriosis in recent decades, the detailed molecular mechanisms of endometriosis has not been fully understood.

In recent years, increasing studies have suggested that *KLKs* are abnormally expressed in a wide variety of cancers, such as prostate cancer and ovarian cancer (17, 18). Prassas et al. (19) performed a cell-based high-throughput screening analysis and highlighted that cardiac glycoside as an anti-cancer medication functioned probably via

remarkably inhibiting the expression level of *KLKs*. Yousef et al. found that seven *KLK* genes (*KLK5-8*, *KLK10*, *KLK11* and *KLK14*) were parallels over-expressed in ovarian cancer, which was confirmed by analyses of multiple databases (20). Additionally, Dong et al. argued that there were higher mRNAs and proteins levels of *KLK5* and *KLK7* in epithelial ovarian cancer. Moreover, a *KLK5* transcript with short 5'-untranslated region (UTR) and a long *KLK7* transcript with a long 3'-UTR region had

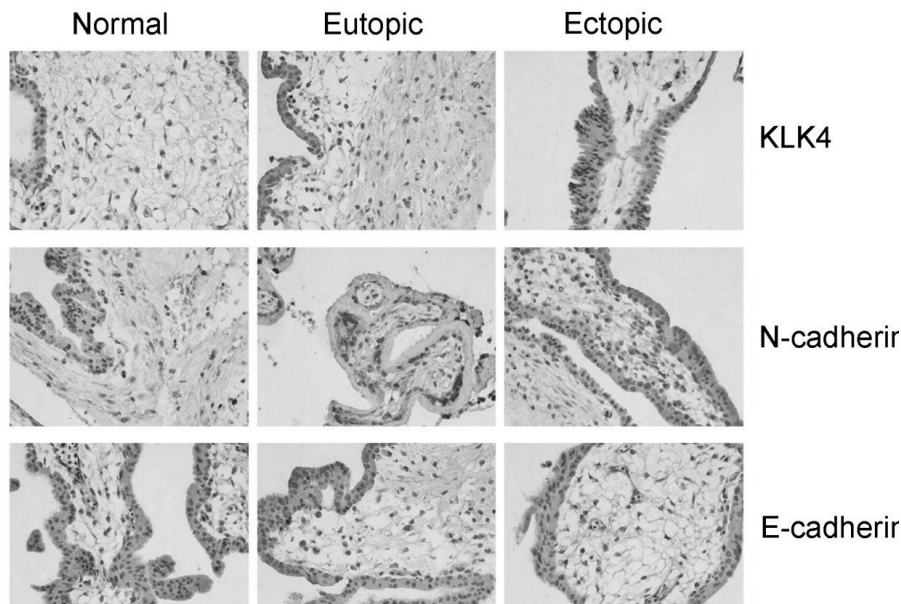


Fig. 1. Representative images of protein expression of *KLK4*, *N-cadherin* and *E-cadherin* in tissue samples collected from eutopic endometrium, ectopic endometrium and normal endometrial tissues.

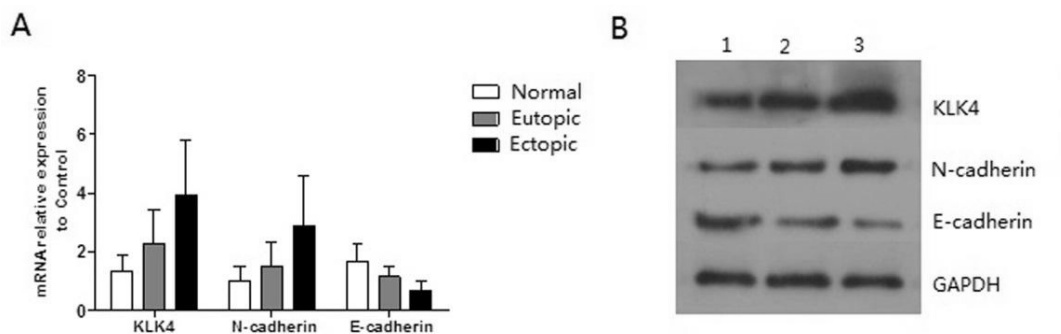


Fig. 2. RT-qPCR results of *KLK4*, *N-cadherin*, *E-cadherin* and *GAPDH* expression in tissue samples collected from ectopic endometrial, ectopic endometrium and normal endometrial tissues. **A)** Results of qPCR. **B)** Results of Western blot assay. *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; RT-qPCR: Reverse transcription-quantitative polymerase chain reaction.

increased levels in two ovarian cancer cell lines (OVCAR-3 and PEO1) compared with normal ovarian epithelial cells, indicating that these two genes possibly participated in the progression of epithelial-derived cancers (21). Notably, a growing number of researchers have demonstrated that *KLK4* plays critical roles in gynecologic cancers, especially in high-grade ovarian carcinoma (22, 23). In the present study, the potential effects of *KLK4* on ovarian endometriosis were investigated and we found that *KLK4* was unregulated in endometriosis, and over-expression of *KLK4* significantly induced the migration and invasion of ESCs. Interestingly, EMT was a prerequisite for the occurrence and development of ectopic lesions in endometriosis (24). Furthermore, EMT was characterized by the loss of an epithelial surface marker E-cadherin and the acquisition of mesenchymal markers such

as N-cadherin (25, 26). Herein, we examined the expression levels of E-cadherin and N-cadherin in ectopic, eutopic and normal endometrium tissues and the results indicated that E-cadherin was under-expressed while N-cadherin was over-expressed in ectopic endometrium tissues compared to normal endometrium tissues. More remarkably, there was a higher E-cadherin level in ectopic endometrium tissues than eutopic endometrial tissues. Conversely, N-cadherin expression was higher in ectopic endometrium tissues than eutopic endometrium tissues. More interestingly, we found that an increased *KLK4* expression enhanced the level of N-cadherin but reduced E-cadherin level in ESCs, suggesting that *KLK4* may have an important role in the regulation of biological behaviors of ESCs. Taken together, we inferred that *KLK4* overexpression increased migration and invasion of ESCs by

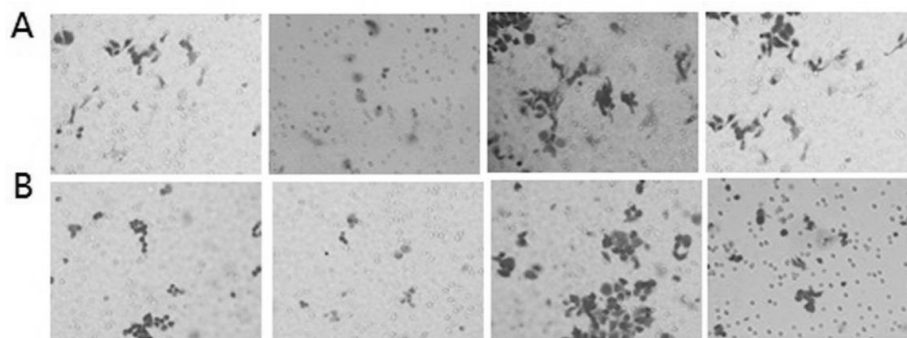


Fig. 3. Identification of endometrial stromal cells by detection of the vimentin expression through immunohistochemistry. **A)** Cell migration. **B)** Cell invasion.

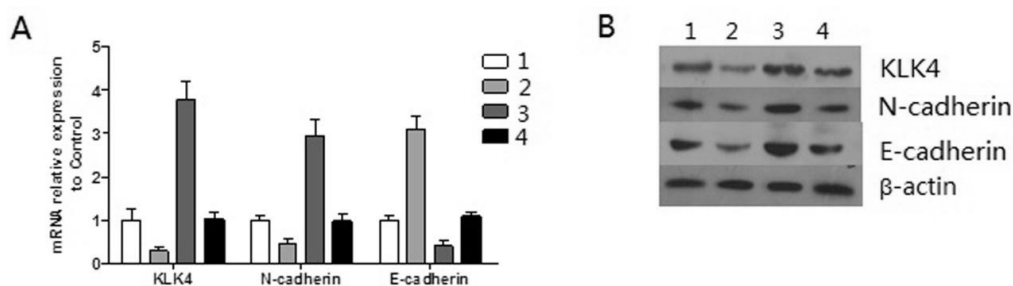


Fig. 4. Expression levels of *KLK4*, *N-cadherin*, *E-cadherin* and β -actin expression and in endometrial stromal cells. **A)** Results of RT-qPCR; **B)** Results of Western blot assay. The expression of β -actin was considered as an internal reference. RT-qPCR: Reverse transcription-quantitative polymerase chain reaction.

inducing EMT, which provided new insights into the potential roles of *KLK4* in the pathogenesis of ovarian endometriosis.

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MiR-543 FUNCTIONS AS TUMOR SUPPRESSOR IN OVARIAN CANCER BY TARGETING TWIST1

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MicroRNAs (miRNAs) have been demonstrated to have promoting or inhibiting effects on the tumorigenesis of multiple cancers, including ovarian cancer (OC), by regulating its downstream target genes. In the presented experiment, our aim was to explore the role of miR-543 in OC cell proliferation and invasion. Results of quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blot revealed that miR-543 have lower expression levels, while Twist homolog 1 (TWIST1) was expressed with higher levels in OC tissues and cells. Furthermore, the effects of abnormal miR-543 expression in OC cell proliferation and invasion were detected by CCK-8 and Transwell assay. According to luciferase reporter assay results, TWIST1 was identified as a downstream target of miR-543 in OC, and a negative correlation was observed between TWIST1 and miR-543 expression by Spearman's correlation analysis in OC tissues. In addition, TWIST1 may reverse the miR-543 suppression effect on OC cell proliferation and invasion. To sum up, miR-543 may promote OC cell proliferation and invasion by targeting TWIST1.

Ovarian cancer (OC) is one of the most common tumors in the female reproductive system, and its early diagnosis is difficult and the prognosis is poor (1). Because the early symptoms of OC are not apparent, most patients are already in the middle or advanced stages when they are diagnosed, and the treatment effect is not ideal in the later stage, therefore the mortality rate is the highest among gynecological tumors (2, 3). The etiology of OC is not yet well understood and may be related to age,

blood type, fertility, environment and genetics (4). OC has a high recurrence rate and complicated pathological mechanism, and the cure rate is approximately 30% (5). Explaining the pathogenesis of OC from the molecular mechanism may provide references for clinical diagnosis and treatment. Therefore, it is urgent to explore the molecular mechanism of OC progression, enrich the regulatory network of occurrence and development, and search for potential therapeutic targets.

Key words: ovarian cancer; miR-543; TWIST1; cell proliferation; invasion

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MicroRNAs (MiRNAs) are non-coding single-stranded ribonucleic acids that are approximately 22 nucleotides in length (6). They regulate a series of biological processes such as cell differentiation, development and homeostasis (6), and are closely related to the occurrence, development, metastasis and prognosis of tumors (7). A large number of studies have shown that miRNAs are closely related to OC progression (8). For example, miR-552 facilitated OC cell proliferation and invasion by repressing phosphatase and tensin homolog expression (9). MiR-363 repressed cellular malignancy and tumor growth by inhibiting NOB1 expression in OC (10). The abnormal expression of miR-543 has been studied in many cancers. Previous studies have reported that miR-543 was downregulated in cervical cancer (CC), glioma and endometrial cancer (EC) and inhibited tumor cellular malignancy (11-13). However, it is overexpressed in gastric cancer (GC), prostate cancer (PC) and colorectal cancer (CRC) and facilitated tumor progression (14-16). Recent studies have found that miR-543 expression was decreased in OC tissues and impeded OC cell invasion which was activated by MMP7 (17). Nevertheless, the function of miR-543 and its potential molecular mechanisms remain unclear in OC.

TWIST1, which belongs to the family of basic helix-loop-helix (bHLH) proteins, is not only an oncogene protein, but also a highly conserved important transcription factor regulating epithelial-mesenchymal transition (EMT), which is closely related to cell apoptosis and the formation, invasion and metastasis of various malignant tumors (18, 19). The role of TWIST1 in tumorigenesis and development has received increasing attention. TWIST1 has been found to be overexpressed in many tumors, including OC (20). Recent studies have shown that miRNAs play an important role in regulating TWIST1 in OC (21-23). Fan et al. found miR-15a-3p impeded OC growth and metastasis via inhibiting TWIST1 expression (21). Moreover, Li et al. determined TWIST1 as a direct target gene of miR-320 to promote OC progression (22). In addition, Zhu et al. confirmed that miR-186 inhibited epithelial mesenchymal migration of OC cells by regulating TWIST1, blocked G1 phase of the cell

cycle and promoted apoptosis of OC cells (23). However, whether TWIST1 function as a target of miR-543 has not been explored in OC.

This study used qRT-PCR, luciferase reporter assay, CCK-8, Transwell and Western blot assays to confirm whether miR-543 can regulate TWIST1 expression and inhibit OC proliferation and invasion, and to explore the function of miR-543 and its possible molecule mechanism.

MATERIALS AND METHODS

Clinical samples

Tumor specimens were collected from 32 patients with OC diagnosed by histopathology from January 2017 to March 2019 in our gynecology department. None of the patients had undergone surgery or chemoradiotherapy. Eighteen cases of ovarian epithelial cysts diagnosed in our hospital during the same period were collected as a benign control group. All patients signed the informed consent form. This study was approved by the ethics committee of our hospital.

Cell culture

The human ovarian epithelial cell line (HOSE) and four OC cells (OV90, SKOV3, OVCAR3 and A2780) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI-1640 medium (Invitrogen, USA) containing 10% fetal bovine serum (FBS, Invitrogen, USA) in 37°C humidified incubator with 5% CO₂.

Cell transfections

MiR-543 mimic, miR-543 inhibitor and controls which intervened miR-543 expression were obtained from GenePharma (Suzhou, China). OC cells were inoculated on 6-well plate and transfected when the cells grew to logarithmic growth stage according to the instructions of the transfection kit of Lipofectamine2000 (Invitrogen, USA). The specific steps are as follows: add 5 µl miR-543 mimic, miR-543 inhibitor or controls into 250 µl serum-free medium respectively. Add 5 µl Lipofectamine2000 into 250 µl serum-free medium. The above liquids were mixed and left at room temperature for 20 min. 1 ml of serum-free medium was added to each well, and above mixed liquid was added for cell culture. In the identification of transfected cells, RNA was

extracted from cells, and the expression of miR-543 was determined by RT-PCR.

Cell proliferation assay

The proliferation of transfected OC cells at the four time points of 1 d, 2 d 3 and 4 d was detected by CCK-8 (Dojindo, Japan) assay. Cells in each group were inoculated on a 96-well plates and cultured in a 37°C incubator containing 5% CO₂. CCK-8 solution (10 µL) was added into each group and incubated for 4 h. The absorbance values (OD450) of each well were measured by a microplate reader. The experiment was repeated three times to draw the cell proliferation curve.

Transwell assay

The cell density of each group was adjusted to 3×10^5 cells/ml with serum-free medium after 48 h transfection, and 200 µl of cell suspension was added to the upper chamber with Matrigel-coated. For lower chamber of 24-well plate, 500 µl medium containing 10% FBS was added. Then cells were incubated at 37°C for 48 h. Finally, invading cells were fixed with 1% paraformaldehyde, and stained with crystal violet for 5 min. The number of cells under the membrane was counted by microscope for statistical analysis. The experiment was repeated three times and averaged.

Dual-luciferase reporter assay

TWIST1 sequences including wild-type (TWIST1-wt) and mutant (TWIST1-mut) containing miR-543 binding sites were cloned into pGL3 Basic vector (Promega, USA). The constructed vector and miR-543 mimics or mimics control using Lipofectamine 2000 (Invitrogen, USA). The relative luciferase activity was detected with Dual-Luciferase Reporter Assay System kit (Promega, USA).

qRT-PCR

Total RNA was extracted by TRIZOL reagent (Invitrogen, USA). RNA concentration and absorption values at 260 nm and 280 nm were determined by uv spectrophotometer. The OD260/OD280 ratio was between 1.9 and 2.0, indicating high purity and meeting the needs of subsequent experiments. Synthesis of cDNA template was performed by reverse transcription in a PCR amplification instrument, and qRT-PCR experiment was conducted with ABI7500 quantitative PCR instrument (Applied biological systems, USA). Relative expression was calculated by the $2^{-\Delta\Delta CT}$ method. U6 and GAPDH was normalized for miR-543 and TWIST1 expression respectively. The experiment was repeated three times. Primers are shown in Table I.

Table I. *Primer sequences for real-time fluorescence quantification PCR*

Gene Name	Primer sequences (5'-3')
miR-543	F CCAGCTACACTGGGCAGCAGCAATTCATGTTT R CTCAACTGGTGTCGTGGA
TWIST1	F GGAGTCCGCAGTCTTACGAG R CCAGCTTGAGGGTCTGAATC
GAPDH	F GCACCGTCAAGGCTGAGAAC R TGGTGAAGACGCCAGTGGA
U6	F TCCGATCGTGAAGCGTTC R GTGCAGGGTCCGAGGT

Western blot

Total proteins were extracted by RIPA lysate (Solarbio, China). Proteins (30 μ g) was separated by 10% SDS-PAGE, and transferred onto PVDF membrane (Millipore, USA) to block with 5% skim milk. The membrane was added with primary antibody (TWIST1 and GAPDH,) and secondary antibody with horseradish peroxidase-labeled. Signals were visualized by enhanced chemiluminescence (ECL, Millipore, USA).

Statistical analysis

All values were expressed as mean $\pm$ SD. All experiments in this study were in triplicated. For statistical analysis the GraphPad Prism 5 software was performed. Value of $P < 0.05$ was considered statistically significant.

RESULTS

miR-543 expression is increased in OC

The expression of miR-543 was detected in OC tissues and cells by qRT-PCR assay. Findings revealed that miR-543 was decreased expression in OC tissues (Fig. 1A). Similarly, its expression was also obviously reduced in OC cells (Fig. 1B). These results indicated that miR-543 expression is low in OC tissues and cells.

MiR-543 suppresses OC cell proliferation and invasion

miR-543 mimic or inhibitor was induced into SKOV3 and OVCAR3 cells to explore its function in OC. qRT-PCR was carried to detect the transfection efficiency (Fig. 2A and B). Then cell proliferation and invasion were measured by CCK-8 and Transwell assays respectively. Results revealed that cell proliferation ability of two OC cell lines was markedly suppressed in miR-543 mimic group, but it was increased by miR-543 inhibitor (Fig. 2C and D). At the same time, the number of invading cells was also markedly reduced by miR-543 mimic, while enhanced by miR-543 inhibitor in two OC cells (Fig. 2E and F). Based on the above results, miR-543 acted as a suppressor in regulating OC proliferation and invasion.

TWIST1 directly targets miR-543

Although the target relationship of miR-543 and TWIST1 has been reported (13), there was still a lack of research in OC. We utilized TargetScan online software to predict their binding sites (Fig. 3A). To verify the prediction, dual-luciferase assay was performed in SKOV3 and OVCAR3 cells. We observed the luciferase activity was markedly decreased in TWIST1-wt cells

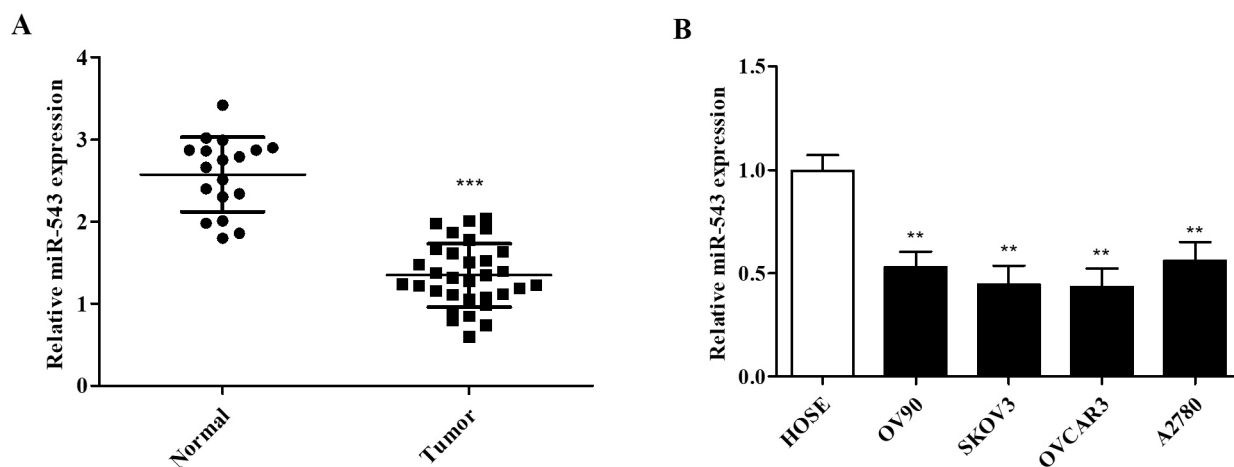


Fig. 1. miR-543's expression in OC. **A)** miR-543 expression was detected in OC tissues and cells. **B)** miR-543 expression was measured in normal cells (HOSE) and OC cells (OV90, SKOV3, OVCAR3 and A2780). ** $P < 0.01$, *** $P < 0.001$.

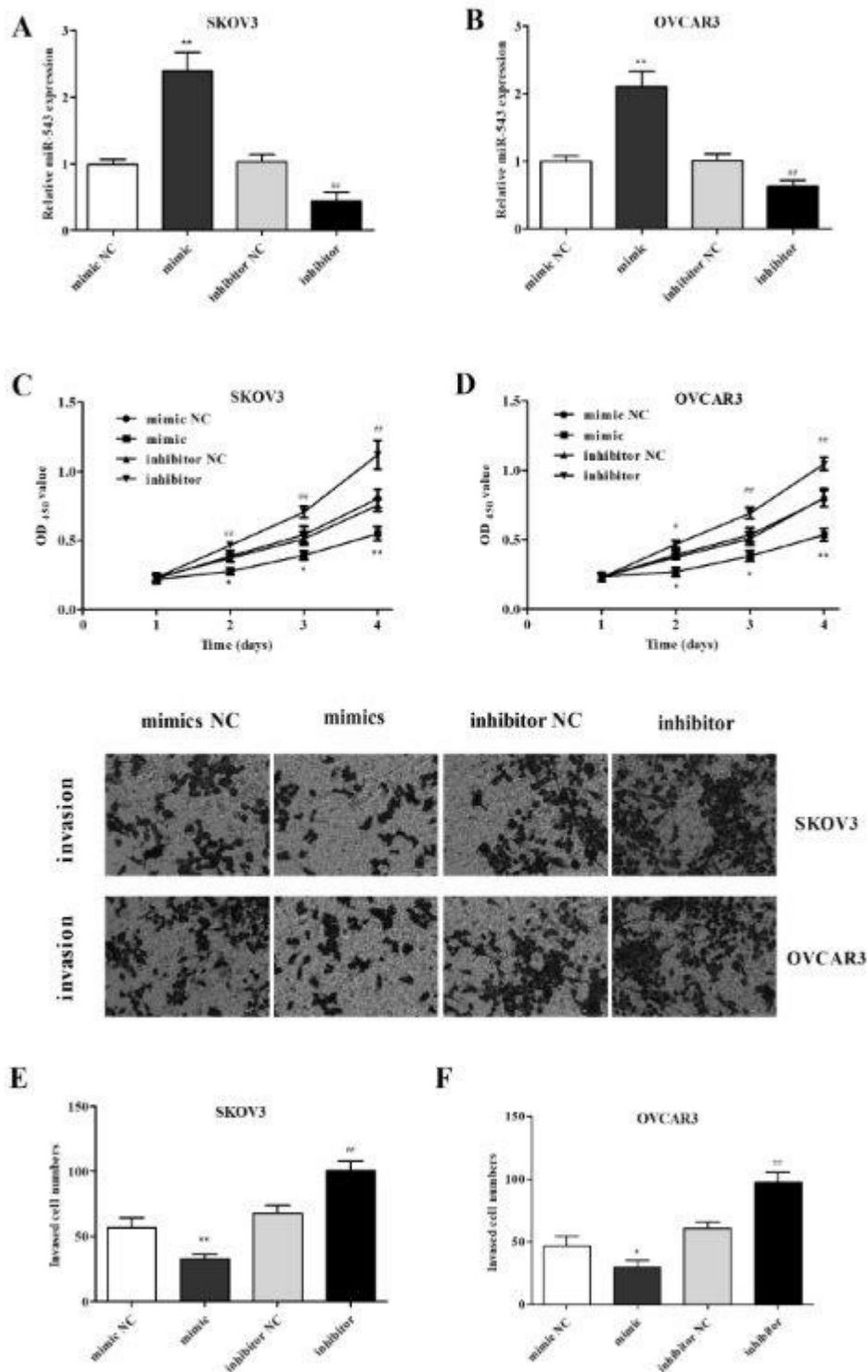


Fig. 2. miR-543 repressed OC cell proliferation and invasion. **A, B**) miR-543 expression in SKOV3 and OVCAR3 cells with miR-543 mimic or inhibitor transfection. **C, D**) Cells proliferation were detected in OC cells with miR-543 mimic or inhibitor transfection. **(E, F)** Invaded cells were detected in OC cells with miR-543 mimic or inhibitor transfection. * $P < 0.05$; ** $P < 0.01$; # $P < 0.05$, ## $P < 0.01$.

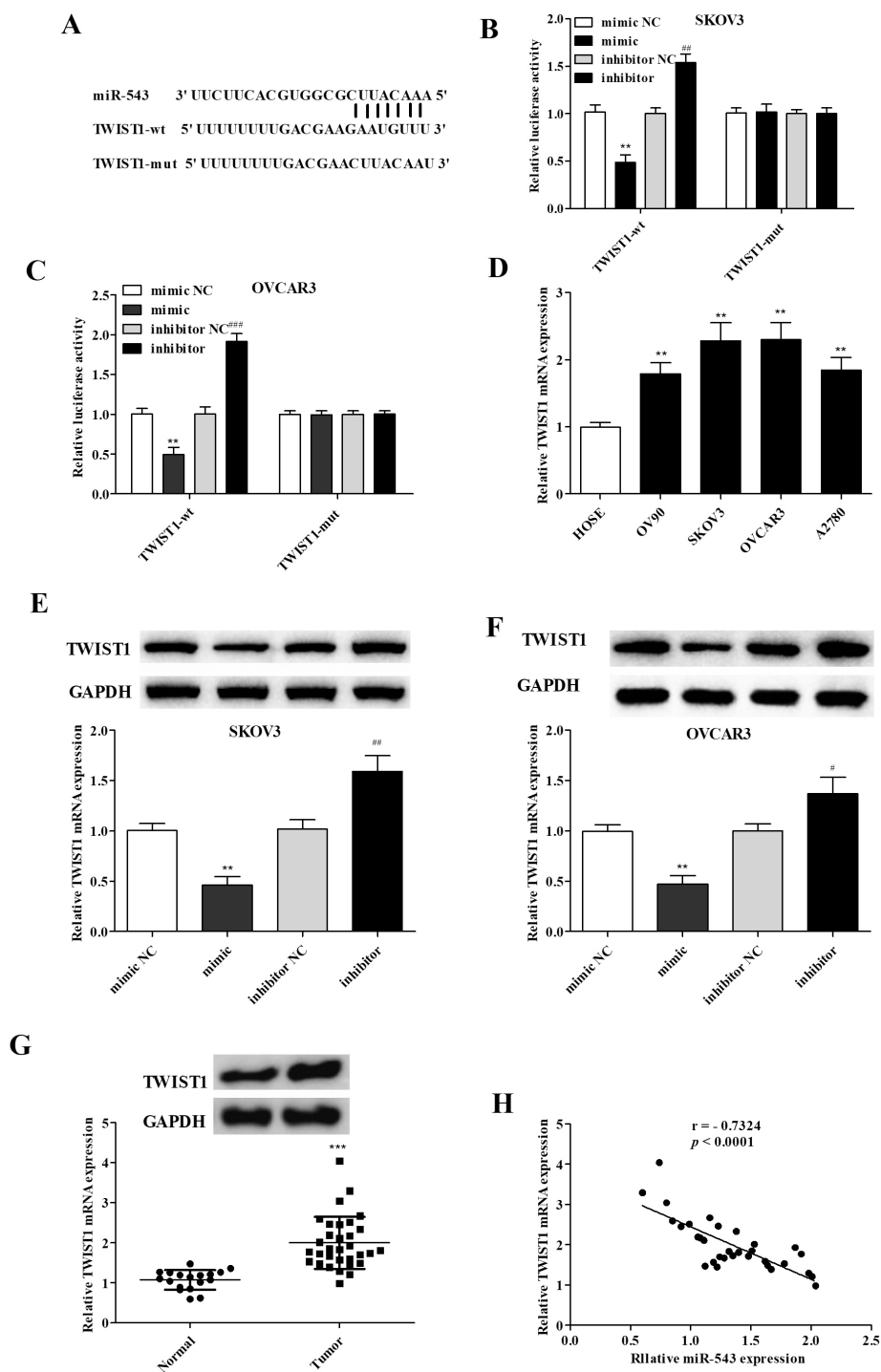


Fig. 3. miR-543 targets *Twist1* in OC. **A)** Binding sites of miR-543 and *Twist1*. **B, C)** Luciferase activity detected in OC cells. **D, E)** *Twist1* expression in OC cells with miR-543 mimic or inhibitor transfection. **F)** *Twist1* expression in OC tissues. **G)** *Twist1* mRNA expression in OC cells. **H)** The relevance of miR-543 and *Twist1* expression. ** $P < 0.01$, *** $P < 0.001$; # $P < 0.05$, ## $P < 0.01$.

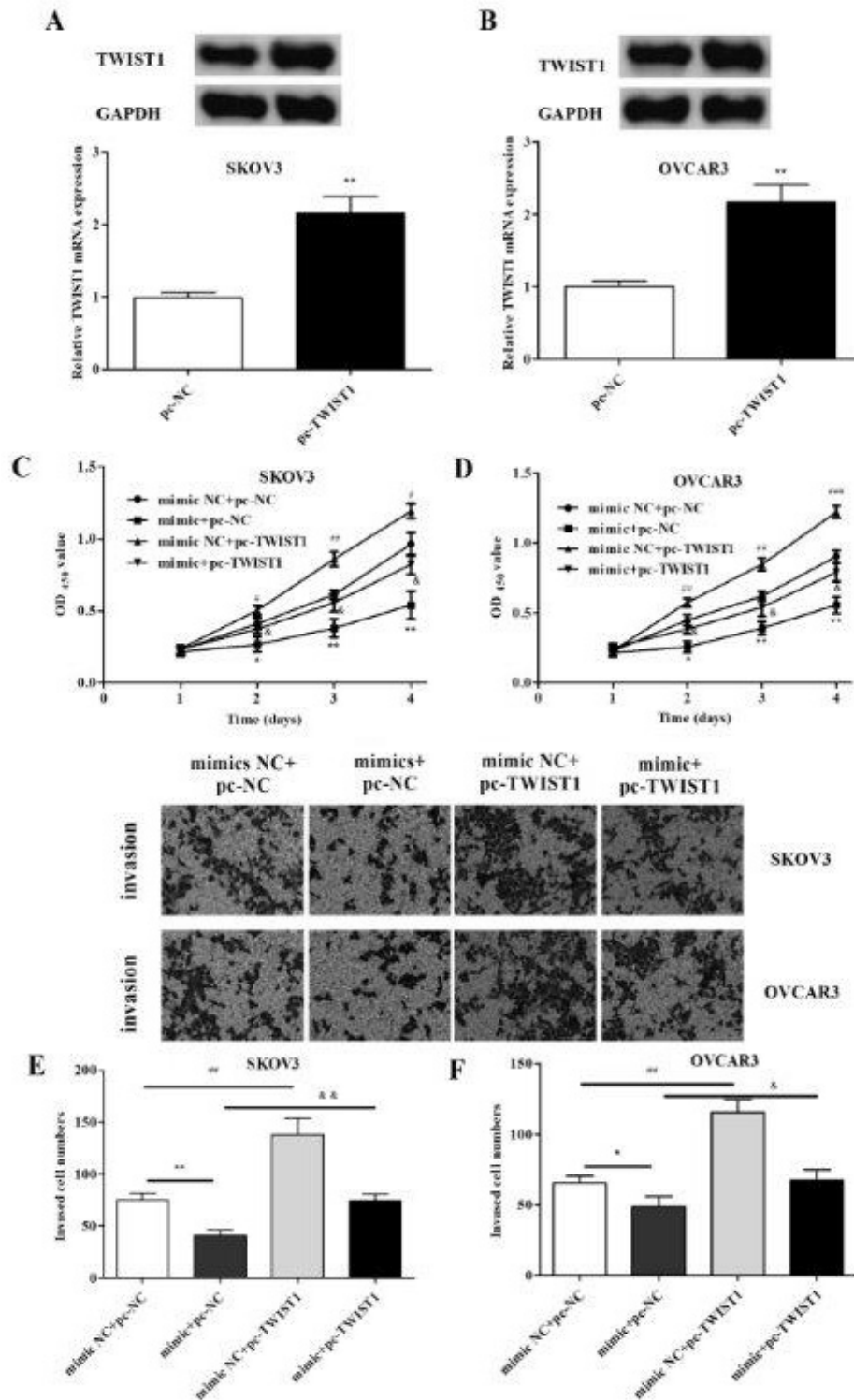


Fig. 4. *Twist1* reversed the miR-543 suppressive effect on OC progression. **A, B)** *Twist1* expression in OC cells after overexpressing *Twist1*. **C, D)** Cell proliferation in OC cells after overexpressing miR-543 and *Twist1*. **E, F)** Invaded cells in OC cells after overexpressing miR-543 and *Twist1*. * $P < 0.05$, ** $P < 0.01$; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$; & $P < 0.05$, && $P < 0.01$.

co-transfected with miR-125 mimic while increased in the presence of miR-543 inhibitor (Fig. 3B and C). Moreover, Twist1 has an increasing expression in OC cells compared with normal cells (Fig. 3D). Moreover, miR-543 mimic could greatly impede TWIST1 expression while miR-543 inhibitor greatly upregulated TWIST1 expression (Fig. 3E and F). Additionally, we found TWIST1 expression was increased and a negative correlation between miR-543 and TWIST1 was observed in OC tissues (Fig. 3G and H). Collectively, miR-543 directly interacted with TWIST1 and repressed TWIST1 expression in OC cells.

miR-543 impedes OC progression via targeting TWIST1

To confirm whether miR-543 regulates OC proliferation and invasion through the regulation of TWIST1, pc-TWIST1 was transfected into SKOV3 and OVCAR3 cells to enhance its expression. Pc-TWIST1 which was used to overexpress TWIST1 were purchased from Genechem (Shanghai, China). As we expected, TWIST1 expression was obviously enhanced in two cell lines after treatment with pc-TWIST1 (Fig. 4A and B). Subsequently, cell proliferation and invasion capabilities were detected using the CCK-8 and Transwell assays. Cell proliferation of two OC cells were promoted by TWIST1 overexpression, but miR-543 mimic could decrease cell proliferation (Fig. 4C and D). Furthermore, pc-TWIST1 could offset miR-543 mimic suppressing effect on OC cell proliferation (Fig. 4C and D). In addition, results of Transwell assay revealed that the number of two cells invaded were decreased by miR-543 mimic but increased by pc-TWIST1 (Fig. 4E and F). Also, pc-TWIST1 was observed to abolish the inhibitory effect on cell invasion induced by miR-543 mimic (Fig. 4E and F). This results suggest that miR-543 represses OC proliferation and invasion through targeting TWIST1.

DISCUSSION

In recent years, increasing attention has been focused on the regulatory role of miRNAs in tumors, especially in studies on the regulatory mechanism. As an important way of regulating gene expression level, the abnormal regulation of miRNAs is closely related to the occurrence and development

of malignant tumors (7, 24). Depending on the target genes they regulate, miRNAs play an oncogene or tumor suppressor role in malignant tumor development (7). OC, which is prone to pelvic, abdominal and retroperitoneal lymph node metastasis, is a common female malignant tumor, the specific mechanism of which is unclear (25). Previous studies have confirmed that miRNAs are involved in the occurrence and development of OC, and multiple miRNAs are expressed abnormally in OC cells and tissues, which can play an important role in the process of cell growth, differentiation, infiltration and metastasis of OC as oncogenes or tumor suppressor genes (8).

In recent years, several studies have revealed that miR-543 is an important factor in the development of cancer and participates in tumorigenesis through various pathways. It was reported that reduced expression of miR-543 was observed in CC and repressed cell growth and metastasis via inhibiting TRPM7 expression (11). Moreover, Xu et al. suggested that miR-543 was downregulated and functioned as a tumor suppressor in glioma (12). In addition, miR-543 expression was also found decreased in EC, and cell proliferation was inhibited by miR-543 forced expression (13). Our results are consistent with these previous studies. Herein, we also revealed that miR-543 directly targeted TWIST1 upregulated in OC through online software prediction.

The crosstalk between miRNA and TWIST1 has also been paid attention to in tumor related studies (26). For example, Yin et al. proposed miR-381 overexpression suppressed gastric cancer cell growth by decreasing TWIST1 expression (27). Similarly, a decreasing expression of miR-186 was also found in prostate cancer, and its upregulation restrained the progression through targeting TWIST1 (28). Analogously, upregulation of miR-543 impeded endometrial cancer cell growth by downregulating TWIST1 expression (13). In OC, miR-15a-3p, miR-320 and miR-186 could repress cellular malignancy by targeting TWIST1 (21-23). Consistent with these findings, miR-543 overexpression inhibited OC cell proliferation and invasion via decreasing TWIST1 expression.

In conclusion, we firstly explored the role miR-543 and TWIST1 in OC and their potential

mechanism in regulating OC cell proliferation and invasion. The results indicated that miR-543 was downregulated in OC, and its overexpression suppressed OC cell proliferation and invasion by inhibiting TWIST1 expression. It also confirmed that TWIST1 was a target of miR-543 and it attenuated miR-543 inhibitory effect on OC cell proliferation and invasion, indicating that miR-543/TWIST1 axis is associated with OC development and might become a biomarker of OC diagnosis and treatment in the future. However, the present study has some limitations which need to be improved. For instance, correlations between miR-543 expression and clinical features of OC were not analyzed on the basis of sufficient tissue samples. Moreover, whether miR-543 could regulate the EMT to promote OC progression by targeting TWIST1. Additionally, *in vivo* animal experiment (tumor formation in nude mice) should be introduced to further confirm the function of miR-543/TWIST1 axis.

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MITOCHONDRIAL AND REDOX DYSFUNCTION IN POST-MENOPAUSE AS RISK FACTOR OF NEURODEGENERATIVE DISEASE: A PILOT STUDY TESTING THE ROLE OF A VALIDATED JAPANESE FUNCTIONAL FOOD

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During the menopause women may experience increased oxidative stress and decreased antioxidant capacity and, together with the decline of neurosteroids, this represents a risk factor for Alzheimer's disease. The aim of the present study was to test a functional food (FPP-ORI, Osato Research Institute, Gifu, Japan) on redox and mitochondrial efficiency in post-menopausal women. The study population consisting of 69 untreated post-menopausal women were given supplements as follows: Group A was given a multivitamin (MV) 1c 2 times a day, and group B was given FPP 4.5 g 2 times a day. Group C consisted of 23 fertile premenopausal women as the control group. The tests carried out on entry, and at 3 and 6 months were erythrocyte redox parameters, plasma oxidated proteins, brain-derived neurotrophic factor (BDNF) and peripheral blood mononuclear cell (PBMC) mitochondria cytochrome c oxidase Vmax activity. Menopausal women showed an increased malondialdehyde (MDA) ($p < 0.05$ vs control) which was normalized by both treatments ($p < 0.05$), but MV failed to do so in the BMI ≥ 26 subgroup ($p < 0.05$). All other redox enzymes and BDNF were significantly lower in menopausal women and they responded only to FPP ($p < 0.05$). Carbonyl protein level was higher in "BMI ≥ 26 " subgroup ($p < 0.05$) and reduced only by FPP ($p < 0.05$). The PBMC cyclooxygenase to citrate synthase activity was reduced ($< 40\%$) in the menopausal group ($p < 0.01$) and only FPP caused a significant restoration ($p < 0.05$). Although preliminary, these data confirm the redox and mitochondrial dysfunction occurring in post-menopause and responsive to FPP but very poorly to high dosage antioxidants. This may lead to potential preventive opportunities in menopause-associated neurodegenerative disease.

A growing body of experimental and clinical data supports the hypothesis that estrogens exert an essential role in regulating both the female brain and body metabolome. This is particularly relevant at a mitochondrial level in high energy-demanding

organs such as brain and heart tissues (1-3). In this subcellular setting, estrogens enhance mitochondrial bioenergetic efficiency and protect from its dysfunction e.g., adenosine triphosphate (ATP) depletion and reduced membrane potential (4). Indeed,

Key words: menopause; redox dysfunction; mitochondria; FPP-ORI; MDA; GPx; SOD1; BDNF; COX activity

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within the brain, estrogen regulates glucose transport, aerobic glycolysis, and the bioenergetic system. Estrogen also modulates several enzymes of relevant importance in mitochondrial bioenergetics, such as glucose transporters, succinate dehydrogenase, pyruvate dehydrogenase, hexokinase, aconitase, alpha ketoglutarate dehydrogenase and Complexes I, III and IV involved in the electron transport chain (ETC) to produce ATP. This leads us to consider why the declining blood estrogen levels observed in menopause coincide with a similar event in brain bioenergetics eventually causing a metabolically impaired phenotype. It has to be recalled that under physiological cellular oxidative phosphorylation, mitochondria generate 1–4% reduced oxygen molecules which, by affecting ETC complexes I and III (5, 6), quickly react to form reactive oxygen species (ROS) (7). ROS are mostly produced in the mitochondria and these organelles are those most intensively damaged when an excess of oxidative stress is generated. Thus, an insufficient complex IV activity, unparalleled by a decline in activity of complexes I and III, as reported in Alzheimer disease (AD) patients, could lead to a relentless increased ROS spillover (8). These abnormalities are of interest considering that they have been clearly documented in AD while, with age progression, female gender represents the main risk factor for AD. Moreover, post-menopausal women show higher oxidative stress and reduced antioxidant capacity (9). As a matter of fact, estrogen receptors (ER) β are located within mitochondria and stimulate mitochondrial function and biogenesis. It has been demonstrated that hormone replacement therapy (HRT) decreases to a certain extent oxidative damage to both DNA and lipids in post-menopausal women (10), and lipid oxidation status may be inversely related to estrogen levels in this cohort of women.

MATERIALS AND METHODS

The aim of the present study was to test a functional food, i.e. Fermented Papaya Preparation (FPP-ORI, Osato Research Institute, Gifu, Japan) endowed with an established basic science and clinical validation on redox and immune system modulatory effectiveness as well

as mitochondrial efficiency (11–14) in post-menopausal women. The FPP used in the present study was obtained from *Carica papaya L.* cultivated in selected non-GMO and organically-treated Hawaiian crops. The properly cleaned fruit was then further subjected for 10 months to a patented yeast fermentation process with pharmaceutical-grade batch-to-batch end-product control also by Electron Spin Resonance at the Osato Research Institute (Gifu, Japan). The final composition of FPP per 100 g is as follows: 90.7 mg carbohydrates, 37 mg glutamic acid, 27 mg aspartic acid, 18 mg leucine, 16.9 mg potassium, 16 mg arginine, 13 mg valine, 11 mg serine, 11 mg glycine, 11 mg phenylalanine, 9 mg tyrosine, 9 mg isoleucine, 8 mg proline, 8 mg treonine, 6 mg lysine, 5 mg histidine, 5 mg methionine, 4.6 mg magnesium, 2.5 mg calcium, 240 μ g niacin, 75 μ g zinc, 17 μ g vitamin B6, 14 μ g copper, 2 μ g folic acid and 2 mg tryptophan. Other claimed “fermented papaya” extracts available on the market were excluded since prior in-house electron spin resonance testing had ascertained their poor-to-nil antioxidant effect in biological systems (Mantello P.ORI Oxidative Stress Research Lab, Gifu, Japan. In-house data).

Study design and population

Sixty-nine post-menopausal non-smoker women who were not on HRT, bioidentical hormone replacement therapy (BHRT) or phytoestrogens, steroids, or ongoing food supplements were enrolled in the study. Participants were recruited using a variety of community-based approaches. The exclusion criteria for the study were: a major depression/anxiety disorder; excessive drinker (>7 drinks/week); illicit drugs user; past or present history of cancer; papaya fruit-allergy heart disease; autoimmune disorders; poor sleeper or suffering from sleep-apnea; attending mind-body relaxation programs. A preliminary selection had been previously carried out to exclude those subjects with Val66Met functional polymorphism of brain-derived neurotrophic factor (BDNF) (Fig. 1) since this has been found to be associated with an impaired release of BDNF (15).

The subjects were divided into two groups, matched for age, duration of menopause diagnosis, BMI, educational level (high-school diploma and higher degree), dietary intake and physical activity (kcal/week), while being instructed to refrain from intense physical exercise. For dietary intake, the web-based version of the National

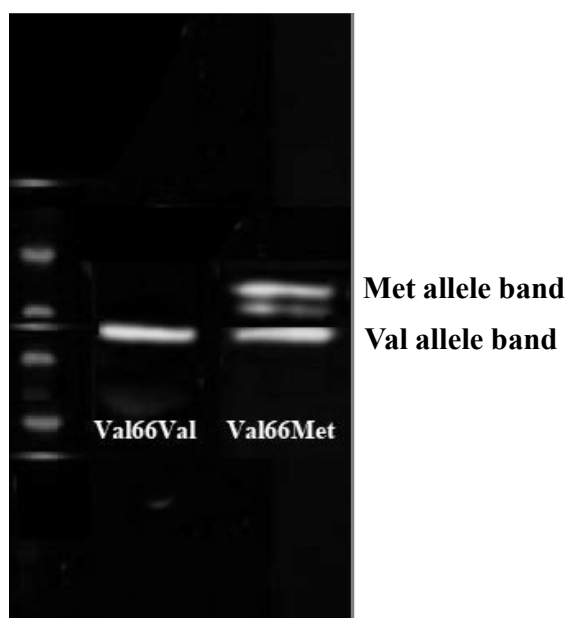


Fig. 1. Western blotting characterization of BDNF genotype. Subjects showing Val66Met genotype were excluded (see text).

Institute of Health Diet History Questionnaire (NIH DHQ) was adopted to assess diet history over the previous month and at each observation check point. The NIH DHQ is a food frequency questionnaire (FFQ) consisting of 124 food items and portion sizes.

A central allocation schedule for randomization was prepared through a computer-generated random sequence of the different treatment allocations. Treatment assignments were then made from separate randomization sequences created for each layer. Supplementation was carried out as follows: Group A was given a multivitamin (MV), (each capsule consisting of vitamin E 200IU, resveratrol 250 mg, coenzyme CoQ10 50 mg, pomegranate 100 mg, alpha-lipoic acid 100 mg, selenium 80 mcg) 1c 2 times a day, and group B was given FPP 4.5g 2 times a day,

orally (sublingually) in the morning and approximately 2 h after lunch. Treatments were maintained for 6 months. Group C, consisting of 23 fertile premenopausal women (age 37–46), served as “young” controls. Essential clinical and anthropometric data are shown in Table I.

All procedures were approved by an independent Ethics Committee for non-pharmacological research (ReGenera Research Group for Aging Intervention, Milan, Italy). All subjects gave written informed consent.

Assays

The participants refrained from alcohol, vigorous exercise or NSAID use 72 h prior to fasting venous blood collection (50 mL).

The genomic DNA was extracted from 5 to 10 ml of peripheral whole blood from all participants, as well as purified from lymphocyte pellets according to GFX Genomic Blood DNA Purification kit (Bio-rad Laboratories, Inc. Hercules, CA, USA). The primer sequences used in the PCR analysis were as follows: APOE rs7412 F:ATGCCGATGACCTGCAGA R:ACTGGCGCTGCATGTCTT Product size (bp) 684; rs429358 F:TCGGAACTGGAGGAACAACR: TACACTGCCAGGCGCTTCT Product size (bp) 260. Genomic DNA (50 ng) amplification comprised 30 cycles (94°C for 5 min, 94°C for 30 s, 65°C for 30 s, and 72° for 30 s), followed by a cycle of 10 min at 72°C for the final chain extension. Moreover, for the analysis of restriction fragment length polymorphism, the amplification product was digested with HhaI, followed by 5% agarose gel electrophoresis at 110 V for 2 h and 30 min, staining with Gel Doc™ (Qiagen), and visualization of the DNA fragments under UV light,

Morning blood samples were taken at 7:00–8:30 a.m. to avoid potential confounding diurnal variation of oxidative stress biomarkers (16). On each examination day, at 1, 3 and 6 months, blood samples were taken for routine testing

Table I. Baseline anthropometric, metabolic, family history and ApoE genotyping parameters

Parameters/Groups at baseline	Premenopause	Menopause + MV	Menopause + FPP
Body weight (kg)	51.4±11.3	56.5±9.0	54.2±12.3
BMI (kg/m ²)	22±4.0	25±3.0	23±5.0
Glucose (mg/dl)	68.2±15.4	62.2±13.4	66.4±11.6
Family history of LOAD	3/23	6/35	7/34
ApoE4 carriers/non-carriers	4/23	9/35	8/39

LOAD: Late onset Alzheimer disease. There was no statistical difference among groups tested.

(glucose, transaminases, haemoglobin, urea, creatinine, LDL and HDL cholesterol, electrolytes) by automated standardized procedures (Hitachi 911 using commercial kits) and for further studies.

Erythrocyte parameters

Whole blood collected in lithium heparin was taken from each subject and kept in chilled vacutainers immediately after its withdrawal followed later by centrifugation at 1600 g at room temperature (500 g for 10 min) to only reticulocytes. These were treated with 10% acetic acid and then centrifuged to obtain erythrocyte pellets. The plasma and buffy layer were discarded, and the erythrocyte sediment was washed 3 times with isotonic saline (9g/L). A stock haemolysate (5 g Hb/dl) was then obtained by processing cells via three freeze-thaw cycles in dry ice and by adding five volumes of ice-cold distilled water. Cell membranes were retrieved by centrifugation, and the supernatant was immediately frozen until the measurement of glutathione peroxidase (GPx), Superoxide dismutase-1 (SOD1) and catalase (CAT) activities. The test cuvette contained 2 ml distilled haemolysate, while a further one contained 1 ml of buffer and 2 ml of haemolysate to serve as standard. The reaction was initiated by adding 8 ml of H_2O_2 (30 mM in the buffer) to the test tube with the extinction decay being calculated at 240 nm.

Lipid peroxidation was assessed as malondialdehyde (MDA) by analyzing the levels of thiobarbituric acid reactive substances USING a colorimetric assay ($\lambda=535$ nm) (23). MDA was calculated according to the following formula: cell supernatant MDA (nmol/mg) = (Experiment group OD - blank control OD) / (Standard group OD - blank control OD) $\times$ Standard concentration / Sample concentration. Each group was repeated in triplicate. The results were expressed as nanomoles of MDA/mL (nmol/ mL).

Measurement of GPx activity was carried out as follows. Briefly, 0.02 ml of heparinised blood was treated with 0.1 ml of 5 mM GSH, 0.1 ml of 1.25 mM H_2O_2 , 0.1 ml of 25 mM NaN3 and phosphate buffer (0.05 mM, pH 7) in a total volume of 2.5 ml at 37°C for 10 min. The reaction was halted by adding 2 ml of 1.65 % H_3PO_3 and the reaction mixture was centrifuged at 1500 rpm for 10 min; 2 ml of supernatant was used for the estimation according to the procedure given under tissue GPx determination. The result was expressed as U/g Hb.

In this method, GPx catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized GSH is at once reduced via oxidation of nicotinammina adenina dinucleotid (NADH) to nicotinamide adenina dinucleotide fosfato (NADP). The decrease in absorbance at 340 nm was measured (Randox Kit) and the activity of GPx was calculated using the formula: Activity of GPx (IU/ gHb) = GPx activity (IU)/ Hemoglobin (g/dl).

Superoxide dismutase (SOD) activity in red blood cell lysate was determined by employing xanthine and xanthine oxidase (Sigma; St. Louis, MO, USA) to generate superoxide radicals reacting with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride to form a red formazan dye. The SOD-1 activity was then measured by the degree of inhibition of this reaction by adding xanthine oxidase at a concentration able to induce a 0.020% change in absorbance per minute at 550 nm (Randox). The activity of SOD was calculated by applying the following formula: Activity of SOD (IU/gHb) = SOD activity (IU)/ Hemoglobin (gr/dl) $\times$ 100.

Catalase activity in the erythrocytes lysate was measured by the kinetic method based on the disappearance rate of hydrogen peroxide (read at 240 nm) and expressed as k/g Hb, (or mU per g Hb). The inter- and intra-assay coefficients of variations (CV) were 2.9% and 6.6%, respectively.

Plasma/serum parameters

To assess the plasma concentration of advanced oxidation protein products (AOPPs) a commercial reagent kit (Thompson, Co., USA) was used following the manufacturer's instructions. The detection method makes 2-4-dinitrophenylhydrazine react with the protein carbonyl groups in an acidic milieu. Briefly, the oxidized protein standards were set by incubating bovine serum albumin (BSA) (50mg/ml) with 0.73 M H_2O_2 and 0.42 mM Fe^{2+} at 37°C for 1 h. The reaction mixture was halted with 40 μM butylated hydroxytoluene and the carbonyl content of the oxidized BSA was analysed spectrophotometrically. Following a dilution with a solution of phosphate-buffered saline (PBS) and native BSA, a final carbonyl content was obtained of 2.0nmol/mg protein and protein concentration of 4mg/ml. A bicinchoninic acid kit (Sigma) enabled the assessment of total plasma protein levels. Following a derivatization step with DNPH, the plate was coated with 200 μl of sample and subjected to

overnight incubation at 4°C. The plate was washed with PBS/Tween (0.05% Tween 20 in PBS) and stopped with 0.1% BSA for 1.5 h. After a further washing passage, a dilution of 1:1500 with 0.1% BSA/0.1% Tween 20 in PBS of biotinylated primary anti-DNP antibody was prepared, incubated at 37°C for 1 h and subsequently added with streptavidin-biotinylated horseradish peroxidase conjugate. After incubation at room temperature for 1 h, the addition of tetramethyl benzidine (Sigma) enabled the color development and after 15–25 min incubation, the reaction was halted by H₂SO₄. The absorbance was read at 450 nm in a spectrophotometer (Genesis-20, Spectronic Inc. USA). Plasma AOPP concentration was calculated as $\mu\text{molAOPPs/l}$ plasma and each sample was assessed in triplicate and recorded an overall 11% of inter-assay coefficient of variation.

Serum levels of BDNF was quantitatively measured by a commercially available sandwich ELISA [BiosensisRapid™ Mature Brain-derived neurotrophic factor (BDNF)], according to the manufacturer's instructions, in 96-well plates by means of a specific human BDNF antibody. Briefly, the plates were coated with 100 μl of mouse anti-human BDNF antibody. Wells were washed three times and 100 μl of standards and samples were added and incubated for 2 h. Wells were then washed with buffer and 100 μl of biotinylated mouse anti-human BDNF antibody was added and incubated for 2 h. Afterwards, 100 μl of streptavidin-horseradish peroxidase was added for 20 min. Then, wells were washed, added with 100 μl of substrate and incubated for 20 min. The absorbance was read within 30 min in a microplate reader (SpectraMax Plus 384, Molecular Device), set at 450 nm, to determine BDNF concentrations in duplicate according to the standard curve, as previously designed by testing different concentrations. Samples were diluted between 1:100–1:150 and BDNF levels were then calculated by non-linear regression from the standard curves. Measurements were repeated in duplicate and value expressed in ng/ml after correcting for sample dilution. The sensitivity of the assay was 20pg/mL with intra-assay and inter-assay variations recorded at 6% and 10%, respectively.

Mitochondria cytochrome (PMC) c oxidase Vmax activity was measured in peripheral blood mononuclear cell (PBMC) with spectrophotometric and fluorometric assays. PBMC isolation was carried out soon after blood samples were diluted 1:1 using a balanced salt solution

(anhydrous d-glucose 5.5 mM, KCl 5.4 mM, CaCl₂ 5 mM, MgCl₂ 0.98 mM, Tris 145 mM and NaCl 140 mM with pH adjusted to 7.6) and processed through density gradient centrifugation by a Histopaque® solution (density 1.077g/m, Sigma-Aldrich, USA). Each time of sampling, four 15 mL centrifuge cuvettes were employed to layer 16 mL of blood on 8 mL of Histopaque®. The suspension was centrifuged (30 min, 225 g) at room temperature and the layer containing the PBMCs at the interface was gently aspirated, washed in PBS (0.7 mM KH₂PO₄, 2.7mM KCl, 7.8 mM Na₂HPO₄, 136mM NaCl; pH7.4) and centrifuged again at 1,800 g for 5 min to remove any platelets. Afterwards, cell supernatants were discarded and the PBMC pellets were maintained at –80°C for further analysis. Enzymes were fully exposed to substrates by freezing and thawing the samples five times to yield optimal activities. Protein concentration was assessed using a DC protein assay kit (Bio Rad, Hercules, CA, USA). Cytochrome oxidase Vmax activity (COX, Complex IV, sec-1/mg) was calculated using standard spectrophotometric procedures in 0.5 ml reaction volume by measuring the oxidation of reduced cytochrome c at 550 nm. Cyclooxygenase (COX) activity was also referenced to its corresponding citrate synthase (CS) activity to correct for potential inter-sample differences in mitochondrial mass. CS Vmax activity (nmol/min/mg) was evaluated spectrophotometrically at 412 nm, monitoring the generation of 5-thio-2-nitrobenzoate after adding 100 μM oxaloacetate at 30°C.

RESULTS

Compared to “young” premenopausal controls, menopausal women, as a whole, showed significantly increased MDA concentration at the entry into the study ($p<0.05$, Table II). Starting from the first month of observation, the FPP group and both multivitamin (MV) and FPP treatment groups from the third month onward determined a statistically significant decrease of this parameter ($p<0.05$) of comparable extent. However, when looking at the subgroup of patients with BMI ≥ 26 , it appeared that MV was ineffective in reducing MDA throughout the study period, whereas FPP yielded a statistically significant reversal to normal level from the third month onwards (Table III, $p<0.05$).

The levels of all other redox enzymes (SOD1, GPx and CAT) were significantly lower in menopausal women, irrespective of BMI, ApoE status and history of late-onset AD (LOAD) and they showed a statistically significant improvement only in FPP-supplemented subjects at the end of the observation period ($p < 0.05$ vs MV-supplemented individuals, Table II).

Plasma levels of carbonyl protein in post-menopausal women showed a wide dispersion of data

which did not bring to any statistically significant difference *versus* premenopausal women controls (Fig. 2). When the “BMI ≥ 26 ” subgroup was taken into account, it appeared that carbonyl protein levels in post-menopausal women were statistically higher than in fertile controls ($p < 0.05$). Moreover, while MV intervention was ineffective, whatever observation period was tested, FPP brought about a significant decrement at 3- and 6-month testing ($p < 0.05$, Table III).

Table II. Time-course erythrocyte level of oxidative stress parameters in post-menopausal women: effect of MV and FPP

Parameters (premenopause control value)	Group	Baseline	1 month	3 months	6 months
MDA nmol/ml (1.1± 0.2)	MV	2.6±0.2*	2.2±0.4	1.3±0.3**	1.3±0.4**
	FPP	2.4±1.3*	2.1±0.2§	1.1±0.2**	1.2±0.2**
SOD1 U/ mgHb (16.9± 1.2)	MV	9.5±2.2	9.7±3.2	10.2±2.5	10.6±1.9
	FPP	10.2±1.6	9.8±2.1	13.4±2.1§	16.3±1.5§
GPx U/ g Hb (57.8± 6.2)	MV	50.5±55.5	52.1±10.1	51.2±8.4	50.2±9.2
	FPP	49.5±9.5	50.4±11.5§	50.5±9.2§	57.4±11.4§
CAT U/mgHb (106.8± 9.2)	MV	78.8±9.5*	82.4±13.2	85.6±11.5	88.4±18.2
	FPP	81.2±10.5*	83.3±11.3§	92.8±15.2	108.2±13.6§

MDA: Malonildialdehyde; SOD1: Superoxide dismutase; GPx: glutathione peroxidase; CAT: catalase; FPP: fermented papaya preparation. *Baseline values vs fertile premenopause control, $p < 0.05$; § vs related multivitamin (MV) value, $p < 0.05$; ** vs baseline values, $p < 0.05$.

Table III. Time-course plasma level of oxidative stress parameters in post-menopausal women: effect of MV and FPP

(premenopause control value)	Group BMI ≥ 26	Baseline	1 month	3 months	6 months
MDA nmol/ml (1.1±0.2)	MV	2.1±2.3	2.2±0.8	2.2±0.6	1.9±0.4
	FPP	1.9±2.5	1.3±0.2	1.2±0.4*	1.2±0.5*
AOPPs μmol/l (30.1±8.2)	MV	36.1±12.3	35.2±8.8	35.6±9.6	36.9±9.4
	FPP	35.9±7.5	34.3±8.2	35.2±5.4	32.2±9.5*

FPP: Fermented papaya preparation; MV: multivitamin; MDA: malonildialdehyde; AOPPs: oxidation protein products. * vs related MV and baseline values, $p < 0.05$.

Compared to the premenopausal women group, subjects in menopause showed a statistically lower plasma concentration of BDNF ($p < 0.01$, Fig. 3). MV treatment did not affect this parameter when observed throughout the study period, whereas FPP yielded a trend improvement which reached a statistical

significance at 3- and 6-months ($p < 0.05$ vs baseline).

Overall, there was a great dispersion of data, not differentiating between hormonal status nor treatment employed (Table IV). However, by reading the values for COX/CS ratio, it appeared that, compared to the premenopausal control group,

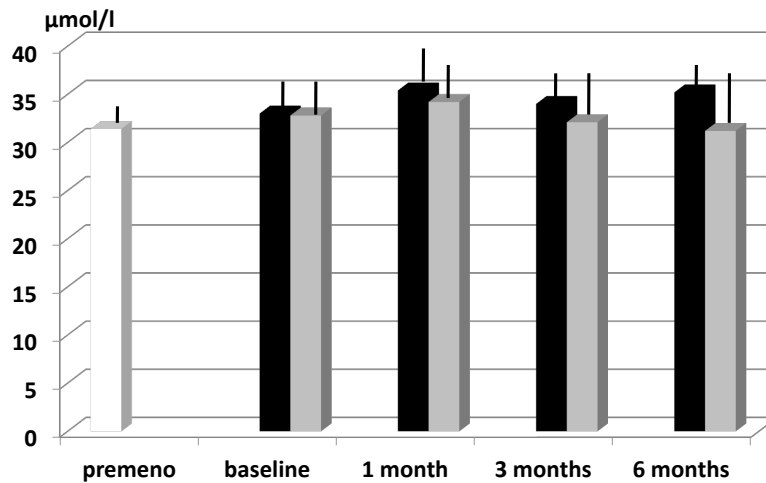


Fig. 2. Plasma oxidation protein products (AOPPs) in premenopause women (premeno, white bar) and women under multivitamin (MV) (black bars) or fermented papaya preparation (FPP) (grey bars) supplementation. No significant difference among groups.

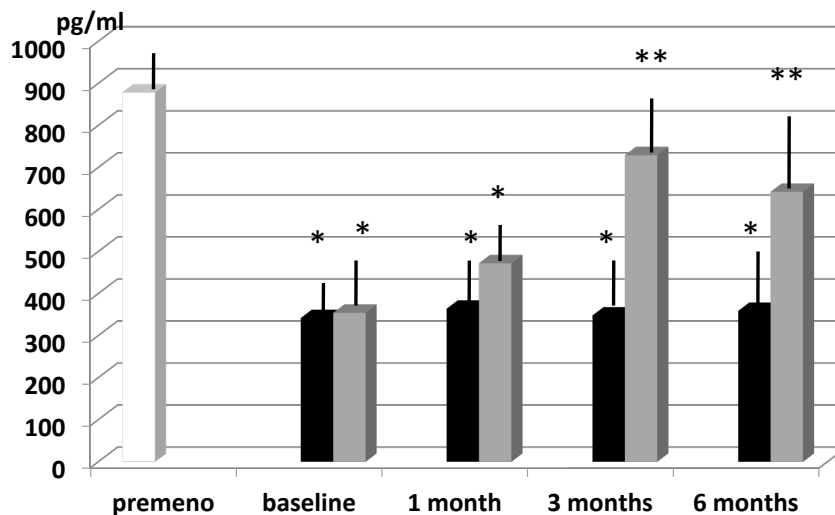


Fig. 3. Plasma brain-derived neurotrophic factor (BDNF) in premenopause women (premeno, white bar) and women under multivitamin (MV) (black bars) or fermented papaya preparation (FPP) (grey bars) supplementation. * $p < 0.05$ vs premenopausal women; ** vs baseline and vs multivitamin (MV)-supplemented group.

the total protein-referenced COX Vmax activity was reduced by more than 40% as an average in the menopausal group at baseline ($p < 0.01$). In particular, when we reanalyzed the time-course of COX/CS ratio after correction for age, such significant effect of FPP intervention was further confirmed (Fig. 4). On the other hand, BMI, ApoE status and history of LOAD did not affect further the significance (data

not shown). MV treatment did not prove to be of any effect, unlike the FPP dietary intervention which yielded a significant restoration at 3- and 6-month observation (Table IV, $p < 0.05$).

DISCUSSION

Although preliminary and ongoing extension

Table IV. Time-course PBMC level of COX, CS and COX/CS ratio in post-menopausal women: effect of MV and FPP

	Group	Baseline	1 month	3 months	6 months
COX sec⁻¹/mg (39.1±19.4)	MV	34.8±22.1	35.5±21.8	36.6±20.4	35.9±21.4
	FPP	36.9± 21.5	34.3± 17.2	37.8± 24.1	36.2± 19.8
CS nmol/min/mg (140.7± 26.7)	MV	139.7±24.2	138.7±29.5	140.5±21.3	141.2±31.3
	FPP	136.2± 22.6	134± 27.2	133.8± 31.2	135.7± 29.7
COX/CS ratio (0.31±0.11)	MV	0.21±0.13*	0.23±0.11*	0.22±0.11*	0.22±0.14*
	FPP	0.22±0.12*	0.26±0.15	0.30±0.16**	0.29±0.11**

FPP: Fermented papaya preparation; MV: multivitamin; COX: cyclooxygenase; CS: citrate synthase.

* vs premenopausal group value, ** vs baseline and vs MV-treated menopausal value.

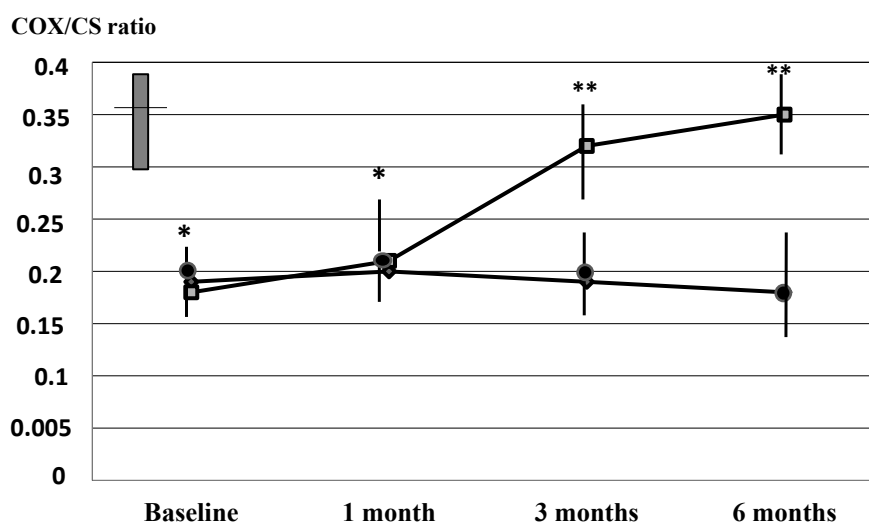


Fig. 4. Time-course measurement of COX/CS ratio in PBMC adjusted by age. Squared dots: fermented papaya preparation (FPP) group, Round dots: multivitamin (MV) group. Upper left grey rectangle: control premenopausal mean value. * vs premenopausal group value, ** vs baseline and vs multivitamin (MV)-treated menopausal value.

of the study is still in progress, these data confirm the reported redox imbalance taking place in post-menopausal women. This occurred whatever their BMI, ApoE status and possible family history of LOAD, although with some degree of association to metabolic and psychometric variables (17, 18). As a redox balance regulator, FPP showed to be significantly more effective than a multicomponent antioxidant formula as well as that reported by using red ginseng (19). These data are not new when considering prior double-blind or vitamin E-controlled clinical studies showing similar results (20, 21) through a well-documented nutrigenomic effect either in healthy young or elderly subjects (22). Overweight itself has been reported to be associated to oxidative stress whatever the age of women (23) and this may explain why only those women with BMI<26 were the ones with significantly higher AOPPs, given the wide scattering of values of this parameter in the pre- and post-menopausal groups. Interestingly, only FPP enabled a normalization of this parameter as well as of MDA which, in this subgroup, did not respond to multicomponent antioxidant supplementation. Indeed, besides a large body of experimental evidence, as pointed out by Taleb-Belkadi et al. (24), overweight has to be framed within a wider inflammatory/dys-metabolic disorder.

Although facing such phenomena requires a multimodal approach, a phytochemical intervention also endowed by brain protective properties has been envisaged (25). In this regard, the antioxidant/anti-inflammatory modulatory effect at gene expression level shown by FPP in the above-mentioned studies is likely to offer a more fundamental action. Of great relevance is also the finding that menopausal women, when analysed after age factor correction against the pre-menopausal group, showed a significant reduction of COX Vmax activity. These data seem to be in agreement with the recent findings reported by Mosconi et al. (26) in platelets of young subjects with family history of LOAD. Although our data as such cannot be held at the moment as endowed with a solid prognostic importance, it is of concern that a decrease in bioenergetic fluxes and mitochondrial enzyme activities together with mitochondrial number has been observed in Alzheimer's disease (42) and is

envisaged to be a candidate for future interventions (27). Our population was not on hormone replacement therapy (HRT), and the intervention with either estrogen or selective β estrogen receptor agonists has been shown to offer some benefit on redox balance, although the data on protection against protein and lipid oxidation are conflicting (28-30). HRT is also known to improve the reported lower BDNF level, as also observed in the present study, and normalized by FPP supplementation. One can hypothesize that FPP-induced activation of Nrf2-mediated redox balance as well as of neurotrophic signaling pathway may prove to yield a potential preventive option in menopause at risk of developing neurodegenerative disease. Also, physical activity is known to bring about an overall protective effect in menopausal women. However, although an active lifestyle is known to induce positive antioxidant enzyme modulation, Farinha JB, et al. (31) have shown that physical activity may be unable to cause significant mitochondrial adaptations when measured by ETC enzymes. FPP has been shown to increase ATP and NADPH concentrations (18) as well as Rac2 gene transcription through increased Sp1 DNA binding activity in PBMC (32). However, molecular studies are still in progress. Given the above data and the very recently confirmed high safety profile of FPP (32), this nutraceutical supplementation offers an interesting opportunity within a wider health-promoting and disease-preventing strategy in menopausal women. A new study using this nutraceutical in post-menopausal women already on HRT is ongoing with the view of revealing any potential synergy.

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LONG-TERM EVALUATION OF AUTOLOGOUS EXTRAORAL BONE GRAFTS AS BONE SUBSTITUTE IN MAXILLARY VERTICAL AUGMENTATION: AN IMMUNOHISTOCHEMICAL STUDY

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The aim of this study was to evaluate calvarial grafts when used as bone substitute for maxillary vertical augmentation prior to dental implantology. Bone specimens were harvested from patients at T0 (time of bone harvesting), 4 months (T1), 6 months (T2) and 15 years (T3) after grafting procedure. Morphostructural analysis and immunohistochemical analyses of bone sialoprotein-2 (BSP2), Collagen I, Vascular endothelial growth factor (VEGF), ERK ½ and nitric oxide (iNOS) inflammatory protein, expression were carried out. Outcomes from T0 specimens were compared to T1, T2 and T3 samples. In immunohistochemical analysis T1 and T2 samples showed the presence of important remodeling phenomena, with areas of bone resorption and apposition, together with new blood vessel formations. The T3 sample showed morphological features very close to native bone, as shown by the disappearance of welding lines. However, small polygonal cells resembling osteoblasts were close to Havers channels. Immunohistochemical analyses showed a drastic decrease of Collagen expression, at T1 and T2, stabilized at T3, in parallel to BSP2 expression increase, which was, however, considerably reduced at T3. Moreover VEGF angiogenic factor was increased at T2, in respect to T0, T1 and T3 which are quite similar. The highest level of Erk1/2 is evidenced in the T2 sample, basal levels of iNOS, related to inflammatory events is highlighted in T0 and T3, in respect to T1. Thus, autologous calvarial bone grafts seem to be suitable as biomaterial for bone regeneration procedures of the jaws. Moreover, the used protocol could be useful for studying bone substitutes in oral surgery and implantology.

Several studies (1, 2) have shown that, in severe atrophies (class V and VI according to Cawood and Howell (3), bone grafts with or without biomaterial (4) that increase the vertical and horizontal volume of the edentulous ridge, are useful. A sufficient volume and quality of alveolar bone must be present at potential implant recipient sites to ensure a predictable long-term outcome in terms of aesthetics and function (5), whereas several other Authors (6-8) proposed minimally invasive techniques to solve this matter.

The present study focuses on the biological and molecular aspects of bone neo-apposition promoted by autologous bone from cranial vault. Several studies also point out that the marginal tissue gap, especially in thin biotypes, is related to bone loss, therefore an accurate measurement of the initial levels and density of bone and soft tissue will influence the choice of the operator (9).

Tissue engineering was initially defined as “an interdisciplinary field that applies the principles of

Key words: bone regeneration; bone; grafting; dental implant; molecular biology; biochemical phenomena

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engineering and life sciences toward the development of biologic substitutes that restore, maintain or improve tissue or organ function” and then as a discipline with “the grand aim of understanding the principles of tissue growth, and applying this to produce functional replacement tissue for clinical use” (10).

In recent years, a variety of bone grafts and biomaterials have been used for augmentation of deficient alveolar ridge before implant treatment in partially and completely edentulous patients, including autogenous, allogenic, xenogenic, synthetic bone, and combinations of these (11, 12). In the panorama of regeneration, an innovative but still experimental alternative, therefore of scientific interest, are stem cells as a possible future means of regeneration combined with a scaffold (13).

One recent review (14) showed that autologous bone is the only bone with triple osteogenic characteristics (osteinduction, osteoconduction, osteogenesis), the other bone substitutes show only one or two of the three characteristics; while the structural composition (micro-macro particles), biocompatibility of biomaterials (release of calcium ions) (15), volume maintenance following bioreabsorption, sterile or readily sterilizable and chemical and physical properties, including porosity, hydrophilicity, and suitable surface topography of the bone substitute affect the choice of the nature of bone grafts (14). Crespi, in 2007, underlined that extraoral grafts have been estimated as a good solution to increase bone lack, obtaining a better quantity of bone with a lower resorption rate than intraoral bone grafts (16). Extraoral sites of autogenous block grafts include, among others, ilium, calvarium, tibia, and rib (17). Mertens affirmed that the calvarial bone graft is a feasible alternative for reconstruction of edentulous maxilla (18). Zizzari et al. in their review affirmed that autologous bone graft is the only bone substitute that has osteogenic potential, as it maintains viable cells from extraction from the donor site to grafting if properly treated and shares the same biologic origin as the recipient organism. This means that rejection will not occur, and neoangiogenesis and bone regeneration are strongly promoted (19).

In an *in-vivo* study (20), the performance of autologous bone in respect to heterologous bone

substitutes was evaluated. Intra and extraoral autologous bone grafts and a porcine-derived heterologous biomaterial were used as bone substitutes before implant-prosthetic therapy in the posterior maxilla. Bone tissue samples from sites regenerated through different methods were taken at the time of implant insertion (about 6 months after the regenerative therapy), and histologic and immunohistochemical analysis was performed to identify the expression of specific molecules involved in bone graft healing and integration. Results from this study indicated that sites treated with autologous bone, after the same period of healing, show a better integration with host tissue because of a greater neoangiogenic and osteoproliferative triggered response, as outlined by the higher levels of bone sialoprotein and vascular endothelial growth factor (VEGF) expression in comparison with levels recorded for porcine-derived heterologous bone graft (20).

Indeed, Hu et al. showed that the vascular endothelial growth factor VEGF, and bone sialoprotein (BSP) play a key role in neoangiogenesis and osteogenesis during inflammation and subsequent bone repair after the placement of the graft (21). Neoangiogenesis and the bone integration process also involve the MAP/ERK pathway, Collagen type I and inducible nitric oxide synthetase (iNOS), and all of these molecules were investigated in the present *ex-vivo* study.

Ito et al. showed that healing of structural grafts is limited due to lack of vascularization and remodeling; in fact, by performing gene expression analyses, they showed that there is a substantial decrease in genes encoding Receptor activator of nuclear factor kappa-B ligand (RANKL) and VEGF during allotransplantation healing. Trying to re-implant them at the allograft, they concluded that RANKL and VEGF are necessary and sufficient for proper bone remodeling (22).

Once the mechanism of osteogenesis is triggered, together with the angiogenesis regulated by VEGF, molecules, such as BSP 2, increase the deposition of the mineral matrix, thus favouring the formation of hydroxyapatite crystals. Experiments on mice have shown that the levels of BSP increased if the bone tissue was subjected to mechanical loading; also in

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(amoxicilline and clavulanic acid, 2g/day for 10 days), a non-steroidal anti-inflammatory (ketoprofen, 400mg/day for 10 days), a glucocorticoid (betametasone, 4mg/day for the first two days, 2mg on the third day) and a local antiseptic (chlorhexidine 0.20% mouthwash 3 times a day). A soft diet and scrupulous oral hygiene were also prescribed to all patients. After the first surgical phase and after removing the sutures after about 10 days, the patients were followed-up monthly with clinical and radiological examinations carried out with periapical X-rays of the treated site.

The insertion of the dental implants was performed in all patients at 4 months from grafting procedure. At this time, at the same point where the implant site was created, a biopsy was harvested with a trephine bur with a diameter of 3 mm and an height of 8 mm. For each patient 3 biopsies were obtained from 3 different implant sites. In each site the biopsy was obtained in the vertical augmented bone. A total of 9 biopsies were collected. This time was considered as the T1 group.

After 2 months from implant placement (6 months from grafting procedure), re-entry procedure for implant exposure was performed; in each patient, one biopsy was harvested with a trephine bur with a diameter of 3 mm and height of 8 mm. A total of 3 biopsies were collected. This time was considered the T2 group. Moreover, a bone biopsy was harvested in one patient who had to undergo implant insertion in a site next to the bone reconstructed with calvarial bone grafts 15 years previously. In the same way as for the T1 and T2 groups, the specimen was collected in the vertical regenerated bone.

Morphological and immunohistochemical analyses

Bone tissue samples, fixed in a buffered solution with 10% formalin phosphate for 72 h, were decalcified in an EDTA solution (MIELODEC kit, Bio- Optica, Milano), dehydrated in solutions with increasing concentrations of alcohol and xylene, and subsequently embedded in paraffin. Sections of 5 µm thickness were then cut, deparaffined in xylene and then in decreasing concentrations of alcohols and processed for haematoxylin-eosin staining and immunohistochemical analysis. In order to highlight the proteins, BSP2, Collagen I, VEGF, MAPK/Erk, iNOS, object of the study, the immunohistochemical analyses were performed on 5 sections of tissue for each experimental sample, using the Ultravision LP Detection System HRP

Polymer & DAB Plus Chromogen kit (Lab Vision Thermo, CA, USA), following the instructions provided by the manufacturer. The sections were incubated with mouse monoclonal antibodies to BSP2, Collagen I, VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA) with rabbit polyclonal antibody to iNOS (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit polyclonal antibody to MAPK/Erk (Cell Signalling Technology Inc., CA, USA). The chromogen used for the development of the reaction was 3,3-diaminobenzidine tetrachloride (DAB), which provided the insoluble oxidation product, stained in orange-brown and precipitated at the reaction site. The nuclei were contrasted with hematoxylin, and the preparations were observed under an optical microscope for both morphological and immunohistochemical examination. Negative controls were obtained by omitting the use of primary antibodies. The sections were observed by means of a Leica DN 4000 optical microscope (Leica Cambridge Ltd, Cambridge, UK) equipped with a Leica DFC 320 camera (Leica Cambridge Ltd, Cambridge, UK) to obtain computerized images.

After digitisation of images from immunohistochemical analysis, a specific software (QWin Plus 3.5 image analysis software, Leica Cambridge Ltd, Cambridge, UK) was used to evaluate the expression of VEGF, Bmp2, collagen, Erk, and iNOS. The analysis of protein expression was carried out by quantifying the brown coloured areas in ten randomly acquired fields for each section of each experimental point. The data obtained by specific software (the QWin software) were recorded in data sheets (Microsoft Excel, Microsoft Corporation, Redmond, WA, USA) and processed for standard deviations and histograms. The statistical significance of the results was evaluated using the Wilcoxon, Mann-Whitney Test, using the statistical analysis software (R Software, version 2.12.1 for Mac, R Development Core Team), and setting the significance level with $p = 0.05$.

RESULTS

The placement of a bone graft, aimed at increasing the volume of the residual crest, induces a reaction at the receiving site. This response, independent of the nature of the donor site, had some features common to the inflammatory process (hyperemia, vasodilatation). In a short time, the graft was

incorporated through the process of angiogenesis and colonization by mesenchymal cells and chemical mediators. Subsequently, these multipotent cells began to differentiate in an osteogenic sense and to proliferate. About 4 weeks after surgery, there was a bone neo-apposition, proven by the fact that osteoblasts are still vital in the graft and activate stem cells in the osteoblastic direction. The development of these reactions can be analyzed by evaluating the variations in the expression of specific proteins included in these processes. In this study it was chosen to analyze the expression levels of molecules closely involved in the graft integration process, such as BSP2, Collagen I, VEGF, MAPK/Erk, iNOS. Analyses were performed on biopsy samples of bone tissue taken from patients on surgical return to the regenerated site at 4 months after bone grafting, i.e. at the time of implant placement, at 6 months, i.e.

at the time of exposure of the screws, and finally, in one patient, at 15 years from the grafting procedure.

The morphological analysis was performed under a light microscope after staining with hematoxylin-eosin, and allowed to highlight the microscopic structure and the mutual relationships created between the native bone tissue and the grafted biomaterial.

In T1 samples (4 months), the presence of native bone tissue and new connective tissue fibers was identified; in fact, in T2 samples (6 months) a mostly compact structure with large areas of remodeling was detected in which it was possible to highlight the presence of vascular neoformations. In addition, there were welding lines with margin of bone resorption and neo-apposition between the pre-existing bone tissue and the graft. In the samples obtained about 15 years after grafting, the

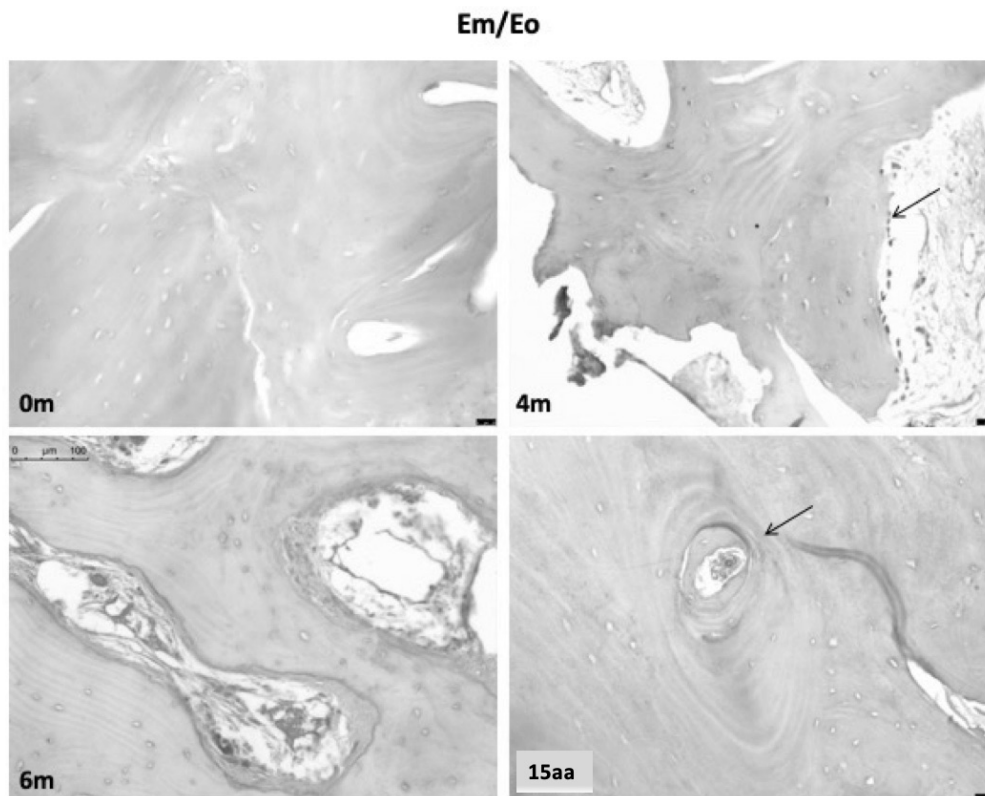


Fig. 1. Optical microscopic observation of bone tissue samples at the graft site obtained 0 (T0) 4 (T1), 6 (T2) months and 15 years (T3) after insertion of the graft from regenerated sites with autologous bone taken from calvaria. Colouring: haematoxylin- eosin. Magnification: 20x - 40x.

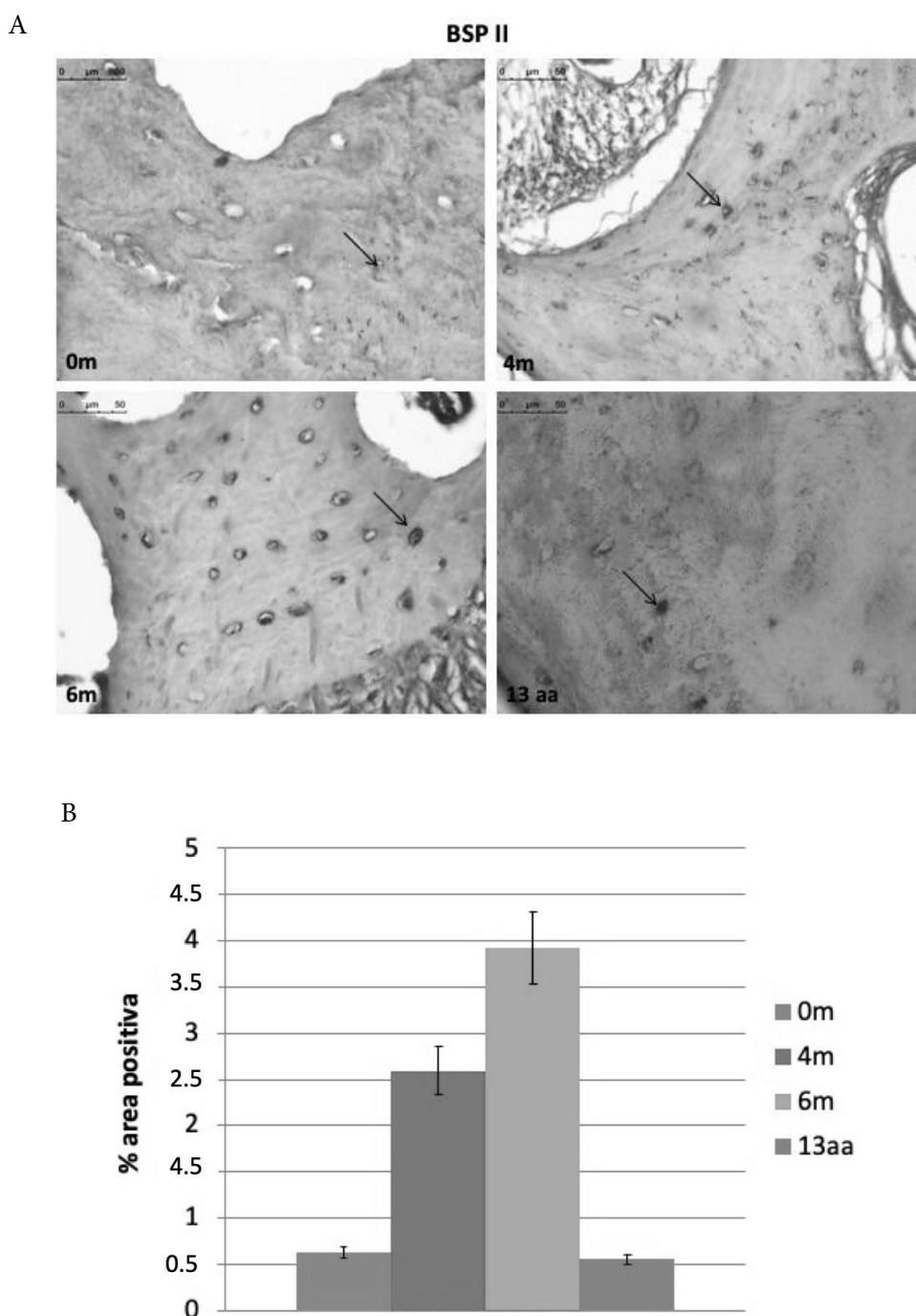
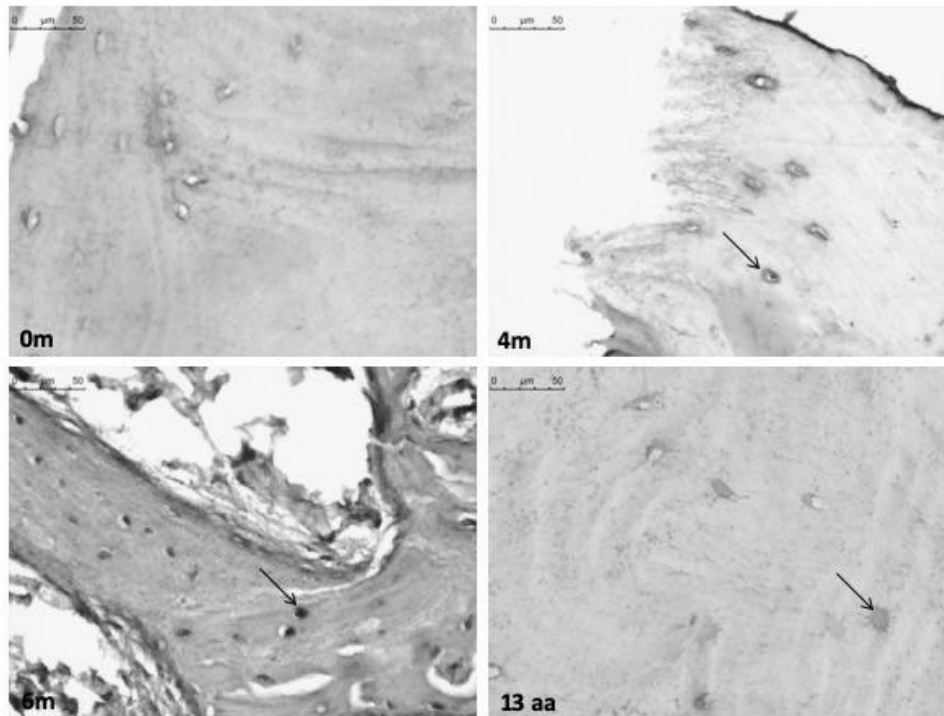


Fig. 2. a) Immunohistochemical analysis of BSP II in bone tissue samples obtained at the time of grafting, 4 and 6 months and 15 years after regeneration with autologous bone graft taken from calvaria. Magnification 20x and histogram obtained from the mean of BSP II values obtained from the samples at different times. **b)** Histogram obtained from the average of BSP II values obtained from sampling at different times, at 4, 6 and 15 years after regeneration with autologous bone graft taken from calvaria. The histogram shows that the levels of BSP are minimal in the early stages; at six months then in T2 we have the peak precisely because it is forming mature bone, in fact it is the protein responsible for the mineralization of the bone.

A

VEGF



B

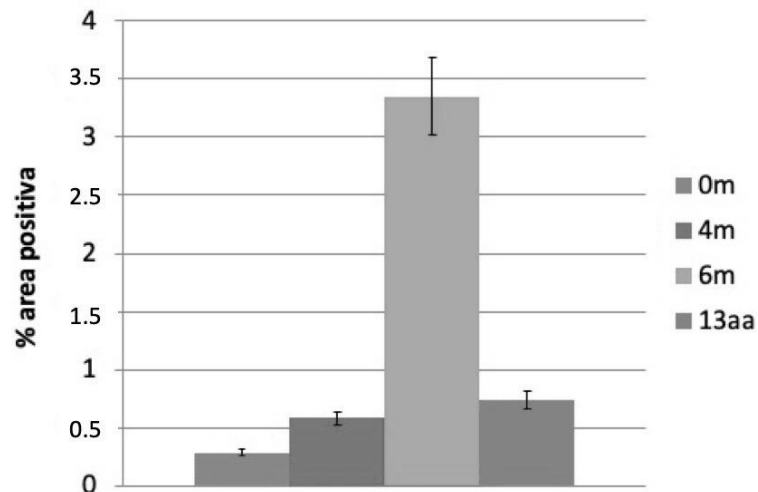


Fig. 3. a) Immunohistochemical analysis of VEGF in bone tissue samples obtained during grafting, 4 and 6 months and 15 years after regeneration with autologous bone graft taken from calvaria. Magnification 20x and histogram obtained from VEGF values from samples taken at different times. **b)** Histogram obtained from the VEGF values of the samples taken at different times during 4, 6 and 15 years after regeneration with autologous bone graft taken from calvaria. The VEGF factor follows the trend of the BSP II, has its peak at six months as noted in the histogram. In fact, it is a growth factor that mediates neoangiogenesis, which, as a process, is parallel to the osteogenesis, mediated specifically by BSP II.

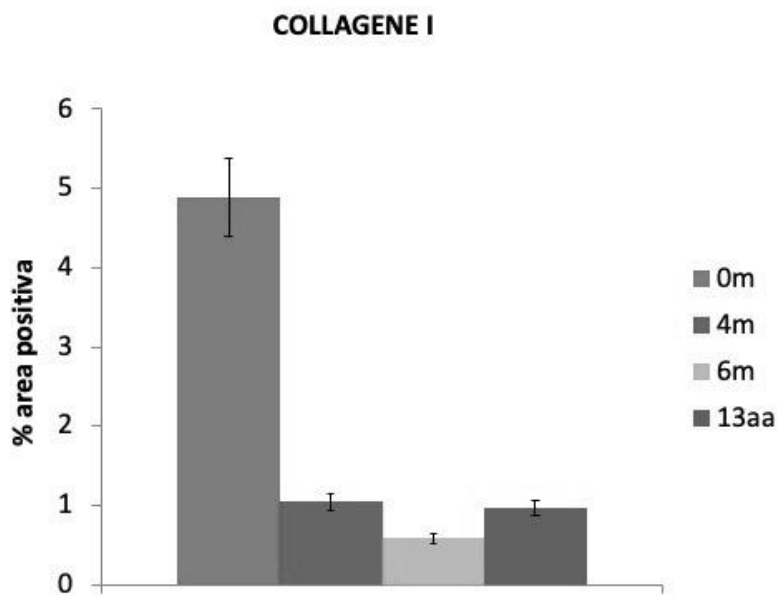


Fig. 4. Histogram obtained from Collagen I values from samples taken at different times, at 4, 6 and 15 years after regeneration with autologous bone graft taken from calvaria. In the histogram we see how collagen I, a molecule involved in the formation of extracellular matrix, is obviously maximum in T0 and progressively reduces in T1 and T2 and stabilizes in T3.

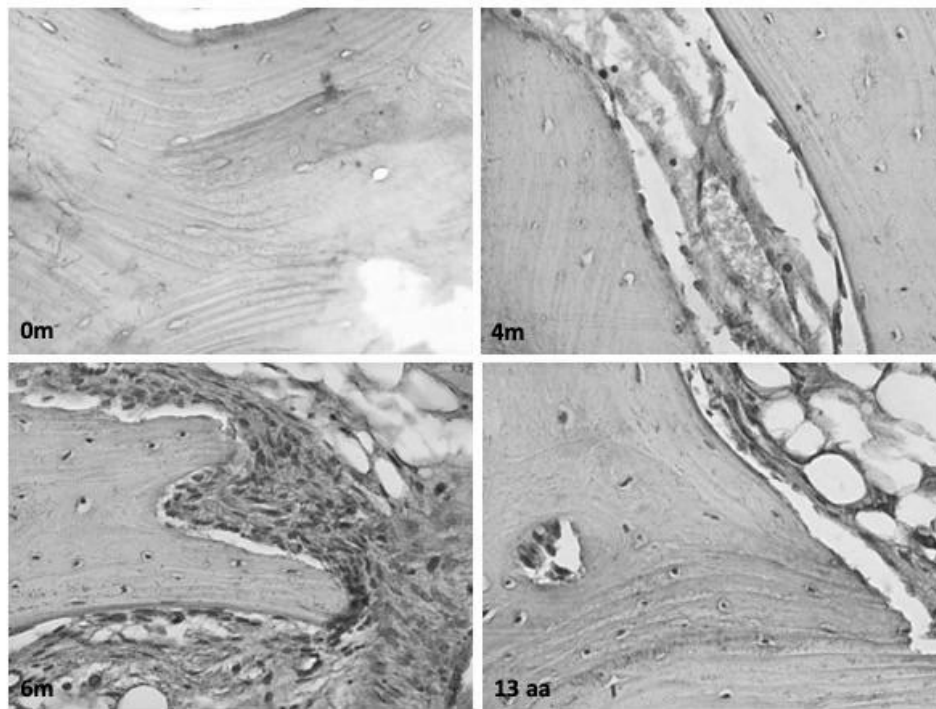
microscopic observation revealed a morphological aspect very similar to that of the native bone, an indication of the formation of cortical bone having the same structural and biomolecular characteristics as the grafted one. In fact, the microscopic analysis showed that the graft had assumed morphological characteristics very similar to the native bone, as can be seen from the disappearance of the welding lines. However, the most interesting thing is that, around the Havers canal, there were small polygonal cells that recall the morphology of osteoblasts, so you could guess that not only the sample was perfectly integrated but you might think that there was still bone apposition (Fig. 1). Immunohistochemical analysis of bone sialoprotein (BSP2) (Fig. 2a-b), an osteogenic marker, showed a peak in the six months following the bone grafting procedure (T2), parallel to the expression of VEGF (Fig. 3 a-b), an angiogenic growth factor and proliferation of endothelial cells in newly formed gaps, as well as the development and maintenance of endothelial fenestrations, which indicated an index of activity and bone remodeling

and of the neoangiogenic potential of host tissue. In fact, the levels of expression of this molecule were significantly higher in T1 and T2 than in T0 and T3, which showed very similar values, and the positivity was evident especially in correspondence of the immature connective tissue interposed between the bone lamellae, where a new vascular network was forming, despite the levels of collagen I (Fig. 4), molecule involved in the formation of extracellular matrix, obviously maximum in T0, being progressively reduced in T1 and T2 and stabilized in T3.

The other molecule considered in this study was the ERK1/2 (Fig. 5 a-b), responsible for the stimulation of angiogenesis and osteogenesis, which was higher in T1 and T2 and less expressed in T0 and T3, supporting the hypothesis that both regulatory VEGF and BSP2. In the present study, the levels of expression of the inflammatory molecule iNOS (Fig. 6 a-b) was higher in the grafts of calvaria after 4 months (T1) compared to other samples, demonstrating the peak of inflammatory activity, which drew in osteoblasts.

A

ERK1/2



B

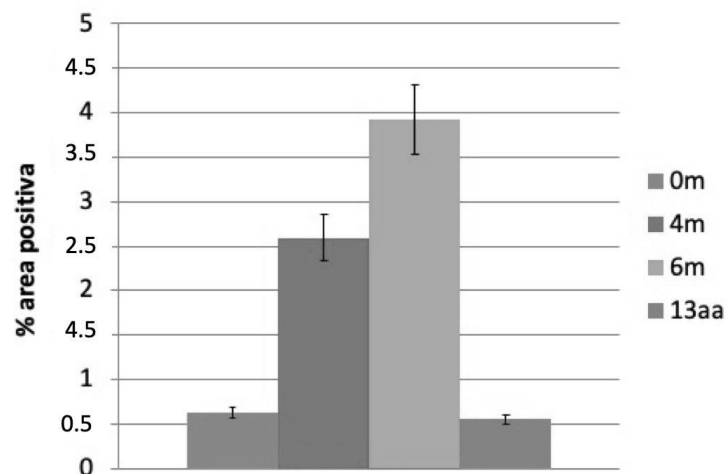


Fig. 5. a) Immunohistochemical analysis of ERK1/2 in bone tissue samples obtained during grafting, 4 and 6 months and 15 years after regeneration with autologous bone graft taken from calvaria. Magnification 20x and histogram obtained from ERK values from samples taken at different times. **b)** Histogram obtained from the ERK values of the samples taken at different times, during 4, 6 and 15 years after regeneration with autologous bone graft taken from calvaria. ERK1/2 is the molecule responsible for the stimulation of angiogenesis and osteogenesis, which is higher in T1 and T2 and less expressed in T0 and T3, supporting the hypothesis that both VEGF and BSP2 are regulatory.

DISCUSSION

Implant prosthetic rehabilitation of the maxillary bones requires a careful approach in order to properly restore the chewing function taking into account anatomical alterations in terms of bone quantity and quality. Autologous bone has advantages for its long-term biological and mechanical properties, but Zizzari et al. showed that there are disadvantages due to its limited availability and the need for a second surgery, resulting in increased morbidity (20). Moreover, residual disability in the withdrawal area may persist, and there is a risk of seizures and osteitis or osteomyelitis (25). Chiapasco, in his review, showed that the morbidity of the intervention related to the removal of bone from the cranial vault is difficult to explain to the patient, and it is necessary to inform him adequately about all the procedures. The author also points out that the use of bone blocks is an operator-dependent surgical technique. In particular, sampling techniques from extra-oral donor sites require specific training in maxillo-facial surgery and knowledge of anatomy (26).

Scheerlinck showed that the calvarial bone, among the various types of grafting of autologous bone, is the one that reports the most serious complications (19.2%); in fact it was an operation that was previously performed with the help of the neurologist (27). Breeze said that in the hospital where he conducted his study the major complications were related to autologous calvarial bone grafting compared to intraoral sites. However, the type of study is not expressly stated and the rate of failure may be related to more serious cases at the start (28). Putters, in a randomized clinical trial study, showed that twenty patients selected with an insufficient volume of maxillary bone for reliable implant placement were assigned randomly to either a calvarial (n=10) or an anterior iliac crest (n=10) bone harvesting group. All patients underwent a maxillary sinus floor elevation procedure combined with widening of the alveolar process using buccal bone blocks. Donor site morbidity was assessed before, during, and at 1 year after the surgery through patient questionnaires, physical examination, and medical records. No perioperative complications

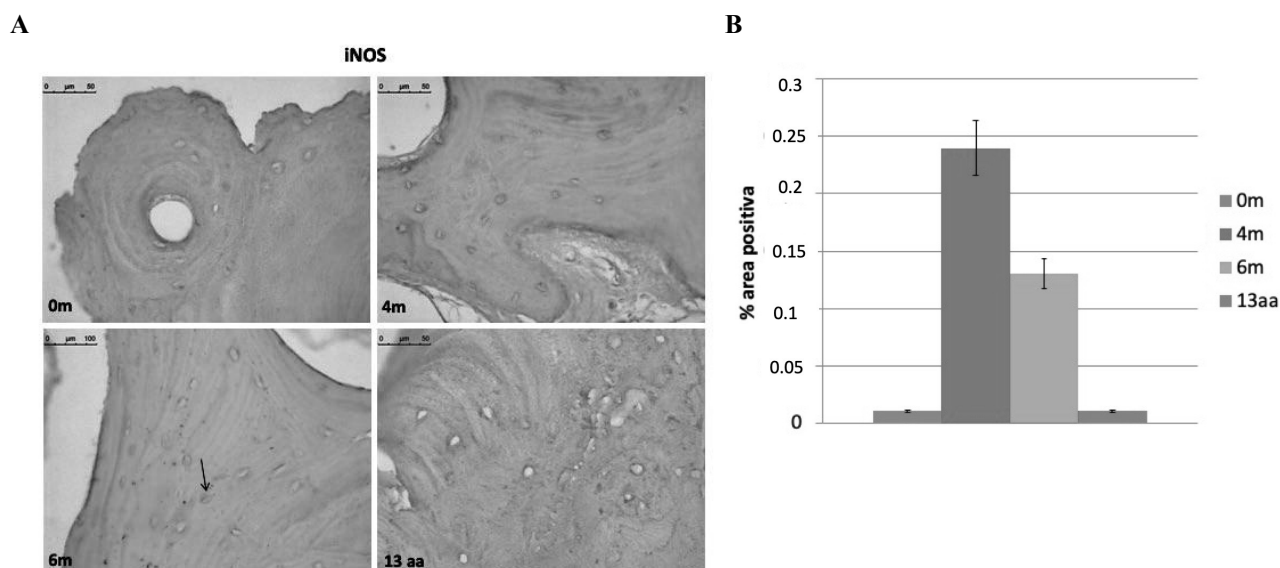


Fig. 6. a) Immunohistochemical analysis of iNOS in bone tissue samples obtained during grafting, 4 and 6 months and 15 years after regeneration with autologous bone graft taken from calvaria. Magnification 20x and histogram obtained from iNOS values from samples taken at different times. **b)** histogram obtained from iNOS values from samples taken at different times, at 4, 6 and 15 years after regeneration with autologous bone graft taken from calvaria. the levels of expression of the inflammatory molecule iNOS are higher in the grafts of calvaria after 4 months (T1), compared to other samples, demonstrating the peak of inflammatory activity, which draws in osteoblasts.

occurred. The anterior iliac crest group reported minor postoperative pain after harvesting. The scars after calvaria harvesting were significantly longer ($P=0.003$), but this was not bothersome for the group of patients. Long-term pain was negligible and satisfaction was high in both groups. Both the calvaria and anterior iliac crest are associated with low long-term donor site morbidity and high patient satisfaction (29).

Many authors have reported that calvarial bone has a minimal resorption rate, good biologic integration, and a more predictable clinical outcome (30). In fact, in the present study, the most interesting thing about the morphostructural analysis is that around the Havers canal, 15 years after grafting, small polygonal cells were placed that recall the morphology of the osteoblasts, suggesting that not only was the sample perfectly integrated, but that there was still bone apposition. In fact, the expression levels of VEGF and BSP molecules were significantly higher in the T1 and T2 groups than in the T0 and T3 groups, which show very similar values, and the positivity was evident especially in correspondence to the immature connective tissue interposed between the bone lamellae, where a new vascular network was forming, despite the levels of collagen I, molecule involved in the formation of extracellular matrix, obviously maximum in T0, are progressively reduced in T1 and T2 and stabilized in T3. Indeed, the expression of these molecules seems to promote the correct arrangement of lamellar bone under load. The other molecule considered in the study is the ERK1/2, responsible for the stimulation of angiogenesis and osteogenesis, which was higher in T1 and T2 and less expressed in T0 and T3. The inflammatory reaction induced in the host organism towards the biomaterial represented a crucial element for the establishment and integration of the grafted tissue. The initial phases assumed an inflammatory phase in which inflammatory cells and macrophages were called to contribute indirectly, through the production of cytokines and pro-inflammatory signals, and directly to the reabsorption of the matrix of grafted tissue in order to create the physical space within which new vascular buds can develop and proliferate and differentiate mesenchymal cells (31). In the present study, the levels of expression of the inflammatory molecule iNOS was higher in the

grafts of calvaria after 4 months (T1), compared to other samples, demonstrating the peak of inflammatory activity, which drew in osteoblasts. At the 15-year follow-up we should expect a bone more similar to the native bone or mainly spongiose bone, instead, it is much more cortical and therefore similar to the structure of the bone taken. Biological factors confirmed this and tell us that at the 15-year follow-up the grafted bone was perfectly integrated, and not of a partial integration only at the interface between graft and native bone, because it did not present those phenomena typical of the initial phases of inflammation: therefore the bone structure has reached its balance as if the operation had not taken place.

However, it remains an operator-dependent surgery, which can be performed without complications when experienced surgeons are involved, but it is not easily repeatable (32). The morbidity of extraoral bone grafts in general remains high and, compared to the new minimal-invasive techniques, it is often a second choice technique (33). In addition, other studies with a higher number of patients are required.

The proposed protocol could be useful for further studies on biomaterials and their future development, also in supporting stem cells of embryonic origin or directly reprogrammed in the laboratory, the induced pluripotent stem cells, already used in studies for degenerative neurological diseases (33). Majorana et al. showed how the combined use of autologous bone and biomaterials could be the treatment of choice for a complete and less invasive treatment (34). Within the limitations of the present study, from the histological and immunohistochemical point of view, autologous bone harvested from the calvaria seems to confirm good integration and, after 15 years, a complete remodeling in the recipient site. Further studies are needed to apply and evaluate the present protocol to tissues regenerated with different bone substitutes.

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LETTER TO THE EDITOR

MECHANISM AND EFFECTS OF miRNA-124 ON STATUS EPILEPTICUS

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To the Editor,

Status epilepticus (SE) refers to frequent recurrence of seizures and incomplete recovery of consciousness between successive epileptic seizures, or the duration of seizures exceeding 30 min which cannot stop by themselves (1). Long-term epileptic seizures may lead to irreversible brain damage with high disability and mortality (2). microRNA(miRNA) is a non-coding short RNA molecule composed of about 22 nucleotides, which affects most biological processes such as development, physiological function and stress responses (3). miRNA-124 is the most abundant class of microRNAs. It has been shown that the expression of miRNA-124 in the hippocampus of rats with chronic epilepsy is reduced, thus affecting epileptic seizures (4).

SPHK1 is one of the key enzymes which catalyze sphingomyelin to sphingosine phosphate (5). SPHK1 is highly expressed in cancer, ischemic stroke, dementia and other central nervous system diseases (6). Meanwhile, it is also an effective target for the treatment of cancer (7). Some scholars have found that miR-124 has a protective effect on ovarian cancer and can negatively regulate the expression of target

gene SPHK1 (8). It is speculated that SPHK1 may be one of the targets of miR-124 in status epilepticus.

At present, epilepsy is still a difficult and complicated disease. Lack of a unified conclusion on its specific pathogenesis and treatment is a problem. Therefore, this study explored the effect of microRNA-124 on status epilepticus and its mechanism, and provides a scientific basis for clinical prevention and treatment of epilepsy.

MATERIALS AND METHODS

Animals

Twenty-four healthy male C57BL/6 mice (purchased from Nanjing Junke Bioengineering Co., Ltd., China) aged 6-8 weeks and weighing about 18-25g were used in the study. All animals were fed in cages with standard rodent chow (4 mice in each cage). The mice were allowed free access to food and water, and there was no significant difference in body weight between groups. Under natural light, room temperature was controlled at 20-26°C and humidity was set at 40-50%. Adaptive feeding was carried out for 2 weeks. The animal experiment conforms to the International Regulations on Animal Protection

Key words: miRNA-124; epilepsy; SPHK1 protein; pilocarpine

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and Management and the protocol was approved by the Ethics Committee of The Affiliated Hospital of Hangzhou Normal University.

Grouping

Twenty-four male C57BL/6 mice were randomly divided into two groups: one blank control group (Con group, 6 mice) and three experimental groups (6 mice in each group) divided into 0h group, 0.5h group and 4h group, respectively.

The mice in the experimental group were intraperitoneally injected with lithium chloride (125mg/kg, Shanghai Yanzhen Biotechnology Co., Ltd., China), and then injected with methyl atropine bromide (1mg/kg, Sigma, USA) intraperitoneally 24 h later. After 30 min, they were intraperitoneally injected with pilocarpine (25mg/kg) (Shanghai Aladdin Biochemical Technology Co., Ltd., China). At the same time, the behavioral manifestations of the mice were observed. According to Racine classification criteria, when the behavioral performance of the mice reached epilepsy grade IV or above and the duration was more than 1 hour, the modeling would be considered successful (9). Moreover, mice that did not meet the standard continued to be injected with pilocarpine at the dose of 10 mg/kg. Mice in 0h group, 0.5h group and 4h group were intraperitoneally given diazepam (10mg / kg, Tianjin Alta Technology Co., Ltd., China) at 0h, 0.5h and 4h after successful modeling, respectively, to terminate the epilepsy, and they were then decapitated.

The mice in the blank control group were given saline intraperitoneally. Twenty-four hours later, methyl atropine bromide (1mg/kg) was injected intraperitoneally; then, 30 min later, intraperitoneal injection of normal saline was given.

Animal behavior observation on behavior of mice in each group

The behavioral changes of mice in the experimental group and the blank control group after each injection were observed and recorded, and the performance among the groups was compared according to Racine grading standard.

The severity of epileptic seizures in animals was classified into five grades according to Racine classification criteria: Grade I, facial convulsions, and chewing; Grade II, facial convulsions, and nodding; Grade III, facial

convulsions, nodding, and forelimb convulsions; Grade IV, standing, generalized convulsions; Grade V, standing, generalized convulsions, and falling.

Extraction of total RNA from tissues by trizol method

Mice in the experimental and blank control groups were sacrificed and placed on ice. The brain was removed quickly by craniotomy. The cerebellum and forebrain tissues were removed, and bilateral hippocampal tissues were separated. They were labeled and stored in liquid nitrogen tanks. The left hippocampus tissues were put into a glass grinder and quickly ground after adding liquid nitrogen, and then frozen repeatedly until ground into powder. One mL Trizol was added to all 100 mg hippocampal tissues (Jiaozuolu Fanfanfang Biotechnology Co., Ltd., China). After mixing according to this proportion, the mixture was poured into an EP (Eppendorf) tube. The pipe was covered and shaken vigorously, so as to mix them evenly and then left to stand at room temperature for 5 min. Afterwards, trichloromethane was added (Trizol: trichloromethane = 5:1) (China Pharmaceutical Group Chemical Reagents Co., Ltd., China), and the pipe was covered and shaken vigorously, so as to mix them evenly and then left to stand at room temperature for 5 min. Then, the tissues were separated by high-speed centrifugation (Allegra X-22, Beckman Kurt Co., Ltd., USA) for 15 min (12,000 g/min, 4°C). The upper solution was absorbed and transferred to a new EP tube. Isopropanol (Trizol: Isopropanol = 2:1) (Tianjin Guangcheng Chemical Reagent Co., Ltd., China) was added, shaken violently and left at room temperature for 5 min then centrifuged (12,000bpm, 4°C) for 10 min. The supernatant was discarded and 75% glacial ethanol (Trizol: 75% glacial ethanol = 1:1) (Tianjin Guangcheng Chemical Reagent Co., Ltd., China) was added. After mixing evenly, it was centrifuged (7500 g/min, 4°C) for 5 min. After discarding the supernatant, the sediment was spread out and dried, and 20 mL DEPC (diethyl pyrocarbonate) water (Shanghai Huyu Biotechnology Co., Ltd., China) was added, to dissolve in a constant temperature water bath at 60°C (Shanghai Jiwei Testing Instruments and Equipment Co., Ltd., China).

Detection of the expression of microRNA-124 in hippocampus of mice by (real-time quantitative polymerase chain reaction)

Specific primers (Guangzhou Reebok Biotechnology

Co., Ltd., China) were utilized to synthesize reverse transcription DNA (RT-DNA) for microRNA samples. The template was total RNA extracted by Trizol method (2 μ g). The RT reaction solution was provided through synthesizing some materials as follows: 2 μ L 5 $\times$ Prime Script Buffer (Bao Bioengineering Co., Ltd., Dalian, China), 0.5 μ L Prime Script RT (Bao Bioengineering Co., Ltd., Dalian, China), 4.5 μ L RT primer (Shanghai Science and Mind Biotechnology Co., Ltd., China), 0.5 μ L Total RNA and RNase Free H₂O (Beijing Baiao Sentai Biotechnology Co., Ltd., China). After reaching the total volume of 10, the mixture was uniformed and centrifuged to the bottom of the tube. Reverse transcriptase was inactivated for 15 min at 42°C and 4 s at 85°C, and stored at 4°C after the end of the reverse transcription reaction. Furthermore, the PCR reagent was configured (25 μ L) through mixing these materials on ice as follows: 12.5 μ L SYBR Premix Ex Taq II 2 $\times$ (Shanghai Shanran Biotechnology Co., Ltd., China), 1 μ L PCR Forward Primer (10 μ M) (Shanghai Jitai Ikesai Biotechnology Co., Ltd., China), 1 μ L PCR Reverse Primer (10 μ M) (Shanghai Yanqi Biotechnology Co., Ltd., China), 2 μ L cDNA template and 8.5 μ L RNase Free H₂O. Moreover, predenaturation was performed for 30 s at 95°C. PCR was performed for 5 s at 95°C and 30 s at 60°C, pending the end of reverse transcription. After 40 cycles of repeated reaction, the dissolution curve was drawn. Relative quantitative method and $2^{-\Delta\Delta CT}$ method were used to calculate the relative value of gene expression (the control gene was U6 gene). ΔCT value = target gene Ct value - control gene Ct value, $\Delta\Delta CT$ value = test sample ΔCT value - control sample ΔCT .

Measurement of protein concentrations by bicinchoninic acid method

For homogenization, the right hippocampus tissues extracted from liquid nitrogen were put into a ground glass, and 1 mL RIPA (Radio Immunoprecipitation Assay) tissue lysate (Hangzhou Haoxin Biotechnology Co., Ltd., China) was added to 100 mg hippocampus tissues and mixed evenly. They were then ground quickly to become homogenate. Afterwards, the mixture was poured into an EP tube, and centrifuged at high speed (4°C, 12 000 $\times$ g/min) for 15 min. The supernatant was absorbed and transferred to a new EP tube. The bovine serum albumin standard (BSA standard, 5mg/ml) was diluted to 100 μ L

with 10 μ L ddH₂O. The RIPA tissue lysate was used to set different concentrations of BCA working fluid (the ratio of reagent A to reagent B was 50:1, Beijing Baioleibo Technology Co., Ltd., China): 0mg/mL, 0.0025 mg/mL, 0.125mg/mL, 0.25mg/mL, 0.5mg/mL, 1mg/mL, 2mg/mL. The diluted protein samples (diluted 20 times by RIPA tissue lysate) were added to 96 well- plates with 20 μ L per well. Three parallel wells were set for each sample and 200 μ L BCA working fluid was added to each well. After fully mixing, the samples were put into an incubator (37°C) for 30 min and cooled. The absorbance was measured at 562nm utilizing a fully automated enzyme labeling system (Molecular Devices, USA). The standard curve was drawn based on the absorbance of the working fluid. Moreover, the protein concentration of the sample was calculated. The protein samples to be tested were mixed with protein sample buffer (3:1, Xi'an Hutt Biotechnology Co., Ltd., China), then boiled in boiling water for 10 min and cooled on ice. Furthermore, they were put in the water bath for 15 min, then taken out and cooled on ice, and finally frozen at -80°C for reserve.

Detection of the SPHK1 protein expression in hippocampus of mice by Western blotting

10% separating gum and 5% concentrating gum were prepared according to the molecular weight of the protein sample. The head of the sample gum was inserted into the sample well, and the rainbow protein marker (Shanghai Bogu Biotechnology Co., Ltd., China) and the protein sample were added to be tested (40 μ L/well). The power supply was connected, and electrophoresis was carried out for 30 min at a constant voltage of 80V. According to the bromophenol blue indicator, the electrotransfer of proteins to the separation gel was observed. Afterwards, the electrophoresis was conducted at a voltage of 120 V. After 1 h, the electrophoresis was terminated when the protein was transferred to the bottom of the gel. According to the selected protein samples, the gel in the film transfer area was selected. In the electrophoresis tank, the blackboard, sponge, filter paper, 3 layers, gel, PVDF (polyvinylidene fluoride) membrane, filter paper, 3 layers, sponge and whiteboard were placed in order. The power supply was turned on. At a constant current of 400mA and a voltage of 100V, the film was transferred for 1 h. The PVDF film was removed and rinsed in the shaker with TBST (T: Tris; B: Buffer; S: Solution; T: Tween) solution for 5 min. At

room temperature, the sealant was shaken (5% skimmed milk) on the shaking table for 30 min and discarded. It was rinsed three times (10 min per time) with TBST solution. The PVDF film was cut according to the position of the protein marker, and anti-dilution solutions were added: rabbit anti-SPHK1 polyclonal antibody (dilution ratio: 1:1000, Bioengineering Co., Ltd., Shanghai, China), and mouse anti-beta-actin monoclonal antibody (dilution ratio: 1:1500, Shanghai Zhenyu Biotechnology Co., Ltd., China). It was placed on a rocking bed (4°C) and shaken lightly overnight. It was rinsed three times (10min/time) with TBST solution. Furthermore, the second anti-rat diluted solutions were added: HRP (Horseradish Peroxidase) labeled sheep anti-mouse IgG (dilution ratio, 1:10000; Nanjing Dean Biotechnology Co., Ltd., China), and HRP labeled sheep anti-rabbit IgG (dilution ratio, 1:6000; Shanghai Yuanmu Biotechnology Co., Ltd., China). It was placed on a rocking bed at room temperature in the dark and shaken lightly overnight. It was rinsed three times (5min/time) with TBST solution. Moreover, the appropriate ECL (electrochemiluminescence) luminescent solution was added, and was placed at the darkroom and then repeatedly beaten. Then the Chemi Doc XRS gel image acquisition system (Bio-Rad, USA) was scanned for preservation. β -actin was utilized as internal parameter index to calculate the gray value of protein bands.

Statistical analysis

SPSS21.0 statistical software was used for data processing and analysis. Quantitative data conforming to normal distribution were described by mean $\pm$ standard deviation. Independent samples *t*-test or rank sum test was applied to compare the two groups, and *chi*-squared test was employed to compare the rates of different groups.

RESULTS

Observation of animal behavior

After intraperitoneal injection of lithium chloride, there was no abnormal reaction in the experimental group. After the injection of pilocarpine, symptoms such as eye congestion, hair erection, drooling and glassy eyes were observed. About 10 minutes later, epilepsy symptoms appeared in all cases one after another, such as sniffing, chewing movement, head backward, tremor, etc. Rhythmic nodding, forelimb

convulsions, semi-standing, and epilepsy continued to occur further. Hindlimb convulsions (HLCs) and falls developed into general tonic-clonic seizure persistence which lasted more than one hour. After diazepam treatment in mice, their epileptic behavioral activities decreased. There was no abnormal reaction in the blank control group.

Detection of the microRNA-124 expression in hippocampus of mice by qRT-PCR

In real-time fluorescence quantitative PCR, the expression level of microRNA-124 in hippocampus of mice at different time points after status epilepticus is shown in Table I and Fig. 1A. The expression of microRNA-124 in the experimental group was significantly lower than that in the blank control group (* $p < 0.001$). The expression of microRNA-124 in the 0.5h group was the lowest (* $p < 0.001$). Moreover, the expression of microRNA-124 in the 4h group was significantly lower than that in the blank control group (* $p < 0.001$). However, compared with the 0h group and the 0.5h group (* p

Table I. Expression level of microRNA-124 and SPHK1 mRNA at different time points of status epilepticus.

Group	miR-124	SPHK1
Con	1.0000 $\pm$ 0.0067	1.0009 $\pm$ 0.0084
0h	0.4278 $\pm$ 0.1868*	1.7874 $\pm$ 0.8365
0.5h	0.1821 $\pm$ 0.1103*	1.3563 $\pm$ 0.3823
4h	0.6033 $\pm$ 0.2744*	0.6015 $\pm$ 0.6011

($\bar{x} \pm s$, * $p < 0.001$)

Table II. The expression level of SPHK1 protein at different time points of status epilepticus

Group	SPHK1 protein
Con	1.7596 $\pm$ 0.2545
0h	1.2053 $\pm$ 0.2006*
0.5h	0.6545 $\pm$ 0.1046*
4h	0.8447 $\pm$ 0.0854*

($\bar{x} \pm s$, * $p < 0.001$)

<0.001), the expression of microRNA-124 increased to a certain extent. Thus, in the status epilepticus, the expression of microRNA-124 decreased, which was inversely proportional to the duration of epilepsy.

Detection of the SPHK1 mRNA expression by qRT-PCR

The expression of SPHK1 in hippocampus of mice was detected by real-time fluorescence quantitative PCR, as shown in Table I and Fig. 1B. Compared with the blank control group, there was no significant difference in the expression of SPHK1 in the hippocampus of mice in 0h group, 0.5h group and 4h group ($p=0.301$). In addition, there was no significant difference between the expression of SPHK1 and the duration of epilepsy ($p=0.214$).

Detection of SPHK1 protein levels in hippocampus of mice by Western blotting

In Western blotting, the expression of SPHK1 protein in hippocampus of mice at different time

points after status epilepticus is shown in Table II and Fig. 1C. The expression of SPHK1 protein in the experimental group was significantly lower than that in the blank control group ($*p<0.001$).

The expression of SPHK1 protein was the lowest in the 0.5h group ($*p<0.001$). It could be seen that the longer the duration of epilepsy, the lower the gray value of SPHK1. Although the expression of SPHK1 in the 4h group in comparison with the blank control group decreased significantly ($*p<0.001$), its expression increased to a certain extent compared with the 0h group and the 0.5h group ($*p<0.001$). Immunoblotting results are shown in Fig.2A.

Correlation between the expression of microRNA-124 and SPHK1 protein in hippocampus of mice

According to the results of real-time fluorescence quantitative PCR and Western blotting, the expression levels of microRNA-124 and SPHK1 in hippocampus of mice with status epilepticus were analyzed by SPSS

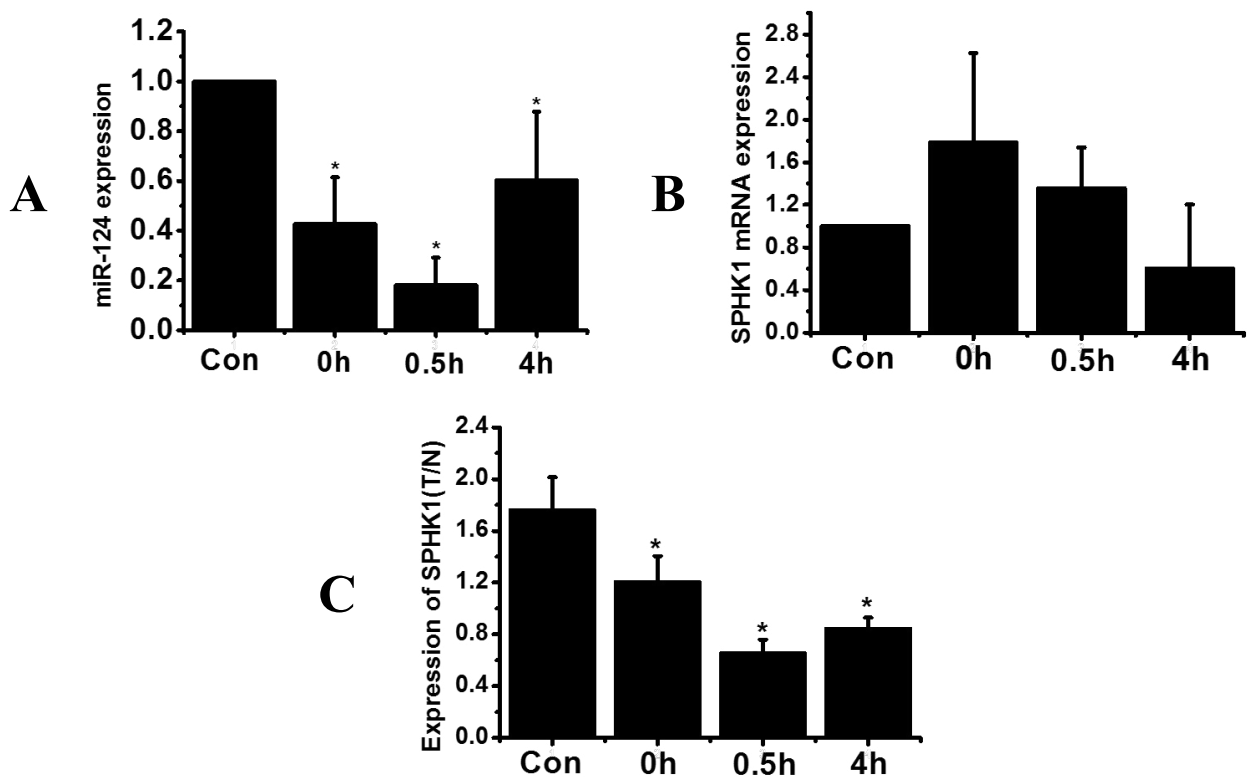


Fig. 1. Real-time fluorescence quantitative PCR and Western blotting results. **A)** Expression level of miR-124 in hippocampus of mice at different time points of status epilepticus; **B)** expression level of SPHK1; **C)** expression level of SPHK1).

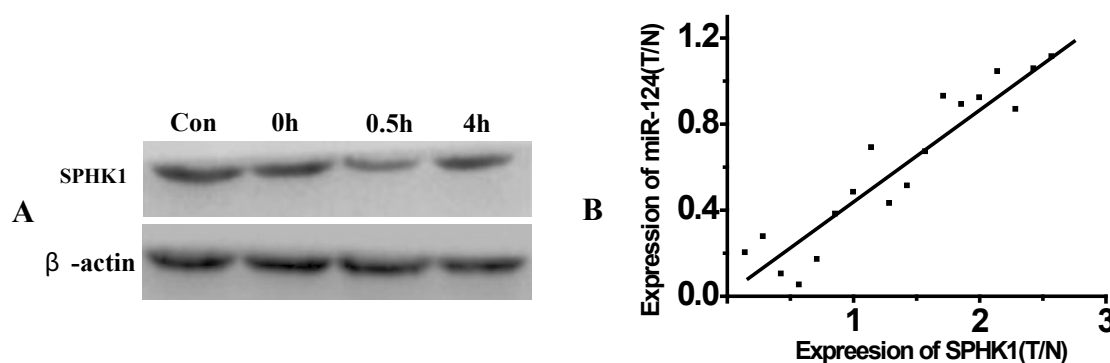


Fig. 2. Expression of SPHK1 in hippocampus of mice at different time points of status epilepticus and the correlation between the expression of miR-124 and SPHK1. **A)** Expression of SPHK1 in hippocampus of mice, and β -actin is the internal parameter index; **B)** positive correlation between miR-124 and the expression of SPHK1).

Pearson correlation analysis. As shown in Fig. 2B, the expression levels of microRNA-124 and SPHK1 were positively correlated with each other ($r=0.897$, $p=0.004$).

DISCUSSION

miR-124 plays a key role in regulating the development and differentiation of neurons, and has physiological functions such as protecting hippocampal neurons, limiting synaptic plasticity and protecting the normal morphology of cones (10). It has been found that miR-124 has a major role in many kinds of central nervous system diseases (11). In the present study, the expression of miRNA-124 was detected in hippocampus of mice with status epilepticus of different epileptic duration. The results showed that the expression of miRNA-124 in the experimental group was significantly lower than that in the blank control group. The expression was lowest at 0.5 h, which was inversely proportional to time. At 4h, the expression of miRNA-124 increased to a certain degree.

SPHK1 can transfer from cytoplasm to plasma membrane under the action of hormones, stimulation, and cytokines as well as growth factors. Furthermore, it can promote the synthesis of sphingosine phosphate and inhibit the expression of ceramide, and consequently regulate downstream signaling pathways, which play a key regulatory role in cell growth, proliferation, differentiation, and apoptosis along with inflammatory reaction (12). In this experiment, the expression of

SPK1 protein in mice of different epilepsy duration was detected. The results showed that the expression of SPHK1 protein in the experimental group was significantly lower than that in the blank control group, and the expression was lowest at 0.5h, which was inversely proportional to the time. At 4h, the expression of SPHK1 protein increased to a certain degree. The correlation analysis showed that there was a significant positive correlation between expressions of miRNA-124 and SPK1.

In conclusion, the down-regulation of microRNA-124 expressions in hippocampus of mice with status epileptics can affect downstream apoptosis-related signaling pathways through reducing the expression of SPHK1, promoting neuronal apoptosis and participating in the process of persistent epileptic seizures.

ACKNOWLEDGEMENT

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LETTER TO THE EDITOR

LUTEINIZING HORMONE: FOLLICLE-STIMULATING HORMONE RATIO, BODY MASS INDEX, DISTURBANCES IN SACCHAROMETABOLISM AND LIPOMETABOLISM INTERDEPENDENCE IN POLYCYSTIC OVARY SYNDROMEJ. CHEN¹, MM. DU² and D. XIAO³

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To the Editor,

Polycystic ovary syndrome (PCOS) is a common gynecological endocrine disorder with an incidence of 5-10% of the population (1). The etiological factors of PCOS are not entirely elucidated, the combination of genetic and environmental factors playing a significant role (1, 2). The clinical manifestations of PCOS are diverse and include polycystic changes in ovaries, often accompanied by insulin resistance (IR) and dyslipidemia (3). The most common endocrine manifestation is the increase in luteinizing hormone (LH)/follicle stimulating hormone (FSH) ratio (LH/FSH) of 2-3 times more than the normal range, which is associated with abnormalities at the Hypothalamic-Pituitary-Ovarian (H-P-O) axis level (4). Another essential clinical manifestation of PCOS is obesity evaluated by body mass index (BMI) and waist-hip ratio (WHR). Clinical manifestations of hyperandrogenism are negatively correlated with LH/FSH ratio, while IR or hyperinsulinism can increase the secretion of LH, thereby increasing the LH/FSH ratio. Other studies on PCOS women showed that LH/FSH ratio is not correlated with IR,

but with disturbances in saccharometabolism and lipometabolism (6). The treatment of PCOS may also affect the changes in LH/FSH ratio as. Both drug therapies and surgical procedures may decrease the LH/FSH ratio. In this study, we aimed to investigate the correlation between serum LH/FSH ratio, BMI and saccharometabolism, and lipometabolism disturbances in PCOS patients and to evaluate the diagnostic values of LH/FSH ratio in the treatment of PCOS.

MATERIALS AND METHODS*Patients*

A total of 116 PCOS female patients who were treated in the Second Affiliated Hospital of Zhejiang University School of Medicine from February 2016 to March 2019 were recruited in the study. On the 2nd to 5th day of menstruation, the patients underwent B-mode ultrasonography to investigate the number and size of ovarian follicles and the diameters of ovaries. For amenorrhea patients, the B-mode ultrasonography was performed on all the days.

Inclusion criteria: abnormal ovulation; performance of

Key words: polycystic ovary syndrome; luteinizing hormone; follicle-stimulating hormone; saccharometabolism and lipometabolism disturbances; obesity

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hyperandrogenism; polycystic changes in ovaries; able to participate in the entire experimental process; availability of complete medical data.

Exclusion criteria: PCOS accompanied by cardiovascular diseases; Cushing syndrome; malignant tumors; thyroid disease; hyperprolactinemia, and other disorders of the endocrine system; history of surgical treatment in the last 3 months; history of treatment with drugs that affect hormones, insulin, saccharometabolism, and lipometabolism over the 3 months prior to admission to hospital; PCOS accompanied by severe heart, liver, and kidney dysfunctions; general infectious diseases; incomplete medical records.

Age, menstruation situation, height, body weight, waist circumference, and blood pressure of the included patients were recorded. The height was measured without any headwear or shoes with an accuracy of 0.5 cm. The weight was measured with an accuracy of 0.1 kg. Before measuring blood pressure, the patients rested in a quiet environment for 5-10 min. The body mass index (BMI, kg/m²) and waist-hip ratio (WHR=waist circumference/hip circumference) were calculated.

The study was approved by the Ethics Committee of The Second Affiliated Hospital of Zhejiang University School of Medicine, and each patient signed an informed consent for participating in the study.

Detection of sex hormone and saccharometabolism biomarkers

Two mL of fasting venous blood samples were collected from each patient in the morning. The blood samples were maintained at room temperature for 30 min and then centrifuged at 3600 r/min for 15 min at 4°C. The supernatant was collected and stored at -80°C until further analysis. Enzyme-linked immunosorbent assay (ELISA) was utilized to detect the levels of Ghrelin and Orexin-A (Amresco, USA). An automatic chemiluminescence instrument (DXI8000; Beckman Coulter Inc., Brea, CA, USA) was utilized to detect the levels of prolactin (PRL), LH, FSH, testosterone (T), and estradiol (E2) and the indicators of saccharometabolism and lipometabolism.

The saccharometabolism was evaluated by fasting plasma glucose (FPG), oral glucose tolerance test (OGTT), and insulin-releasing test. After fasting for 8 h, the patients underwent OGTT in the morning. Three days before the OCGTT test, the patients had regular physical activities and a normal diet. For OGTT test were as patients received 75 g glucose diluted in 250-300 mL of drinking water, and the blood samples were collected before administration and at 1 and 2 h after glucose administration to detect the FPG level, fasting insulin level, 1-h insulin level (INS-1h), 2-h plasma glucose (PG-2h) level, and 2-h insulin level (INS-2h), respectively.

The lipometabolism was evaluated by total cholesterol (TC), triglyceride (TG), free fatty acids (FFA), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C).

Clinical findings used to assign patients to different groups

Changes in BMI: underweight (BMI<18.5), normal body weight (18.5≤BMI<23), overweight (23≤BMI<25), and obese (BMI≥25). In addition, waist circumference (WC) > 80 cm or WHR > 0.85 indicated obesity.

Hyperandrogenism: T≥0.7 ng/ml; clinical manifestations of hyperandrogenism included hypertrichosis, acne, and acanthosis nigricans. Hypertrichosis mainly affected sexual hair, in which the pubic hair was prolonged to crissum and groin, and long hair was observed around the mammary areola.

Saccharometabolism disturbances: FPG in the range of 3.9~6.0 mmol/L indicated normal saccharometabolism; 6.1~6.9 mmol/L indicated impaired fasting glucose (IFG); FPG ≥7.0 mmol/l indicated diabetes mellitus. In terms of OGTT, PG-2h < 7.7 mmol/l indicated normal plasma glucose levels; 7.81~1.0 mmol/l indicated impaired glucose tolerance (IGT) levels; the normal ranges of standard OGTT results were FPG < 6.1 mmol/l and OGTT-2h < 7.8 mmol/l.

IR: the homeostasis model of IR (HOMA-IR) was utilized as follows: fasting insulin (mIU/L) × FPG (mmol/L)/22.5; the value of HOMA-IR ≥ 2.69 indicated IR.

Dyslipidemia: any one of the following criteria indicated dyslipidemia: TC ≥ 6.19 mmol/l, LDL-C ≥ 4.14 mmol/l, TG ≥ 2.27 mmol/l, and HDL-C < 1.04 mmol/l.

Statistical analysis

All the data were analyzed by SPSS 22.0 software (IBM, Armonk, NY, USA). The measured data were expressed as mean ± the standard deviation ($\bar{x} \pm s$). The statistical analyses were performed by independent samples *t*-test. The significance of the results was set at P=0.05, in which P<0.05 indicated statistical significance.

RESULTS

Group comparison showed that with the increase in BMI, LH levels and LH/FSH ratio showed apparent descending trends. INS at 0 and 1 h increased with the increase in BMI. TG levels, FFA and LDL-C, increased with the increase in BMI, while HDL-C, ghrelin, and orexin-A decreased respectively (Table I).

A direct correlation between the BMI increase and the incidence of saccharometabolism

disturbances was observed ($P<0.05$). Similarly, in terms of lipometabolism, a direct correlation was seen between the BMI increase and the incidence of lipometabolism disorders ($P<0.05$) (Fig.1).

The LH levels and LH/FSH ratio significantly decreased with the decrease of T levels, while other clinical parameters showed no significant differences between the groups (Table II).

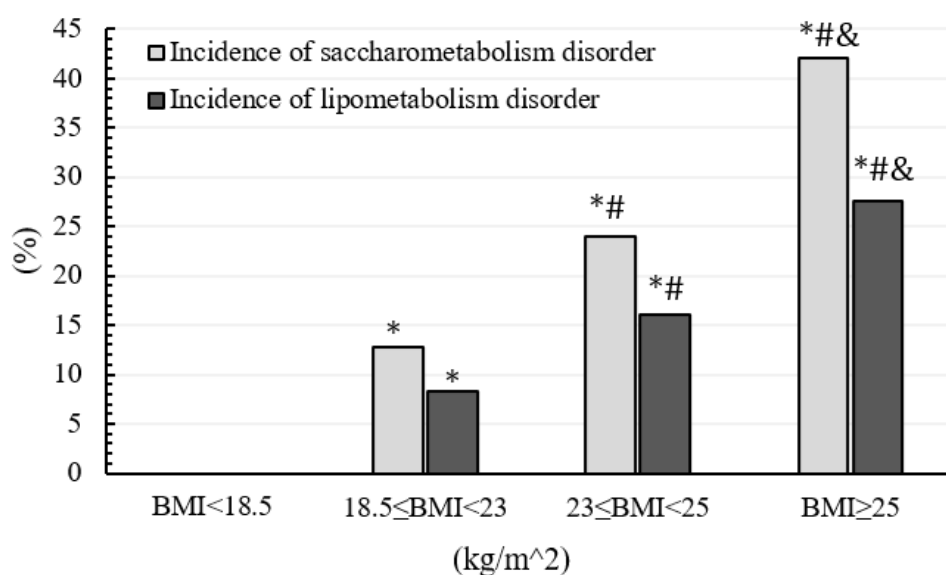
Table I. Organs that may be more frequently affected by toxicity after infusion of pro-inflammatory cytokine-releasing CAR-T cells.

Clinical findings		Underweight (n=3)	Normal body weight (n=94)	Overweight (n=25)	Obesity (n=76)	T $\geq$ 0.7ng/mL	T<0.7ng/mL
Basic parameters	Age (years)	25.35 $\pm$ 2.25	26.65 $\pm$ 4.54	26.35 $\pm$ 5.15	26.57 $\pm$ 5.51	25.78 $\pm$ 4.45	27.38 $\pm$ 4.19
	Year of incidence	7.52 $\pm$ 4.31	6.11 $\pm$ 2.75	7.47 $\pm$ 3.18	6.16 $\pm$ 2.54	6.82 $\pm$ 2.18	7.16 $\pm$ 3.02
	Menarche age (years)	13.64 $\pm$ 1.34	13.73 $\pm$ 1.15	13.82 $\pm$ 0.74	13.96 $\pm$ 1.45	13.48 $\pm$ 1.43	13.35 $\pm$ 1.62
	BMI (kg/m ²)	17.57 $\pm$ 0.45	21.42 $\pm$ 1.56	23.24 $\pm$ 0.86 [#]	28.72 $\pm$ 1.90 [#]	24.42 $\pm$ 3.74	24.05 $\pm$ 3.36
	WHR	0.78 $\pm$ 0.17	0.81 $\pm$ 0.14	0.85 $\pm$ 0.12 [#]	0.96 $\pm$ 0.15 [#]	0.86 $\pm$ 0.15	0.83 $\pm$ 0.13
Hormones	E2 (pg/mL)	50.45 $\pm$ 8.15	49.72 $\pm$ 9.54	50.23 $\pm$ 11.56	50.73 $\pm$ 10.34	49.26 $\pm$ 10.36	49.32 $\pm$ 10.45
	LH (IU/L)	14.72 $\pm$ 7.41	15.07 $\pm$ 6.46	14.28 $\pm$ 7.56	10.88 $\pm$ 5.27 [#]	14.87 $\pm$ 4.46	12.73 $\pm$ 5.42*
	LH/FSH	2.03 $\pm$ 0.93	2.46 $\pm$ 1.14	2.36 $\pm$ 1.02	1.99 $\pm$ 0.74 [#]	2.36 $\pm$ 1.25	2.16 $\pm$ 0.65*
	PRL (nmol/L)	13.69 $\pm$ 1.82	15.38 $\pm$ 2.10	14.52 $\pm$ 2.73	14.81 $\pm$ 2.57	15.54 $\pm$ 1.35	15.10 $\pm$ 2.02
	T (ng/mL)	0.65 $\pm$ 0.21	0.71 $\pm$ 0.31	0.75 $\pm$ 0.39	0.77 $\pm$ 0.26	-	-
Biomarkers of saccharometabolism	FPG (mmol/L)	4.47 $\pm$ 0.47	4.98 $\pm$ 0.81	5.15 $\pm$ 0.72	5.26 $\pm$ 0.57	4.94 $\pm$ 69.15	5.07 $\pm$ 0.75
	PG 2h (mmol/L)	5.83 $\pm$ 1.25	6.51 $\pm$ 1.27	6.41 $\pm$ 1.57	6.75 $\pm$ 1.84	6.34 $\pm$ 1.35	6.41 $\pm$ 1.49
	INS 0 (mIU/L)	14.16 $\pm$ 4.86	14.36 $\pm$ 6.46	15.84 $\pm$ 6.47	22.26 $\pm$ 10.45 [#]	17.76 $\pm$ 9.54	17.38 $\pm$ 8.26
	INS 1h (mIU/l)	93.04 $\pm$ 52.28	96.58 $\pm$ 55.27	97.93 $\pm$ 53.05	141.66 $\pm$ 68.35 [#]	114.05 $\pm$ 61.52	113.96 $\pm$ 57.26
	INS 2h (mIU/l)	91.62 $\pm$ 7.69	86.16 $\pm$ 53.54	98.37 $\pm$ 51.57	32.86 $\pm$ 81.83 [#]	104.75 $\pm$ 62.69	104.82 $\pm$ 57.32
Biomarkers of lipometabolism	TC (mmol/l)	4.15 $\pm$ 0.51	4.12 $\pm$ 0.65	4.21 $\pm$ 0.59	4.52 $\pm$ 0.46	4.23 $\pm$ 0.74	4.39 $\pm$ 0.57
	TG (mmol/l)	0.97 $\pm$ 0.22	1.04 $\pm$ 0.38	1.02 $\pm$ 0.61	1.51 $\pm$ 0.37 [#]	1.92 $\pm$ 0.37	1.36 $\pm$ 0.31
	FFA (mmol/l)	635.65 $\pm$ 48.29	612.52 $\pm$ 55.23	783.34 $\pm$ 72.47	893.57 $\pm$ 92.75 [#]	831.64 $\pm$ 37.18	807.73 $\pm$ 28.37
	LDL-C (mmol/l)	2.41 $\pm$ 0.39	2.44 $\pm$ 0.42	2.56 $\pm$ 0.44	3.26 $\pm$ 0.47 [#]	2.71 $\pm$ 0.56	2.69 $\pm$ 0.59
	HDL-C (mmol/l)	1.49 $\pm$ 0.31	1.42 $\pm$ 0.22	1.35 $\pm$ 0.25	1.19 $\pm$ 0.21 [#]	1.35 $\pm$ 0.26	1.33 $\pm$ 0.23
Serum parameters	Ghrelin (ng/l)	94.18 $\pm$ 9.83	99.66 $\pm$ 10.48	88.03 $\pm$ 8.28 [#]	71.04 $\pm$ 8.73 [#]	82.05 $\pm$ 8.25	85.15 $\pm$ 7.56
	Orexin-A	400.82 $\pm$ 30.18	421.38 $\pm$ 49.86	338.27 $\pm$ 42.68 [#]	266.75 $\pm$ 37.57 [#]	331.14 $\pm$ 45.26	326.15 $\pm$ 43.25

Note: [#] $P<0.05$ compared with Normal body weight; * $P<0.05$ T $\geq$ 0.7ng/mL compared with T<0.7ng/mL.

Table I. Organs that may be more frequently affected by toxicity after infusion of pro-inflammatory cytokine-releasing CAR-T cells.

Clinical findings		Coefficient of correlation	P-Value
Basic parameters	Age (years)	0.016	0.776
	Menarche age (years)	0.021	0.720
	Systolic blood pressure	0.046	0.480
	Diastolic blood pressure	0.117	0.080
	BMI	-0.218	0.001
	WHR	-0.539	0.001
Hormone	T	0.138	0.039
	E ₂	0.018	0.750
	PRL	0.008	0.868
Biomarkers of saccharometabolism	FPG	0.066	0.314
	PG 2h	0.000	0.948
	INS 0	0.044	0.496
	INS1h	0.028	0.651
	INS2h	0.072	0.273
Biomarkers of lipometabolism	TC	0.012	0.812
	TG	0.055	0.413
	FFA	0.061	0.327
	LDL-C	0.036	0.565
	HDL-C	0.047	0.467
Serum parameters	Ghrelin	0.013	0.803
	Orexin-A	0.039	0.547
HOMA-IR		0.055	0.398

**Fig. 1.** The incidence of saccharometabolism and lipometabolism disorders. * $P < 0.05$ compared with $BMI < 18.5 \text{ kg/m}^2$; # $P < 0.05$ compared with $18.5 \leq BMI < 23 \text{ kg/m}^2$; & $P < 0.05$ compared with $23 \leq BMI < 25 \text{ kg/m}^2$.

BMI and WHR were negatively correlated with LH/FSH ratio, T was positively associated with LH/FSH ratio, and there was no correlation between LH/FSH and blood lipid, plasma glucose, insulin, and other analyzed parameters (Table II).

DISCUSSION

Our results showed that the incidence of saccharometabolism and lipometabolism disturbances increased with an increase in BMI, therefore it could be concluded that the control of body weight can be the primary step in PCOS treatment. The expression of Ghrelin in ovarian granulosa cells could indirectly reflect the ovarian functions (7). Orexin-A is a neuropeptide, participating in energy metabolism and insulin secretion (8). A previous study reported that the secretion of Orexin-A is downregulated in PCOS and negatively correlated with the occurrence and development of PCOS (9) which is in accordance with the present study where the serum levels of Ghrelin and Orexin-A in obese PCOS patients were remarkably lower and decreased with the increase of degree of obesity.

The appearance of hyperandrogenism is an important clinical manifestation of PCOS (10, 11). Our findings showed that the level of testosterone was positively correlated with LH/FSH ratio. This can be explained by the effect that increased androgen levels have on inhibiting the effects of estrogen and progestogen on LH, leading to an increased LH/FSH ratio (12). Increased LH levels may stimulate the follicular stroma and the cells to secrete excessive androgen and promote the synthesis and secretion of ovarian androgen continuing the cycle and increasing the level of testosterone. Increased T level, on the other hand, inhibits the maturity of follicles, limiting the formation of dominant follicles and decreasing the level of estradiol. Another finding of our study was that BMI was negatively correlated with LH/FSH and positively correlated with saccharometabolism and lipometabolism disturbances. Therefore, it could be concluded that for patients with lower LH/FSH ratio, especially those with high BMI that are associated with saccharometabolism and lipometabolism

disturbances, future therapeutic strategies should highly focus on the regulation of the metabolism disorders in order to prevent severe complications.

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LETTER TO THE EDITOR

EXPRESSION OF ANNEXIN A3 IN OSTEOLASTS AND OSTEOSARCOMA HOS CELLS

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To the Editor,

Osteosarcoma (OS) is one of the most common malignant bone tumors originating from mesenchymal cell lines. The tumor growth rate is very rapid, and lung metastasis occurs early, leading to deterioration of the general condition (1). At present, chemotherapy or radiotherapy is most common for the treatment of osteosarcoma, but 30-50% of patients still suffer lung metastasis or tumor recurrence with poor prognosis (2, 3). Therefore, it is necessary to find a new target for the treatment of osteosarcoma.

In recent years, studies have shown that Annexin A3 (ANXA3) is closely related to progression of lung adenocarcinoma, ovarian cancer and gastric cancer (4-6). ANXA3 is an annexin which has a high affinity with phosphatidylserine and can bind to phosphatidylserine on the outside of cells and on the cell membrane of early apoptotic cells. Pan et al. showed that ANXA3 can activate JNK (c-Jun N-terminal kinase) pathway in hepatocellular cancer stem cells, and its expression in hepatocellular cancer cells is positively correlated with JNK pathway (7). It is hypothesized that the same mechanism may exist in osteosarcoma cells.

To sum up, it has been shown that the expression of ANXA3 is closely related to occurrence, development and metastasis of a variety of tumors, but there are few studies on the relationship between

ANXA3 and osteosarcoma. In this study the role of ANXA3 in osteosarcoma cells and its mechanism were explored in order to verify the relationship, with the aim of facilitating the early diagnosis and intervention of osteosarcoma and reduce mortality.

MATERIALS AND METHODS

Cell culture

The human osteosarcoma HOS cell strains and U2OS cell strains (Shanghai BinSui Biotech, China) were cultured at 37°C, 5% CO₂ (Shanghai SANTN Instrument Corporation, China); the culture medium was renewed every 3 days. When the confluency reached 80%, the cell strains were digested and passaged by pancreatin (1 mL, Beijing LvYuan BoDe Biotech Corporation, China). Once the cells started to become round and crystal, and the gaps between cells became larger, the digestion was terminated by using DMEM high glucose complete medium (4 mL, Wuhan Biofavor Biotech Corporation, China). The cells were blown away and the pancreatin solution became turbid. Afterward, 5 mL of cell suspension were pipetted into an autoclaved centrifuge tube for centrifugation at 1000 g for 5 min. The supernatant was discarded, and 5 mL of DMEM complete medium was added to count the cells. The cells were passaged at a ratio of 1:3. The cell suspension was placed in a culture dish to observe the agglomeration of the cells; in order to prevent the cell density from being excessively large, after being passaged,

Key words: Annexin A3; osteoblast; osteosarcoma HOS cell; osteosarcoma U2OS cell; cell apoptosis

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the cells were placed in an incubator for further culture.

The hFOB1.19 human SV40 transfection osteoblasts (Shanghai YaJi Biological Technology Corporation, China) were cultured at 33.5°C, 5% CO₂, and the culture medium was renewed every 3 days. When the confluency reached 80%, the cell strains were digested and passaged by 1 mL of pancreatin. Once the cells started to become round and crystal, and the gaps between cells became larger, the digestion was terminated by using DMEM/F12 complete medium (4 mL, Tianjin TaiZe XingYe Biotech Corporation, China). The cells were blown away and the pancreatin solution became turbid. Afterward, 5 mL of cell suspensions were pipetted into an autoclaved centrifuge tube for centrifugation at 1000 g for 5 min. The supernatant was discarded, and 5 mL of DMEM/F12 complete medium was added to count the cells. The cells were passaged at a ratio of 1:3. The cell suspension was placed in a culture dish to observe the agglomeration of the cells; in order to prevent the cell density from being excessively large, after being passaged, the cells were placed in the incubator for further culture.

Immunofluorescence assay

The osteosarcoma HOS and U2OS cells were seeded at a cell density of 1×10^5 /well on a 6-well plate. When the confluency reached 60%, the medium was discarded, the cells were washed three times with 0.01M of phosphate buffer solution (PBS, Wuhan Booute Biotechnology Corporation, China) for 1 min each. Then, 1 mL of formaldehyde (100%, Shanghai SanShu Biotechnology, China) was added dropwise to each well. After 5 min, the formaldehyde solution was removed by filter paper and the cells were washed three times with 0.01M of PBS for 3 min each. The PBS was discarded, and 800 μ L of Triton X-100 (0.1%, Tianjin TaiZe XingYe Biotech Corporation, China) was added to each well by using a pipette. After 5 min, the Triton X-100 was discarded, and the cells were washed three times with 0.01M PBS for 1 min each. The cells were blocked by bovine serum albumin (Shenzhen Otwo Biotech, China) for 1 h, and washed three times with PBS for 1 min each. 1 μ g/mL of annexin A3 antibody (Nanjing saihongrui Biotechnology Co., Ltd., China) was added and placed overnight in a shaker at 4°C. Afterward, the primary antibody was aspirated and discarded. The cells were washed three times with PBS, 1 min each time. 2 μ g/mL Goat anti-mouse IgG (Beijing Qiangxin Borui

Biotechnology Co., Ltd., China) was added and placed at room temperature for 1 h. The secondary antibody was then aspirated and discarded; the cells were washed three times with PBS for 3 min each. Then, 800 μ L of DAPI (Chongqing Pulike Biotechnology, China) was added to each well. After 3 min, DAPI was removed by a filter paper and the cells were rinsed with ddH₂O (Changsha Dalfun Biotechnology, China). The cells were transferred to slides, and pipetted with fluorescence decay resistant medium (Nanjing Sbjbio Corporation, China). The slides were sealed and stored at 4°C away from light. TCS SP8 laser confocal microscopy (Leica, Germany) was used to observe the results of cellular immunofluorescence.

RNA extraction and RT-PCR

A total of 2 mL of RZ cell lysis buffer (Guangzhou Whiga Technology, China) were added into the flask, and a Pasteur pipette was used to disperse the wall of the flask until the solution was transparent and viscous. The sample was transferred to an EP tube and stored at room temperature. After 5 min, the tube was added with 400 μ L of chloroform (Henan Xinxiang Zhongyuan Organic Chemical, China) and was fully oscillated for 15 s. The tube was placed at room temperature for 3 min; then, it was centrifuged (12000rpm/min, 4°C) for 10 min, and the solution at the top of the tube was pipetted into another EP tube. 1.2 mL of absolute ethanol (Tianjin ShengTongTai Chemical, China) was added and mixed well; then, the tube was transferred to the CR3 adsorption column, centrifuged (12000rpm/min, 4°C) for 30 s, and the supernatant was discarded. Then, the tube was added with 500 μ L of de-protein solution RD (Shanghai HePeng Biotechnology, China), centrifuged (12000rpm/min, 4°C) for 30 s, and the supernatant was discarded. The tube was added with 500 μ L of de-protein solution RW (Shanghai HePeng Biotechnology, China), centrifuged (12000rpm/min, 4°C) for 30 s, and the supernatant was discarded. The de-protein solution RW was added and the operation was repeated again. The adsorption column was placed in a collection tube, centrifuged (12,000rpm/min, 4°C) for 30 s, and the supernatant was discarded. The adsorption column was placed in another clean EP tube, and added with 60 μ L of RNase-Free ddH₂O (Beijing Bioss Antibodies, China), left for 2 min at room temperature, and centrifuged (12,000rpm/min, 4°C) for 2 min. Next, 5 μ L of the RNA sample was diluted with RNase-Free ddH₂O in a ratio of

1:100. NanoDrop 2000 nucleic acid concentration analyzer (Thermo Fisher Scientific, USA) was used to determine the concentration of RNA extracted from cells.

The extracted total cell RNA was used as the template, for the gDNA removal reaction 2 μ L of 5 $\times$ gDNA Eraser Buffer were supplemented with RNase Free H₂O to a total volume of 10 μ L, and for the reverse transcription reaction 2 μ L of 10 $\times$ FastRT Buffer, 2 μ L of RT primer Mix, and 2 μ L of FQ-RT Primer Mix were supplemented with RNase Free H₂O to a total volume of 10 μ L. The reverse transcription reaction system was added to the gDNA removal reaction system, mixed uniformly, incubated at 42°C for 15 min, and then incubated at 95°C for 3 min. After the reaction was terminated, it was stored at -20°C. The annexin A3 primer was designed by using Oligo 6 software and was subjected to RT-PCR assay according to the following reaction system: 5 μ L of SYBR Green, 2 μ L of Template, 0.4 μ L of Primer, and 2.6 μ L of ddH₂O. The relative value of gene expression was calculated by using the relative quantitative method.

Western blot analysis

Forty-eight h after the siRNA (small interference RNA) transfection of osteoblasts, U2OS cell lines, and HOS cell lines, RIPA cell lysis buffer (Beijing Solarbio Science & Technology Corporation, China) was added to the cells to extract the total protein in the cells; in addition, the protein concentration in the cells was determined by the polybutylcyanoacrylate method. The protein sample to be tested was mixed with the protein loading buffer and the PIPA lysis buffer that contained the protease inhibitor; the volume and mass of the sample to be tested were equal. The sample was put in boiling water for 10 min; then, it was cooled in an ice box for 3 min and centrifuged (12,000 g) for 2 min. In accordance with the molecular weight of the protein samples to be assayed, the 10% separation gel and 4% stacking gel were prepared. The Rainbow Protein Marker (Shanghai Bogoo Biotechnology, China) and the protein samples to be assayed (25 μ g) were added into the electrophoresis lanes. The power supply was connected, and the sample was submitted for electrophoresis with a constant voltage of 80V for 30 min; next, the constant voltage was shifted to 120V, and the electrophoresis was continued for another 90 min. The PVDF membrane was placed in the Bio-Rad standard wet membrane transfer device for 2 h with a constant current of 260 mA. At room

temperature, the membrane was shaken for 2 h with (5% skimmed milk), after which the blocking solution was discarded, and the membrane was rinsed three times with TBST solution for 10 min each. Next, the membrane was added with annexin A3 antibody and stood overnight; then, the primary antibody was recovered, and the membrane was rinsed three times with TBST solution on a shaker for 10 min each. The membrane was added with the second antibody dilution (horseradishase-labeled goat anti-rabbit IgG) (Beijing DingGuo ChangSheng Biotechnology, China) and incubated for 1 h. The Chemi Doc XRS gel image acquisition system (Bio-Rad, USA) was used to scan and photo the membrane. The β -actin (Shanghai YouYu Biotechnology, China) was used as the internal reference index to calculate the gray value of the protein bands.

Flow cytometry

HOS cells in the logarithmic growth period were seeded on a 6-well plate at a cell density of 3×10^5 /well, and siRNA651, siRNA732, siRNA1094, and negative control and positive control siRNA were separately transferred into the cells. The liposome 2000 transfection reagent (Guangzhou Whiga Technology, China) was mixed with Opti-MEM medium (Thermo Fisher Scientific, USA) and allowed to stand for 5 min; then, it was added with the Opti-MEM diluted siRNA (Shanghai Biosamite Biotechnology, China) and kept in the dark for 20 min, and the mixture was added to the 6-well plate and the solution was renewed 6 h later. After 48 h, the cell culture medium was transferred to a centrifuge tube and washed with PBS; the washing solution was collected in a centrifuge tube. The pancreatin was added to digest the cells. After the digestion was terminated, the cell suspension was centrifuged (1000rpm/min) for 5 min, added with the pre-cooled PBS for resuspension, and centrifuged (1000rpm/min) for 5 min; afterward, the supernatant was discarded, and 100 μ L of 1 $\times$ binding buffer, 5 μ L of recombinant human Annexin V/PE, and 5 μ L of 7AAD were added and mixed well; the tube was stood in a dark room for 15 min and added with 400 μ L of 1 $\times$ binding buffer. The apoptosis of cells was detected and analyzed by a flow cytometer (Beckman Coulter, USA).

Statistical analysis

SPSS 22.0 statistics software was used for statistical analysis of the data; all quantitative data were submitted for normal distribution test and homogeneity test of

variance and were expressed as the mean number $\pm$ standard deviation ($\bar{x} \pm s$). One-way ANOVA was used for comparison between groups; if the normal distribution and variance were consistent, the LSD method was used; otherwise, the SNK-q test was used. Pearson correlation analysis was used to analyze the correlations between two parameters, $P < 0.05$ indicated the statistical significance of the difference.

RESULTS

Detection of annexin A3 expressed in osteosarcoma cells by immunofluorescence

As shown in Fig. 1, under the laser confocal microscope, annexin A3 was obviously expressed in osteosarcoma HOS and U2OS cells. Fig. 1A shows the expression of annexin A3 in osteosarcoma HOS cells; Fig. 1B shows the stained nuclei of osteosarcoma HOS cells, and Fig. 1C shows the cell fusion of osteosarcoma HOS cells. In addition, Fig. 1D shows the expression of annexin A3 in osteosarcoma U2OS cells, and Fig. 1E shows the stained nuclei of osteosarcoma U2OS cells, Fig. 1F showed the cell fusion of osteosarcoma U2OS cells.

Detection of relative annexin A3 expression in mRNA by RT-PCR

Detection results of the relative annexin A3 expression in mRNA by RT-PCR are shown in Fig. 2A. It was seen that compared with osteoblasts, the expression levels of annexin A3 in osteosarcoma HOS cells and U2OS cells were obviously increased, and the differences were statistically significant ($*P < 0.05$). In addition, the increase of annexin A3 expression in HOS cells was the most obvious.

Detection of relative annexin A3 expression in proteins by Western Blot

Results of Western blot are illustrated in Fig. 2 C-E, in which the relative expressions of annexin A3 based on protein level are shown in Fig. 2C and D. It was seen that compared with osteoblasts, the expression levels of annexin A3 in osteosarcoma HOS cells and U2OS cells were obviously increased, and the differences were statistically significant ($*P < 0.05$). The results of Western blot further proved the detection results of RT-PCR. After being transfected by iRNA-651, siRNA-732,

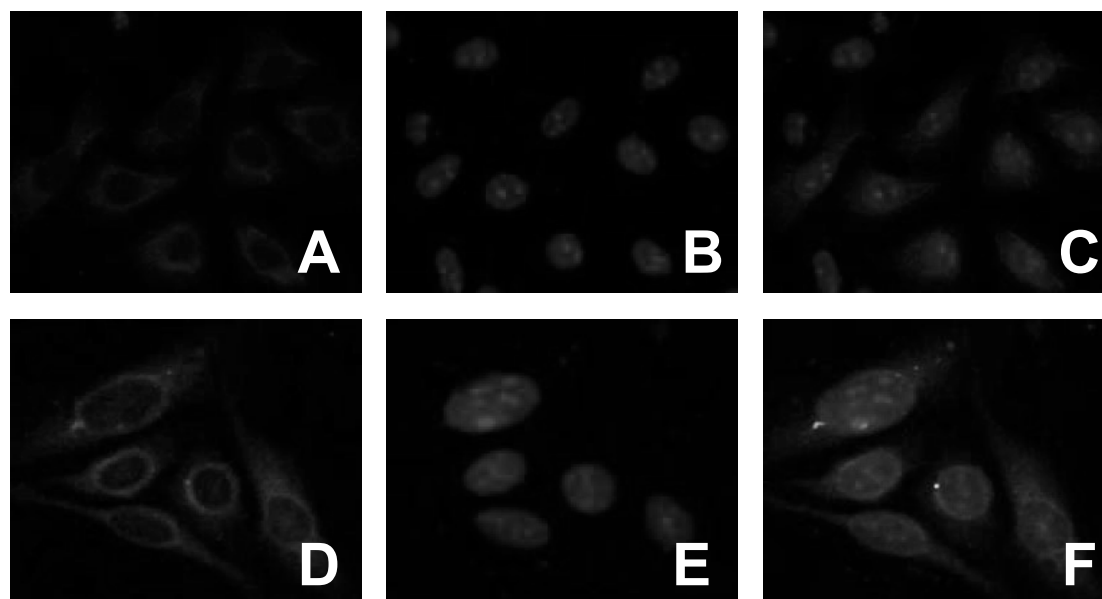


Fig. 1. Expressions of annexin A3 in cell line HOS and U2OS. **A)** Expression of annexin A3 in osteosarcoma HOS cells; **B)** Stained nuclei of osteosarcoma HOS cells; **C)** Cell fusion of osteosarcoma HOS cells; **D)** Expression of annexin A3 in osteosarcoma U2OS cells; **E)** Stained nuclei of osteosarcoma U2OS cells; **F)** Cell fusion of osteosarcoma U2OS cells.

and siRNA-1094, the expression of annexin A3 in transfected HOS cells was obviously decreased, and the differences were statistically significant (* $P < 0.05$), in which the expression reduction of annexin A3 in HOS cells transfected by iRNA-651 was the most obvious, as shown in Fig. 2 E and F.

Detection of osteosarcoma cell apoptosis after transfection by flow cytometry

In terms of the flow cytometry, the apoptosis of transfected osteosarcoma cells is shown in Fig. 2B; it

was seen that once the expression of annexin A3 was down-regulated, after being transfected by iRNA-651, siRNA-732, and siRNA-1094, the apoptosis rate of transfected HOS cells was obviously increased compared with before transfection, and the differences were statistically significant ($P < 0.05$).

DISCUSSION

Overexpression of ANXA3 promotes the

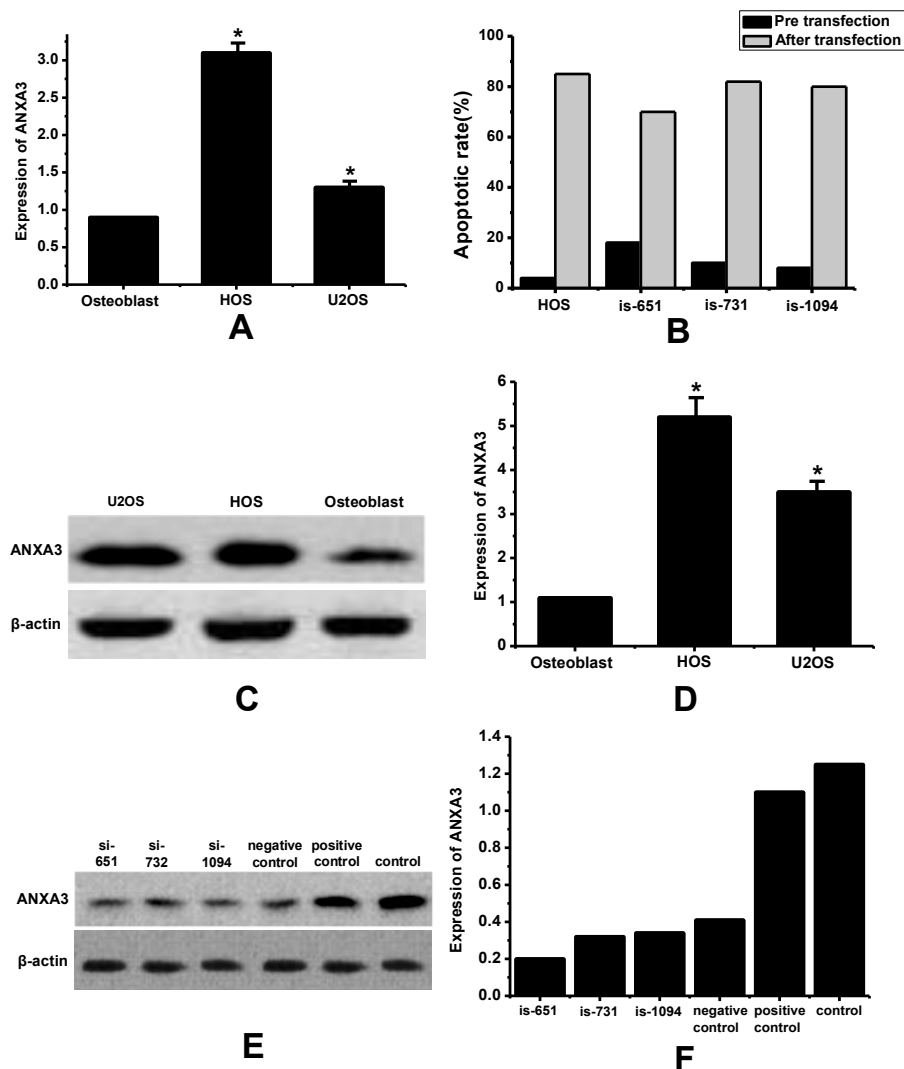


Fig. 2. The expression of annexin A3 and the apoptosis of osteosarcoma cells. **A)** Relative expression of annexin A3 gene at mRNA levels; **B)** Apoptosis of osteosarcoma cells before and after transfection; **C)** Expression levels of annexin A3 in osteoblasts, osteosarcoma HOS cells, and osteosarcoma U2OS cells, β-actin was the internal index of antibody; **D)** Relative expression levels of annexin A3 based on the protein level; **E)** Cell annexin A3 expression in HOS cells transfected by iRNA-651, siRNA-732, and siRNA-1094; **F)** the expression levels of annexin A3 in transfected HOS cells.

proliferation and metastasis of ovarian cancer, liver cancer and prostate cancer (7-9). Yin et al. showed that the increased expression of ANXA3 may be a resistant response of ovarian cancer to chemotherapy (10). Zeng et al. thought that ANXA3 can be used as a marker for early diagnosis of prostate cancer (11). In this study, the significance of ANXA3 expressions in osteoblasts and osteosarcoma HOS cells was explored. The results showed that the expression of ANXA3 in osteosarcoma cells was significantly higher than that in osteoblasts. This suggested that ANXA3 may also be involved in the formation of osteosarcoma cells. In this study, the relationship between the expression of ANXA3 and osteosarcoma cells was further explored. The apoptotic rate of HOS cells increased significantly when the expression of specific genes was eliminated by RNA interference. It is indicated that the high expression of ANXA3 is related to the proliferation of osteosarcoma cells. Therefore, ANXA3 may be a new therapeutic target for osteosarcoma. By intervening in the expression of ANXA3 in osteosarcoma cells and osteosarcoma stem cells, it is expected to become a new immunotherapy for osteosarcoma.

In conclusion, ANXA3 is highly expressed in osteosarcoma cells, which can promote apoptosis by downregulating the expression of ANXA3 in HOS cells. Therefore, ANXA3 is expected to become a new therapeutic target for osteosarcoma, which facilitates the early diagnosis and intervention of osteosarcoma.

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LETTER TO THE EDITOR

EVALUATION OF THE ANTI-INFLAMMATORY ACTIVITY OF A *MIMUSOPS ELENGI* (LINN)-INCORPORATED HERBAL PRODUCT: A ZYMOGRAPHIC ANALYSIS

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To the Editor,

Periodontitis represents a specific inflammatory response to microbial residents of the subgingival biofilm (1). Emerging evidence strongly states that it is the inflammatory response of the host that facilitates the tissue destruction, and the variability of host responses is accountable for much of the variability in the clinical manifestation of periodontitis (2). It is now well accepted that large consortia of cytokines, cell-signaling molecules and matrix metalloproteinases (3), particularly MMP-8 and MMP-13 and gelatinases MMP-2, and MMP-9 play a major role in destruction of tissues in periodontitis.

Gelatin zymography is a simple, reliable, and sensitive method used to monitor gelatinase (MMP-2 and MMP-9) expression and activation in a variety of biological samples (cells, tissues, and fluids). Gelatin zymography enables detection of gelatinolytic activity in biological samples using sodium dodecyl sulfate (SDS)-polyacrylamide gels impregnated (co-polymerized) with gelatin (4).

Periodontal treatment involves various modalities

to reduce the bacterial load which limits the host response against inflammation-induced degradation of periodontal tissues. Recently various herbal extracts have been tried as mouthwashes and irrigating solutions as adjuvants to mechanical plaque control with potential results (5, 6). Being herbal, they do not cause many side effects and can be used safely (7). This is the first study of its kind that aimed to assess the anti-inflammatory properties of the unique mix, approved by the Food and Drug Administration (F.D.A), obtained from the important herbs. The product incorporates the potential medicinal plant, *Mimusops elengi* (8), as a major component, apart from other vital plant products with medicinal value. Hence, in the present study, an attempt was made to assess the anti-inflammatory efficacy of a new polyherbal preparation (PH) available as Bakul dant powderTM in comparison with tetracycline hydrochloride (TTC HCl).

MATERIALS AND METHODS

A total of thirty patients using convenience sampling,

Key words: matrix metalloproteinases, polyherbal, tetracycline hydrochloride, gelatin zymography, inflammation

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both male and female aged between 25-60 years visiting the Division of Periodontics and Community Dental Sciences, College of Dentistry, King Khalid University were enrolled for the purpose of the study. Prior to the initiation of the study, ethical clearance was obtained from the Institutional Ethics Committee (Approval No.SRC/ETH/2018-19/116). A written informed consent, in a language known to them, was obtained from all patients prior to the study. This analytical study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000.

The inclusion criteria were: patients with chronic periodontitis teeth indicated for extraction, with clinical attachment loss ≥ 4 mm and probing pocket depth (PPD) ≥ 5 mm at more than 30% of sites with ≥ 20 functional teeth; subjects having 25-60 years of age as they were easier to approach and convince to participate compared to a younger subjects - also, this age group was selected due to the fact that in ages ≤ 25 years, attachment loss of ≥ 4 mm in at least two teeth signifies aggressive periodontitis (9).

The exclusion criteria were patients with any systemic diseases/condition, pregnant women, smokers, patients with a history of dental treatment in the previous 3 months, patients on any antibiotics or anti-inflammatory agents 3 months prior to the study.

Sample collection

Gingival tissue samples of 2-3mm were obtained from patients clinically diagnosed as having chronic periodontitis in teeth indicated for extraction. Tissue samples were collected from marginal tissues before the tooth was extracted. All gingival samples following excision were briefly washed under saline and then transferred into small sterile plastic vials. The tissue sample was minced completely; 5 ml of tris buffer was added and centrifuged at 3000 rpm for 15 min, and stored at -20°C for further use.

Preparation of the polyherbal plant product and procedure

It is noteworthy that the formulated polyherbal product (Bakul dant powderTM) is approved for its use by the Food and Drug Administration (F.D.A, India Permission No. 1174). The plant materials used in the preparation of the polyherbal product are the popular plants used in ancient and traditional medicine. The significant plants were selected and prepared in appropriate concentrations based upon previous ethno-botanical literature and potential

medicinal values. The collected plants were identified at the HiTech laboratories, Pune, India, by a senior doctor of Ayurveda (Herbal medicine), who authenticated the proportion of each plant material to be incorporated in making the final product. Each component in the herbal formulation was selected after meticulous understanding of their pharmacological actions so that the combination is a product of paramount benefit.

The most potential parts of the plants, judged by relying on the traditional concept for the parts to be used for the treatment of diseases, were selected for the study (10). All collected plant materials were air-dried at room temperature under shade, and subsequently pulverized into fine powder in a pulverizer. Each powdered plant material was then mixed in adequate proportions based on their potential medicinal benefits. The parts used and proportions of each plant material are listed in Table I. As reported in previous studies, there can be limited antibacterial potency due to the extraction methods employing various chemicals and crude extracts to get the plant extracts (11). It is therefore suggested that pure plant compounds isolated from plants might exhibit better antibacterial activity. Owing to this concept, the native pure form of the plants was used as a mixture to retain their vital properties in natural form.

The electrophoresis apparatus was cleaned in warm water and the glass plates in methanol. The plates were set up. First the large plate and then two spacers (little petroleum jelly on both sides of the spacers applied) and then the small plate was set on the top. The plates were assembled into clamp and gently screws were tightened. The agarose gel was heated and poured between the two glass plates just to seal the bottom surface and then left for 5-10 min. The resolving gel (10 ml) was prepared using 3.3 ml of acryl amide – Bis-acrylamide, 1.25 ml of resolving gel buffer stock, 100 μl of 10% (SDS), 500 μl of 1.5% APS, 1 ml of gelatin, 3.8 ml of water and 10 μl of tetramethylethylenediamine (TEMED). The appropriate resolving gel mixture was mixed between the glass plates avoiding bubbles. The plates were filled to about 80%, leaving space for the stacking gel and comb. It was overlayed with a small amount of water to achieve a completely flat interface between the resolving gel and the stacking gel, and allowed to set for about 45 min. While the resolving gel is setting, the stacking gel (10ml) was prepared using 1.7 ml of Acrylamide – Bisacrylamide,

1.12 ml of stacking gel buffer, 100 µl of 10% SDS, 500 µl of 1.5% Ammonium Persulfate (APS), 6.5 ml of water and 10 µl of TEMED. When the resolving gel was set, the excess water was poured off and a wash was added between the plates with distilled water. The stacking gel was poured in and the comb was inserted avoiding bubbles. It was allowed to set for about 30 min. When the stacking gel was set, the comb was gently removed. The wells were washed with distilled water and the gels were assembled onto the electrode/gasket section of the gel apparatus. The top and bottom of the tank were filled with the reservoir buffer i.e., the upper tank with 100 ml and lower tank with 150 ml.

The gingival tissue sample was minced completely after allowing it to thaw to room temperature. Two ml of the extraction buffer was then added and centrifuged at 3000rpm for 15 min at 4°C, and stored at -20°C for further use. 10% of the test polyherbal product was prepared for the study, i.e., 10mg/ml of the test product (PH) dissolved in dimethyl-sulphoxide. After centrifugation,

the supernatant was used for mixing with the inflamed tissue sample (MMP sample). Then from the dissolved compound, 50 µl was mixed with the 50 µl MMP sample. Likewise TTC HCl (positive control) was mixed and incubated for 1 h. While loading, the incubated samples were mixed with equal volume of 2x non-reducing buffer. This non-reducing buffer contains bromophenol blue that guides the movement of the samples in the gel, and glycerol gives weight to the samples that helps to settle into the wells. Negative control used was MMP sample only.

The upper and lower tanks of gel electrophoresis apparatus were filled with the reservoir buffer. The electrodes were connected; a lid was put on the tank and it was run at about 50V for 15 min and then 100V until the bromophenol blue reached the bottom of the plates. After electrophoresis, the apparatus was disassembled and the gel was gently removed and put into a plastic dish and the gel was washed with zymogram renaturing buffer i.e. 2.5% Triton x-100 for 1 h to remove SDS from the gel

Table I. Plant material used and their proportions.

Botanical name	Local name	Parts used	Proportions used (%)
<i>Mimusopselengi</i> (Linn)	Bakul saal	Bark	22
<i>Azadirachtaindica A</i>	Neem saal	Bark	11
<i>Acacia nilotica</i>	Babul saal	Stem	11
<i>Acacia catechu</i>	Khairsaal	Bark	11
<i>Terminalia chebula</i>	Hirda	Fruit	11
<i>Terminalia bellirica</i>	Bahera	Fruit	11
<i>Emblica officinalis</i>	Amalaki	Fruit pulp	11
<i>Alum</i>	Turti	Hydrated double sulfate salt of aluminium	2
<i>Syzygiumaromaticum</i>	Lavang	Dried flower buds	5
<i>Rock salt</i>	Saindhav	Mineral	5

Table II. Comparison of mean percentage inhibition of MMP-2 among the groups.

Particulars	PH (Test solution)	Tetracycline HCl (Positive control)	Negative control (MMP sample)	Anova test	p value
Range	71-83	85-97	7-12		
Mean	80	95	10	40.47	<0.01*
SD	3.56	2.42	1.48		

*: statistically significant

Table III. Comparison of mean percentage inhibition MMP-9 among the groups.

Particulars	PH (Test solution)	TTC HCl (Positive control)	Negative control (MMP sample)	Anova test	p value
Range	54-71	77-97	0		
Mean	56	90	0	52.75	<0.01*
SD	5.16	2.96	0		

*: statistically significant

and allow proteins to denature. The zymogram renaturing buffer was decanted and the gel was incubated in zymogram incubation buffer at 37°C overnight. Staining was carried out with Coomassie blue R-250 for 1 h. Gels were then destained with an appropriate Coomassie R-250 destaining solution for about 2 h. After staining, the background stains blue with Coomassie stain where the gelatin is degraded; white bands appear indicating the presence of gelatinases. The lower bands were gelatinases-A (MMP-2) which is about 72KD while the upper bands were gelatinases-B (MMP-9) which runs at about 95KD.

Photographic analysis

Enzymatic activities were observed as clear bands of gelatin destruction against the blue background (Fig. 1). To measure the relative enzyme levels, clear zones were scanned and the percent inhibition was analyzed. The photographic analysis was carried out by a UV trans-illuminator attached to a camera and the image was viewed on the desktop directly, captured and saved.

Statistical analysis

Data were tabulated and examined using the Statistical Package for Social Sciences Version 20.0 (IBM SPSS Statistics for Mac, Armonk, NY: IBM Corp, USA). Descriptive statistical analysis was carried out in the present study. Results on continuous measurements are presented as Mean $\pm$ SD. The statistical power calculation was based on the assumption that the data were normally distributed. ANOVA test was used to determine the significant difference between the groups ($p < 0.05$).

RESULTS

Table II depicts the range, mean values and the standard deviation (SD) values for the PH, TTC

HCl (positive control) and MMP samples (negative control) for inhibition of MMP-2. The mean values showed 95% reduction in the MMP-2 activity with the addition of TTC HCl, 80% reduction in the MMP-2 activity with the addition of the PH and 10% reduction in the MMP-2 activity with the negative control to the gingival tissue samples, respectively, under identical conditions. This signifies that the maximum anti-inflammatory activity was shown by TTC HCl followed by the PH in respect to reduction of MMP-2 levels.

The range, mean values and the SD values for the test solution and TTC HCl for inhibition of MMP-9 are shown in Table III. The mean values showed 90% reduction in the MMP-2 activity with the addition of TTC HCl, 56% reduction in the MMP-9 activity with the addition of the test solution and 0% reduction in the MMP-9 activity with the negative control to the gingival tissue samples, respectively, under identical conditions. Therefore, the maximum anti-inflammatory action was shown by TTC HCl followed by the PH in respect to the reduction of MMP-9 levels.

When the mean percentage difference of inhibition of MMP-2 and MMP 9 by TTC HCl and the test solution was compared by using ANOVA, it was found to be statistically significant.

DISCUSSION

MMPs are known to be the major enzymes implicated in the degradation of the extracellular matrix (3). The role of MMPs is commonly assessed by measuring their gene expression, protein amount, and proteolytic activity using gel zymography (12). Herbal formulations have long

been known to have anti-inflammatory properties and have been continuously investigated in the field of dentistry to treat periodontal diseases (5, 6). As these formulations are widely acclaimed for their minimal side-effects and cost-effectiveness, their use has increased in recent years. We investigated the inhibitory effects on the gelatinolytic activity of MMP -2 and MMP-9 by the new polyherbal product i.e. *Mimusops elengi* (Linn), most frequently known as “Bakul” which has been considered a potential herb with different parts of the plant suggested to possess different therapeutic uses (8).

In the present study, the mean inhibition of MMP-2 and MMP 9 by tetracycline was found to be greater when comparing the PH. Subair et al. in their study (7) had examined the efficacy of a formulated herbal anti-gingivitis agent (Bakul, Babul and Vajradanti) compared to the 0.2% chlorhexidine in gingivitis cases followed by scaling procedure and found that herbal formulation showed good acceptance and could be used as an alternative for people interested in natural products for the maintenance phase of gingival diseases. However, there are no

studies assessing the anti-inflammatory activity of such a *Mimusops elengi* (Linn) incorporated herbal product using gelatin zymography. To the best of our knowledge, the present study is the first of its kind utilizing zymography for the detection of anti-inflammatory activity of the important medicinal herb incorporated polyherbal product, so that it can be effectively and safely used by the patients with gingivitis and periodontitis.

The present in vitro study was found to be successful in determining the anti inflammatory effect on MMP-2 and MMP-9 in patients with periodontal disease, which opens avenues for conducting further research to validate the efficacy of this herbal formulation in periodontal diseases.

It can be concluded from the above results that this herbal adjuvant could be effective as an anti-inflammatory agent in periodontal diseases with good acceptance and could be used effectively. Future studies should be carried out using the split mouth technique using herbal and allopathic adjuvants. Further microbiological and immunological studies should be carried out to check the efficacy of such herbal formulations. It may be suggested that clinical trials with larger sample size are required to verify the efficacy of the polyherbal product. Herbal formulations should be considered as a valuable asset and further investigated for its efficacy in the management of periodontal diseases.

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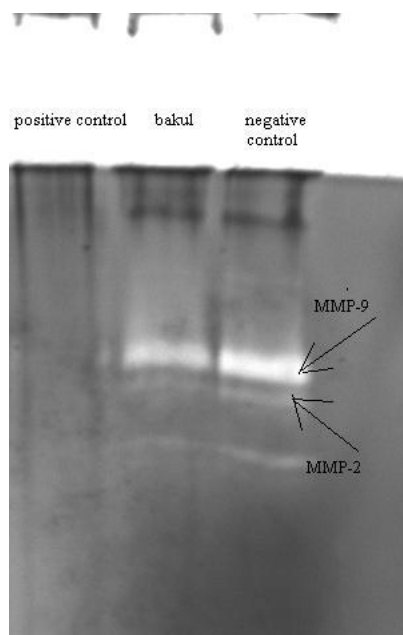


Fig. 1. Expression of MMP-2 and MMP-9 by gelatin zymography assay after the treatment with the polyherbal preparation (PH) [Bakuldant powder], TTC HCl (positive control) and MMP sample (negative control).

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LETTER TO THE EDITOR

EFFECT OF EXPLANTS AND GROWTH HORMONES ON DIRECT REGENERATION OF *SILYBUM MARIANUM* IN VITROZ. JABEEN¹, KHALIL-UR-RAHMAN¹, M.A. ZIA¹ and N. JAHAN²¹Department of Biochemistry, University of Agriculture Faisalabad, Pakistan; ²Department of Chemistry, University of Agriculture Faisalabad, Pakistan

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To the Editor,

Silybum marianum, also known as milk thistle, has been used as a hepatoprotective herb against liver ailments for 2000 years. Silymarin is a biomarker of *S. marianum*, a mixture of seven flavonolignans and flavonoid (1). The hepatoprotective activity of milk thistle is well documented among all medicinal plants (2).

Herbal therapy has attracted great attention as an alternative approach to cure liver disease. High quantities of Silymarin as a potential medicinal bioactive against liver disease are therefore needed (3). There are different traditional constraints, including spiny leaves and flowers, and the plant is cultivated in rows and the use of combine harvesters reduces production to 40% (4). Plant tissue culture is considered to be one of the best strategies for the hyper-production of valuable secondary metabolites on a large scale. The sufficient availability of plant material and secondary metabolites throughout the year is the main advantage of *in vitro* micropropagation of plants.

MATERIALS AND METHODS

Direct organogenesis is the method of tissue culture in which the plant is derived from any part

of the explant using different growth regulators. Different *in vitro* generated explants, including leaves and shoots, have been evaluated for their ability to induce direct organogenesis and subsequent proliferation. The regeneration medium was fortified with different combinations of BAP, Kin, NAA and GA3 and the percentage of direct organogenesis was calculated after five weeks of cultivation.

Regeneration medium

Regeneration media were prepared in Murashige and Skoog (MS) basal medium fortified with growth hormones including BAP, Kin, NAA (1mg/L each) as shown in Table I. Direct regeneration was optimized in different media to regenerate the entire plant. Six different direct induction media (DRIM) were used to check the regeneration capacity of leaf and shoot explants (Table I).

During direct regeneration, leaves and roots were induced from the same induction media. Shoots and leaves were cultivated on a direct regeneration induction medium and shooting days, number of shoots and regeneration frequency were recorded. Rooted plantlets were exposed to bavistin solution (1%) to prevent contamination prior to transfer to soil-containing plastic pots. They were acclimatized under controlled temperature (25±2°C)

Key words: *Silybum marianum*; silymarin; plant tissue culture; direct regeneration; secondary metabolites

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and covered with transparent plastic bags to maintain humidity. The survival rate (percentage) of the plantlets was measured (5).

Statistical analysis

In the tissue culture experiment, 10 replicates per treatment were used and the data obtained was

Table I. List of medium used for direct regeneration.

Media Type	Media composition	Explants	Replicate
DRIM-1	MS+0.5mg/L BAP+1.5mg/L GA3+1mg/L TDZ	Leaf, Shoots	10
DRIM-2	MS+1mg/L GA3	Leaf, Shoots	10
DRIM-3	MS+1mg/L NAA+2mg/L GA3	Leaf, Shoots	10
DRIM-4	MS+TDZ (11 μ M)	Leaf, Shoots	10
DRIM-5	MS+1mg/L BAP+2mg/L NAA	Leaf, Shoots	10
DRIM-6	MS+1mg/LNAA	Leaf, Shoots	10

DRIM: direct regeneration induction media; BAP: 6-Benzylaminopurine; GA3: Gibberellic acid; TDZ: Thidiazuron; NAA: Naphthaleneacetic acid

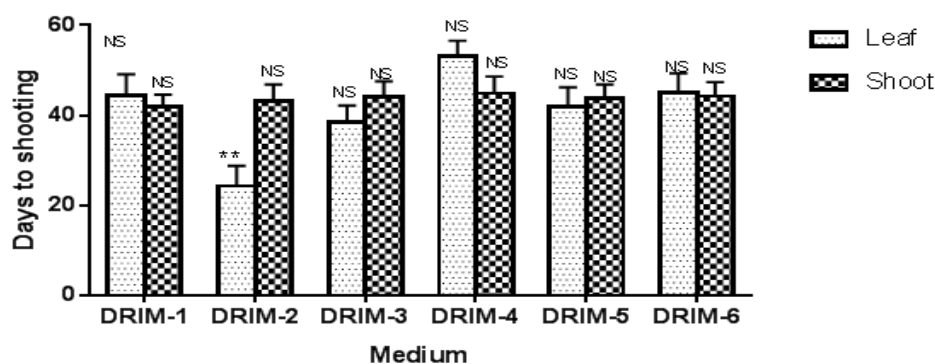


Fig. 1. Days to shooting from leaf and shoot explant on different media. ** significant difference between the different media; ^{NS} non-significance difference between the different media. Values are presented as mean $\pm$ SEM (n=10).

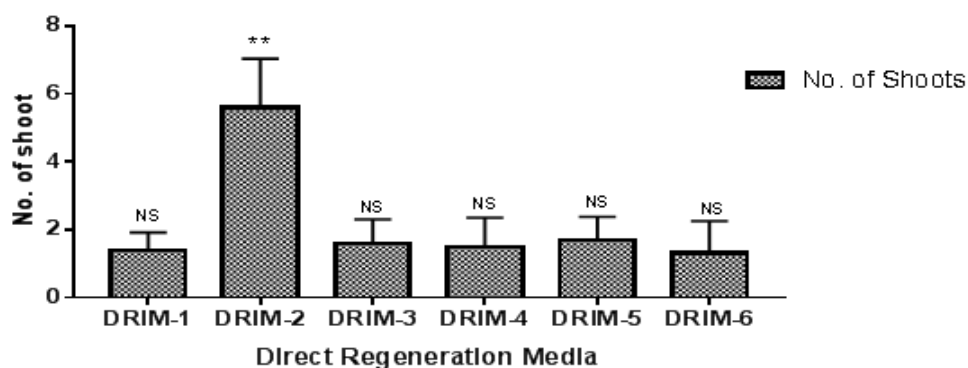


Fig. 2. Number of shoots from leaf explant on different media. ** significant difference between the different media; ^{NS} non-significance difference between the different media. Values are presented as mean $\pm$ SEM (n=10).

statistically analyzed using Graphpad Prism 7.1 software. ANOVA and Turkey Multiple Comparison Test were used to compare different treatments for their regeneration capacity.

RESULTS

The direct regenerative potential of the leaf and shoot explant was examined in six different regenerative media. Leaf explant showed effective initiation of shoot within a minimum period of time i.e. 24 ± 4.47 days after inoculation on MS medium fortified with 1mg/L GA3 (DRIM-2) as displayed in Fig. 1. The yellowish leaf blades were separated and discarded to minimize the movement of the leaf explants from the medium and successive elongation of the shoot buds. These regenerants were then subcultured on the same MS medium supplemented by 1mg/L GA3 and produced a maximum number of shoots/explants i.e., 6.5 ± 1.43 compared to other media (Fig. 2). Improved direct organogenesis response was found in shoots grown on DRIM-1 medium containing a combination of 0.5mg/L BAP, 1.5mg/L GA3 and 1mg/L TDZ which produced shoots after 42 ± 2.62 days (Fig. 1). The maximum regeneration frequency ($93.33 \pm 2.367\%$) was achieved by the leaf explant on DRIM-2 (MS complemented with 1mg/L GA3) compared to the other media. While the MS medium supplemented by 0.5mg/L BAP+1.5mg/L GA3 + 1mg/L TDZ (DRIM-1) induced the lowest regeneration frequency

($20 \pm 0.21\%$) from leaf explant (Fig. 3). The results showed that DRIM-2 was the best medium for direct somatic embryogenesis compared to the other media as shown in Fig. 2.

Finally, the direct regeneration plantlets were moved to sterilized soil and placed under controlled conditions in the growth room for two weeks prior to transfer to pots. A total of 25 *in vitro* rooted plantlets were planted in plastic pots, of which 20 (80%) survived. In addition, no morphological variations were examined as compared to the parent plant.

Leaf and shoot explants were checked for their ability to regenerate directly into the entire plant. It was shown from the results that the leaf has more potential to regenerate the whole plant compared to the shoot explant. Direct regeneration induced the shoots and roots simultaneously, but there was a difference in the days of initiation and the frequency of regeneration, as shown in Fig. 1, 2, 3. The ability to regenerate directly was also influenced by the medium composition. The MS medium supplemented by 1mg/mL GA3 (DRIM-2) showed the highest regeneration capacity compared to the other media.

DISCUSSION

There are two modes of plant regeneration, indirect (with) or direct (without), based on the participation of callus. Direct organogenesis appears to be superior to indirect organogenesis, as indirect regeneration from

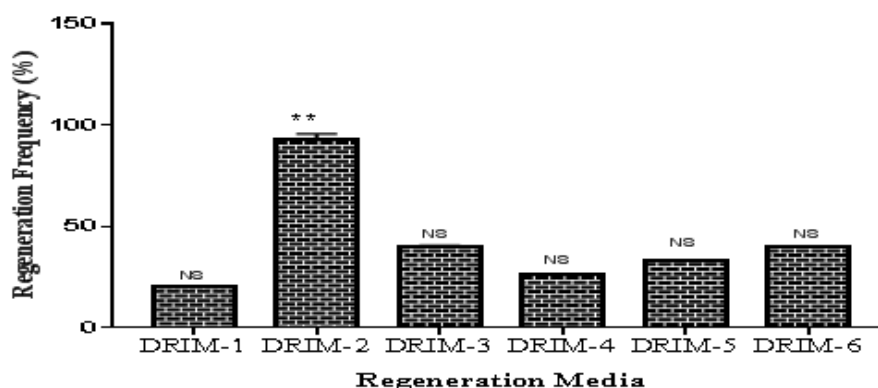


Fig. 3. Regeneration frequency of leaf explant at direct regeneration induction medium. ** significant difference between the different media; ^{NS} non-significant difference between the different media. Values are presented as mean $\pm$ SEM ($n=10$).

callus may differ from parent plants. The ability to regenerate directly was also influenced by the medium composition. The MS medium supplemented by 1mg/mL GA3 (DRIM-2) showed the highest regeneration capacity compared to the other media. The maximum number of shoots was also observed on MS+1mg/mL GA3. While the minimum regeneration capacity in terms of days to organogenesis, number of shoots and regeneration frequency was observed on MS+1mg/LNAA+2mg/L GA3 medium as shown in Figs. 1, 2, 3. In the present study, GA3 may have been responsible for effective direct organogenesis from leaf explants. It has also been reported that leaf explant has the maximum efficacy of direct organogenesis compared to another explant (6). In addition, Arif et al. 2014 (7) also reported that leaf explant was more effective for direct regeneration compared to nodal and internodal explants.

The optimal medium was reported to be MS+3.0mg/L GA3 and 1.0mg/L NAA for direct organogenesis of milk thistle from leaf explants (5). However, BAP+NAA could be more suitable for direct organogenesis as 60 percent direct organogenesis frequency was observed on the MS medium supplemented by 1mg/L BAP and 2mg/L NAA (8, 9). However, in the current study, leaf explant was less responsive to direct regeneration when NAA was used in combination with GA3, TDZ, and BAP. In the present study, 20 (80%) out of 25 plantlets were successfully acclimatized with no morphological changes compared to the parent plants. Al-Hawamdeh et al. (10) also discussed the successful acclimatization of rooted milk thistle transplants with almost 70% survival; 22 (74%) of the 30 plantlets of Abbasi et al., survived (11). According to Yu-weilv et al. (5), 46 of the 50 milk thistle plantlets *in vitro* survived. El Sheriff et al. (12) also recorded a 100 percent survival rate for *in vitro* plantlets.

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LETTER TO THE EDITOR

COMPARATIVE PERFORMANCE-BASED STUDIES OF SECOND GENERATION ANTICOAGULANT RODENTICIDES BROMADIOLONE AND FLOCOUMAFEN IN *MUS MUSCULUS*

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To the Editor

Rodents not only destroy agricultural crops but can also spread several diseases in humans such as endemic rickettsiosis, plague, spirochetosis, leishmaniasis, leptospirosis, and tick-borne encephalitis (1). Chronic rodenticides, also called anticoagulant rodenticides, are generally preferred over acute rodenticides to control rats and mice populations (2). The active components of these pesticides are Indandione derivative and 4-Hydroxycoumarin. Only a small portion of these active components, ranging from 0.005 to 25%, is used in baits. Anticoagulants prevent blood clotting factor resulting from enzymatic lack of vitamin K which helps in clotting. Anticoagulants were discovered in 1940s, their quick results and efficacy make them a dominant method of rodent control (3). To date, two types of anticoagulant-based rodenticides have been introduced: first generation and second-generation (4). First generation anticoagulants include warfarin, diphacinone

and chlorophacinone while second generation are brodifacoum, difenacoum, bromadiolone and difethialone. Rodents developed resistance against first generation anticoagulants after the latter were used frequently, hence second-generation anticoagulants were introduced as a more effective alternative (5, 6). Bromadiolone, $C_{30}H_{23}BrO_4$, is a secondary rodenticide commercially available under the trade name of “MooshMoosh” and is greenish blue in color. It is also available with alternative names such as Pest-off and Talon. Bromadiolone was discovered in 1967 for commensal rat and mice population. Its solubility is 20mg/L in water, 8mg/L in ethanol and 730g/L in dimethyl formamide (7). Flocoumafen is another secondary anticoagulant rodenticide available under the common name of Storm and is an off-white color and has a molecular formula of $C_{33}H_{25}F_3O_4$ -coumarin with a mechanism of action akin to that of warfarin. The presence of 40-coumarin on side chain makes flocoumafen 100-fold more effective compared to warfarin (5). The

Key words: rodenticides; complete blood count; efficacy

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current study was designed to compare and contrast bromadiolone and flocoumafen rodenticides and evaluate their efficacy and utility.

MATERIALS AND METHODS

A total of 50 healthy albino mice (*Mus musculus*) aged four weeks and weighing 25-30 g were included in this study. The mice were raised in sterile stainless-steel cages in a ventilated animal room under experimentally controlled conditions (temperature: $24\pm 2^{\circ}\text{C}$; humidity: $60\pm 10\%$ and light: 12 h/dark cycle). Twelve mice were

selected for testing their bromadiolone and flocoumafen tolerance. Because 100% survival was required, the animals were placed in different cages and acclimatized for a few days before bromadiolone or flocoumafen treatment. To assess the level of tolerance, acute toxicity test was carried out by the "no choice method". Two group of animals were selected for testing the acute toxicity test; one group consisting of 6 mice for bromadiolone tolerance and another group of 6 mice for flocoumafen tolerance. Oral toxicity study was conducted to determine the effects of bromadiolone and flocoumafen on albino mice at various intervals. New groups were assigned to

Table I. *Survival rate of mice after bromadiolone acute intoxication at different time intervals.*

BROMADIOLONE ACUTE INTOXICATION					
Exposure time	Bait consumption (g)	Dose ingested (mg)	Total number of mice	Number of alive mice	Number of dead mice
6 hours	0.8 ± 0.09	4 ± 0.47	6	6	-
12 hours	1.5 ± 0.21	7.7 ± 1.07	6	6	-
24 hours	3.5 ± 0.41	17.3 ± 2.05	6	6	-
48 hours	8 ± 0.58	39.8 ± 2.9	6	6	-
72 hours	12.1 ± 0.52	60.5 ± 2.59	6	5	1
96 hours	16.1 ± 0.54	80.6 ± 2.71	5	4	1
120 hours	20.7 ± 0.5	103.7 ± 2.52	4	2	2
144 hours	24.4 ± 0.31	122.1 ± 1.57	2	-	2

Table II. *Survival rate of mice after flocoumafen acute intoxication at various time intervals.*

Exposure time	Bait consumption (g)	Dose ingested mg/kg	Total number of mice	No of alive mice	Number of dead mice
6 hours	0.8 ± 0.12	4 ± 0.58	6	6	-
12 hours	1.4 ± 0.25	7.2 ± 1.27	6	6	-
24 hours	3.7 ± 0.29	18.6 ± 1.43	6	6	-
48 hours	6.7 ± 0.35	33.7 ± 1.77	6	6	-
72 hours	13.7 ± 0.33	68.3 ± 1.63	6	6	-
96 hours	18.3 ± 0.34	91.5 ± 1.71	6	5	1
120 hours	23.3 ± 0.34	116.7 ± 1.7	5	3	2
144 hours	28 ± 0.47	140.1 ± 2.35	3	-	3

animals. The animals of both categories i.e. bromadiolone and flocoumafen were divided into 4 groups, named G1 to G5, each consisting of 3 mice. G1 was the control group which received no anticoagulant doses and were fed on normal feed. G2 was treated for 6 hours, G3 for 12 hours, G4 for 24 hours and G5 for 48 hours in either category. Blood samples were taken from mice in both categories through sinus puncture for hematology analysis. The blood was drawn into clean sterile vials containing EDTA as anticoagulant. After every single treatment, mice in both categories (bromadiolone and flocoumafen) and control group were sacrificed, after which their kidneys

and livers were dissected for comparative analysis. Weight of the kidney and liver was determined and compared for mice in both categories along with blood count i.e. RBCs, WBCs (neutrophils, basophils etc.), platelets.

RESULTS

Survival rate against Bromadiolone remained 100% up to 48 hours but declined subsequently with time. The survival rate was 83% at 72 hours, 66% at 96 hours and 50% at 120 hours. After 144 hours all the mice were dead (Table I). Flocoumafen toxicity

Table III. Effects of bromadiolone on complete blood count of *Mus musculus* at different time intervals.

Parameters	Group 1 (control)	Group 2 (6 hours)	Group 3 (12 hours)	Group 4 (24 hours)	Group 5 (48 hours)
WBCs ($\times 10^3/\mu\text{L}$)	10.26 $\pm$ 0.15 ^D	10.4 $\pm$ 0.1 ^D	13.23 $\pm$ 0.68 ^C	16.13 $\pm$ 0.41 ^B	21.80 $\pm$ 0.69 ^A
RBCs ($10^{12}/\text{L}$)	7.96 $\pm$ 0.15 ^A	8.06 $\pm$ 0.05 ^A	7.86 $\pm$ 0.22 ^A	7.80 $\pm$ 0.26 ^A	7.50 $\pm$ 0.86 ^A
Hgb (g/dL)	14.6 $\pm$ 0.55 ^A	14.2 $\pm$ 0.32 ^{AB}	14.16 $\pm$ 0.05 ^{ABC}	13.53 $\pm$ 0.47 ^{BC}	13.26 $\pm$ 0.75 ^C
HCT (Vol%)	38.1 $\pm$ 0.15 ^A	37.2 $\pm$ 0.26 ^A	36.06 $\pm$ 3.87 ^{AB}	31.8 $\pm$ 0.15 ^{BC}	33.46 $\pm$ 0.89 ^C
MCV (fl)	49.3 $\pm$ 0.55 ^A	45.4 $\pm$ 0.53 ^B	46.13 $\pm$ 0.41 ^B	45.4 $\pm$ 0.53 ^B	37.1 $\pm$ 1.22 ^C
MCHC (g/dL)	38.2 $\pm$ 0.17 ^B	40.5 $\pm$ 0.50 ^A	38.36 $\pm$ 0.40 ^B	36.7 $\pm$ 0.64 ^C	35.84 $\pm$ 0.20 ^D
MCH (pg)	17.51 $\pm$ 0.5 ^C	18.27 $\pm$ 0.11 ^B	19.3 $\pm$ 0.32 ^A	19.16 $\pm$ 0.15 ^A	18.53 $\pm$ 0.40 ^B
Neutrophils (%)	20 $\pm$ 2.0 ^A	21.26 $\pm$ 1.1 ^B	21.8 $\pm$ 1.04 ^C	22.16 $\pm$ 1.04 ^C	22.06 $\pm$ 0.60 ^C
Lymphocytes (%)	55 $\pm$ 5.0 ^C	68.6 $\pm$ 3.51 ^B	68.53 $\pm$ 0.50 ^B	71.40 $\pm$ 1.21 ^B	76.8 $\pm$ 0.91 ^A
Monocytes (%)	2.6 $\pm$ 1.52 ^B	03 $\pm$ 1.0 ^B	3.66 $\pm$ 0.57 ^{AB}	4.16 $\pm$ 0.15 ^{AB}	5.2 $\pm$ 1.31 ^A
Eosinophils (%)	2.6 $\pm$ 2.08 ^A	4.3 $\pm$ 0.57 ^A	4.16 $\pm$ 0.29 ^A	5.33 $\pm$ 0.57 ^A	04 $\pm$ 2 ^A
PLT (/cmm)	384.3 $\pm$ 51.0 ^A	216 $\pm$ 76.5 ^{AB}	317 $\pm$ 55.0 ^{AB}	192.66 $\pm$ 51.5 ^B	194 $\pm$ 59.22 ^B

Table IV. Effects of flocoumafen on complete blood count of *Mus musculus* at different time intervals.

Parameters	Group 1 (control)	Group 2 (6 hours)	Group 3 (12 hours)	Group 4 (24 hours)	Group 5 (48 hours)
WBCs ($\times 10^3/\mu\text{L}$)	10.26 $\pm$ 0.15 ^A	8.43 $\pm$ 0.4 ^B	8.83 $\pm$ 0.28 ^B	10.4 $\pm$ 0.50 ^A	10.80 $\pm$ 0.26 ^A
RBCs ($10^{12}/\text{L}$)	7.96 $\pm$ 0.15 ^A	7.65 $\pm$ 0.56 ^A	6 $\pm$ 0.21 ^B	5.80 $\pm$ 0.26 ^B	4.76 $\pm$ 0.25 ^C
Hgb (g/dL)	14.6 $\pm$ 0.55 ^A	14.46 $\pm$ 0.41 ^A	11.4 $\pm$ 0.50 ^B	11.63 $\pm$ 0.55 ^B	11.96 $\pm$ 1.0 ^B
HCT (Vol%)	38.1 $\pm$ 0.15 ^A	33.3 $\pm$ 4.16 ^{AB}	36.06 $\pm$ 3.87 ^A	28.3 $\pm$ 0.36 ^C	29.76 $\pm$ 0.25 ^{BC}
MCV (fl)	49.3 $\pm$ 0.55 ^B	47.63 $\pm$ 0.47 ^C	47 $\pm$ 0.52 ^D	51.5 $\pm$ 0.51 ^A	51 $\pm$ 1.76 ^{AB}
MCHC (g/dL)	38.2 $\pm$ 0.17 ^B	38.5 $\pm$ 0.5 ^B	37.9 $\pm$ 0.51 ^A	37.5 $\pm$ 0.45 ^A	37.1 $\pm$ 1.10 ^A
MCH (pg)	17.51 $\pm$ 0.5 ^C	18.1 $\pm$ 0.17 ^C	19.56 $\pm$ 0.51 ^B	21.46 $\pm$ 0.50 ^A	21.2 $\pm$ 0.8 ^A
Neutrophils (%)	22 $\pm$ 2.0 ^A	22.23 $\pm$ 0.25 ^B	22.2 $\pm$ 2.17 ^B	22.5 $\pm$ 0.5 ^B	24.06 $\pm$ 0.60 ^B
Lymphocytes (%)	55 $\pm$ 5.0 ^C	72 $\pm$ 2.0 ^B	77 $\pm$ 3 ^{AB}	77 $\pm$ 3.0 ^{AB}	82.33 $\pm$ 3.21 ^A
Monocytes (%)	2.6 $\pm$ 1.52 ^A	3.67 $\pm$ 0.57 ^{AB}	04 $\pm$ 2.64 ^{AB}	4.66 $\pm$ 0.57 ^B	6.67 $\pm$ 2.51 ^B
Eosinophils (%)	2.6 $\pm$ 2.08 ^B	05 $\pm$ 1.0 ^{AB}	5.67 $\pm$ 1.52 ^A	5.33 $\pm$ 1.15 ^{AB}	4.3 $\pm$ 1.52 ^{AB}
PLT (/cmm)	384.3 $\pm$ 51.0 ^A	404 $\pm$ 177.07 ^A	353 $\pm$ 42.02 ^A	274 $\pm$ 75.0 ^A	253 $\pm$ 40.70 ^A

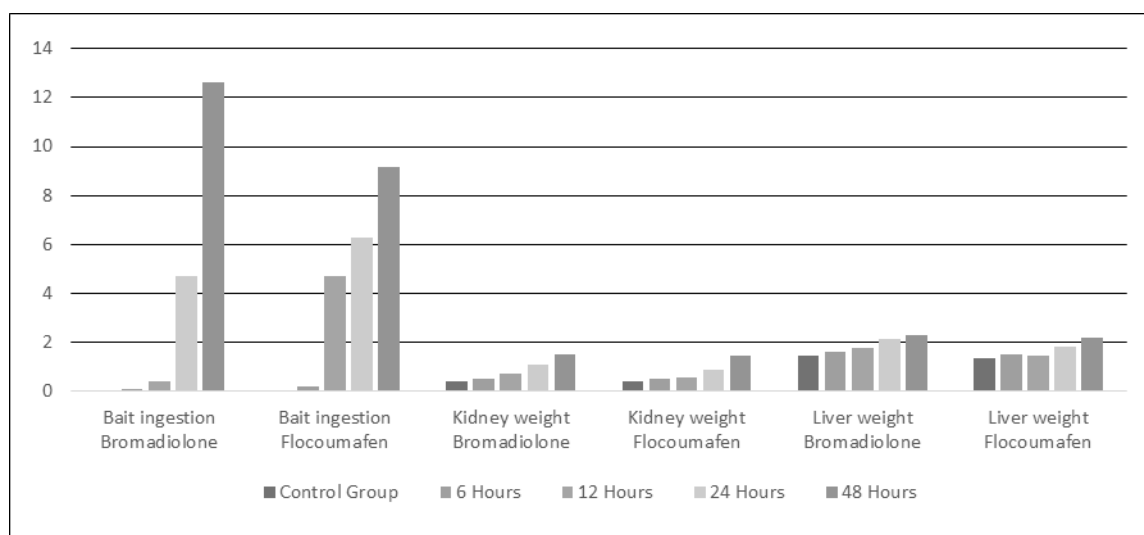


Fig. 1. Comparative action of bromadiolone and flocoumafen on *Mus musculus* bait ingestion, kidney weight and liver weight.

demonstrated a 100% survival rate till 72 hours and then gradually declined, falling to 0% after 144 hours (Table II). The blood count of mice in both categories was then compared. Mice which ingested bromadiolone bait showed significance variation for different parameters in blood of different groups (Table III). WBC increased from 10.26 ± 0.15 to 21.80 ± 0.69 from group 1 to group 6, respectively, whereas RBCs decreased from 7.96 ± 0.15 to 7.50 ± 0.86 from group 1 to group 6, respectively. While all other parameter, such as Hgb (14.6 ± 0.55 to 13.26 ± 0.75), HCT (38.1 ± 0.15 to 33.46 ± 0.89), MCHC 38.2 ± 0.17 to 35.84 ± 0.20) decreased with time, neutrophils (20 ± 2.0 to 22.06 ± 0.60), monocytes (2.6 ± 1.52 to 5.2 ± 1.31) lymphocytes (55 ± 5.0 to 76.8 ± 0.91) and eosinophils (2.6 ± 2.08 to 4.0 ± 2) showed increase in number. Platelets decreased drastically.

Mice treated with Flocoumafen bait showed significant variation in different parameters of different groups (Table IV). Similar trends of blood cell counts were observed in the case of bromadiolone. There was an increase in WBC, and a decrease in RBCs and platelets after bromadiolone ingestion in mice.

Bait consumption was more for Flocoumafen compared to bromadiolone. Decreasing trend in liver

and kidney weight was observed in both groups with the passage of time compared to the control group. Moreover, Flocoumafen showed more of a decrease in organ weight compared to bromadiolone (Fig. 1).

DISCUSSION

The main component of bromadiolone is dicoumarol. Dicoumarol delays prothrombin formation and thus anti-coagulates the blood. Delay in clotting has also been observed in a 40-year-old female who accidentally ingested bromadiolone (8). Bromadiolone is also involved in the degeneration of liver and kidney, resulting in the delay of excretion and reduction in platelet count. The dicoumarol acts as an inhibitor to block the vitamin K cycle (3).

In this study, baiting with bromadiolone induced mortality after 48 hours whereas in the cases of flocoumafen, mortality began after 72 hours of baiting. Similar results have been reported by Bhattacharyya and Borah (2), who studied efficacy of four SGARs as bromadiolone, brodifacoum, difenacoum and Flocoumafena on *Mus musculus*. They found bromadiolone more efficient in action than flocoumafen; therefore, our results are in agreement with theirs.

Based on oral toxicity test results, it is concluded

that bromadiolone is more efficient than flocoumafen. Bromadiolone degrades cofactors required for blood clotting more efficiently than flocoumafen. It can be assumed that due to availability of antidote and minimal non-target attractions, second generation rodenticides can be considered more useful in homes, offices, educational institutes, shops and agricultural fields.

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LETTER TO THE EDITOR

RARE COMBINATION OF INFECTIVE ENDOCARDITIS IN A DRUG ABUSER WITH HENOCH-SCHOENLEIN PURPURA

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To the Editor,

The course of infective endocarditis (IE) is frequently complicated by heart failure (HF), uncontrolled infection, embolic events, infectious aneurysms, acute renal failure, rheumatic complications, splenic abscess, myocarditis, pericarditis, etc (1). Henoch-Schonlein purpura (HSP) is very rare in IE. The aim of the paper is to present a case with such a combination and to suggest possible practical implications.

Case report

A twenty-nine-year-old female with a history of drug abuse (heroin, methadone) was admitted because of dyspnea, fever, cough and arthralgia, to the Department of Pulmonology. Her prior medical history includes Hepatitis C. The chest X-ray showed diminished transparency in medium and lower lung fields, abscess apically and shadows in costophrenic sinuses, as well as very enlarged cardiac silhouette. A multi-slice computer tomography revealed pneumonia with multiple abscesses as well as both pleural and pericardial effusions, without signs of pulmonary embolism. Her laboratory values were

as follows: WBC $9.6 \times 10^9/L$ (normal range, NR 4.0-9.0); RBC $2.5 \times 10^{12}/L$ (NR 3.80-5.30); Hgb 99g/L (NR 110-170); Hct 31% (NR 36-56); Plt $274 \times 10^9/L$ (NR 120-380); Creatinine 103.2 μ mol/L (NR 53.0-115.0); BUN 12mmol/L (NR 2.5-7.5); Glycemia 7.9mmol/L (NR 3.9-6.1); AST 51U/L (NR 10-31); ALT 32.8U/L (NR 10-32); LDH 589.4U/L (NR 220-450); CRP 108mg/L (NR 0.0-5.0); Proteins 56.4g/L (NR 62.0-81.0) Albumins 23.5g/L (NR 35-52); Procalcitonine (PCT) 118ng/mL (NR 0.0-0.05); D-dimer 1761 μ g/L (NR <550); Brain natriuretic peptide (BNP) 4432pg/mL (NR 0.0-100.0).

Klebsiella was identified in sputum, Acinetobacter and Klebsiella in repeated blood cultures and Enterobacter in urine cultures. Blood gases showed: pH 7.18; PaCO₂ 18mmHg; HCO₃ 6.7mmol/L; BE -19.8mmol/L; PaO₂ 100mmHg; SpO₂ 96%. She was treated with antibiotics according to antibiogram (including: meropenem, vancomycine, levofloxacin, metronidazole).

Her clinical course was complicated by an acute kidney injury (BUN concentration up to 24.3mmol/L, Creatinine up to 504.4 μ mol/L, acidosis). Meanwhile, the echocardiogram showed verrucae on the tricuspid

Key words: infective endocarditis; Henoch-Schonlein purpura; rare combination

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valve (dimension 14.9mm x 5.3mm) accompanied by severe tricuspid regurgitations (right ventricular systolic pressure 46 mmHg). The left ventricle (LV) was slightly enlarged with preserved ejection fraction (58%); moderate to severe aortic and mitral regurgitation were also present. Pericarditis was confirmed (maximum 13 mm in diastole).

IE was diagnosed according to Duke's criteria: two major (verrucae confirmed with the echocardiography and positive blood cultures) and two minor criteria (intravenous drug abuse, fever). The patient was transferred to the Cardiology Department and continued to receive antibiotics as suggested by antibiogram (including: meropenem, vancomycin, levofloxacin, metronidazole, doxycycline and cilastatin) together with fluconazole, eubiotic and corticosteroids.

Solid LV contractility allowed an intensive volume load and the renal function improved (BUN 10.0mmol/L; Creatinine decreased from 504.4 μ mol/L to 93.7 μ mol/L at discharge). During the further clinical course, the size of verrucae on the tricuspid valve had diminished (dimension 11mm x 7mm) (Fig. 1).

On the 13th day purpuric lesions became obvious on her hands, trunk and legs (Fig. 2). The clinical picture, confirmed by histopathology, and presence of symptoms of arthritis and kidney disease, confirmed the diagnosis of HSP.

The patient was discharged without visible verrucae both by echocardiography and MRI, with mild tricuspid and mitral regurgitation, without aortic insufficiency, and with discrete pericardial effusion. Blood cultures were negative. Laboratory values at discharge were: Troponin 13ng/L (NR 0.0-40ng/L), BNP 82.2pg/mL (reduced from 4432pg/mL), CRP 0.9mg/L, PCT 0.19ng/mL (diminished from 118ng/mL).

DISCUSSION

We have presented a patient with severe dominantly right-sided IE, typical for intravenous drug abusers, complicated by heart and renal failure. Underlying causative agents *Klebsiella* and *Acinetobacter* belong to the Gram-negative group of non-HACEK (*Haemophilus* species, *Actinobacillus*, *Cardiobacterium*, *Eikenella* and *Kingella* species) microorganisms that are less common causes of IE,

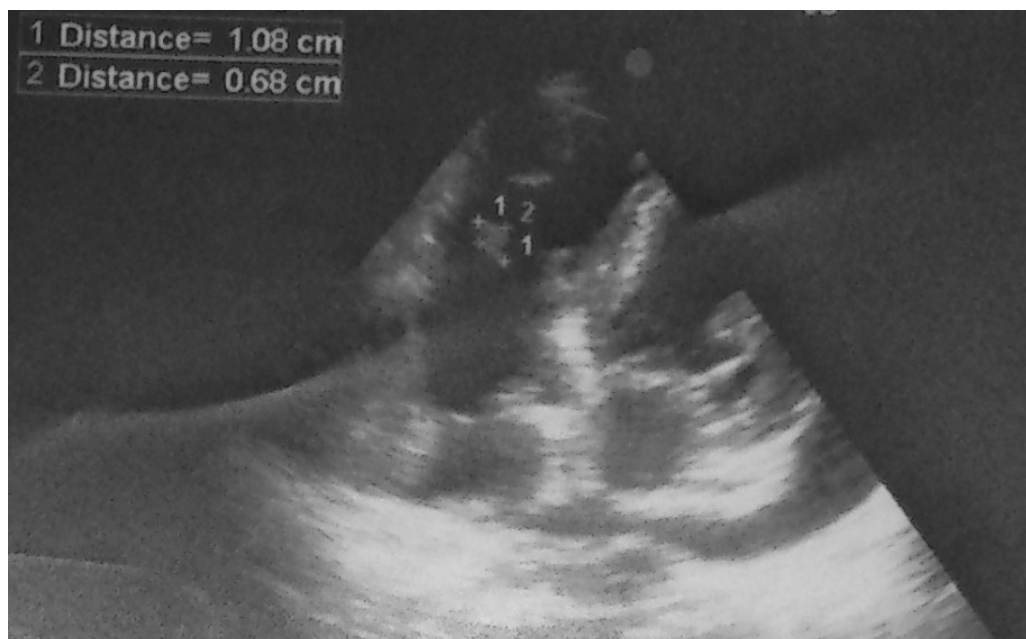


Fig. 1. Verrucae on the tricuspid valve (dimension 11mm x 7mm), accompanied by severe tricuspid regurgitation.

even in drug abusers (2). An uncommon feature in IE in our patient was the clinical finding of vasculitis. The distribution of the rash with typical palpable purpura in this case, was similar to that seen in classic HSP. It is primarily a childhood disease that is typically self-limited, but 10% of the cases occur in adults, when features and outcomes may vary. HSP is usually a hypersensitive reaction to bacteria (streptococcae or mycoplasma pneumoniae) or viruses, but some drugs may also act as antigens. HSP is usually associated with arthritis, nephritis, gastrointestinal disorders, and sometimes with lung hemorrhage, and respiratory tract infection may precede its onset. Purpuric lesions usually appear on the legs and buttocks, but may also be seen on the arms, face and trunk, as in the case of our patient.

The association of IE and HSP has been reported only in a few patients in the medical literature (3-6). None of them was an intravenous drug abuser, nor had other relevant microbiological features and comorbidities, which makes our case unique. The literature search revealed only one case report of cutaneous rash due to leukocytoclastic vasculitis associated with pneumonia caused by *Klebsiella pneumoniae* (7) and no similar

cases of vasculitis associated with *Acinetobacter* or *Enterobacter* infection.

IE patients frequently exhibit positive ANCA tests (Antineutrophil cytoplasmic antibodies directed against proteinase-3 ANCA-PR3 or myeloperoxidase-ANCA-MPO) and mimic ANCA-associated vasculitis (8). In a recent study, 18% of patients with IE had cytoplasmic ANCAs and/or perinuclear ANCAs and 8% had PR3-ANCAs or MPO-ANCAs, some with very high titers (8). There is evidence of their association with prolonged bacterial or Hepatitis C virus (HCV) infection and heroin addiction in patients with IE. Cutaneous and renal blood vessel vasculitis in ANCA-positive patients with IE is frequent but not consistent. In patients with persistently normal levels of alanil transferase and serological evidence of HCV infection, both polyclonal IgG and either polyclonal or monoclonal IgM rheumatoid factor (RF) (mixed cryoglobulinemia), could be found. HCV exerts a chronic stimulus on the immune system, which may lead to the proliferation of B cell clones producing IgM with RF activity. In 10-42% of the patients, cryoglobulins were associated with a systemic vasculitic disorder of the small and, less frequently, medium-sized arteries and veins. It is



Fig. 2. Typical palpable purpuric lesions on buttocks and dorsal side of hands.

characterized by the deposition of immune complexes containing the RF, IgG, HCV RNA, and complement on endothelial surfaces, eliciting vascular inflammation with clinical signs as palpable purpura similar to HSP, renal involvement, arthropathy, peripheral neuropathy or Raynaud phenomenon.

Renal lesions in IE can range from microscopic hematuria to glomerulonephritis and acute kidney injury. Acute renal failure occurs in approximately one-third of IE patients and predicts a poor outcome. Causes are numerous: immune complex and vasculitic glomerulonephritis, renal infarction, hemodynamic impairment due to HF, severe sepsis, or after cardiac surgery, antibiotic toxicity (acute interstitial nephritis), notably related to aminoglycosides, vancomycin and even high dosage of penicillin, nephrotoxicity of contrast agents used for imaging (1).

In the presented case, renal insufficiency was probably the result of a complex interplay of infection, IE itself, hemodynamic compromise in HF, toxicity of antibiotics and immunologic changes in kidney vessel walls induced by immune dysregulation due to HCV-induced cryoglobulinemia, ANCA vasculitis, and heroin addiction (9). In our case, the aggressive steroid therapy led to complete renal recovery and disappearance of cutaneous purpuric lesions, which is a regular treatment of purpura.

We report an exceptionally rare case of IE with HSP. This association represents an additional threat to the renal function and warrants its close follow-up, together with the minimization of drugs that are potentially nephrotoxic. Likewise, the onset of HSP may be an alarming sign for searching for possible causes and comorbidities other than those usually expected.

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LETTER TO THE EDITOR

A RARE CASE OF RING CHROMOSOME 3 SYNDROME

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To the Editor,

Ring chromosome 3 syndrome is a rare cytogenetic malformation with clinical heterogeneity. Only 13 such patients have been reported to date. Mukerjee and Burdette reported ring chromosome 3 for the first time in 1966. Its clinical features in patients vary, mainly manifested as growth retardation (including prenatal and postnatal retardation), mental retardation, mental-motor developmental retardation, microcephaly, and particular facial features (including micrognathia, ptosis, a sagging mouth, and a widened root of the nose) (1).

MATERIALS AND METHODS

This study reports a rare case of ring chromosome 3 syndrome. A written informed consent was obtained from the parents of the patient to publish this case report.

High-precision exon PLUS detection was performed on the child and her parents. Firstly, 2-3 ml of peripheral venous blood was collected from the proband and her parents with EDTA anticoagulant tubes, and then genomic DNA was prepared according to the operational requirements of the "Salt Extraction Blood DNA Extraction Kit". One µg of genomic DNA was obtained and broken with a Q800R ultrasonic shredder. The peak value of electrophoresis was approximately 350 bp, then end-to-end repair was

performed; the 3' end was added with A and both ends were added by connectors with a specific sequence. Different samples were connected by barcodes with different sequences and the library with a connector was amplified by polymerase chain reaction (PCR). Qubit was performed to determine the concentration of the library. Three hundred ng was taken from each sample to mix and eight samples served as a mixed unit. The mixed unit sample was added with one human exon libraries for hybridization capture, the product was captured and purified, then amplified by post-PCR. The size of the library was determined by agarose gel electrophoresis. The concentration of the library was determined by Qubit3.0 and fluorescence quantitative PCR. After the quality test, the library was diluted to the sequencing concentration of the machine.

Sequencing analysis was performed using the Illumina Nextseq500 sequencing platform. The average coverage depth of the patient was 245±136X, coverage regions of > 10X accounted for 99.8%, and coverage regions of > 20X accounted for 99.5%. The sequencing results were analyzed by bioinformatics methods.

G banding (320-400 bands): counting analysis: the number of medium-term cells was 30.

RESULTS

The patient was a girl, 11 years and 3 months

Key words: chromosome 3; chromosome microarray; ring chromosome

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old, who was admitted to our hospital due to “slow growth and development for more than 10 years”. The annual growth rate (AGR) was unknown. Admission examination results showed that the patient’s height was 120.5 cm ≤ 3 SD, her stature was well proportioned, and her intelligence was normal. The child usually had a poor appetite and had less food intake than normal. The child had no headache, vomiting, convulsion, and ambiguity of vision and personality changes. The patient was the parent’s first child and she was delivered through spontaneous labor at full-term and the birth weight was 2 kg. The child had no suffocation rescue history. The anterior fontanelle was closed after birth. The child was fed artificially and complimentary food was given on time. Furthermore, the child’s exercise, language, and mental developments were behind those of

children at the same age. The child was in the fourth grade of elementary school and had poor study achievements. The parents of the child did not have consanguineous mating and had no family history of genetic diseases. Admission examination results: the patient’s height was 120.5 cm and weight was 15 kg; her head circumference was 44 cm and she had microcephaly, a wide rooted nose, and a sagging mouth. A brown, bean-sized excrescence could be observed behind the right ear. Bilateral breasts were at stage B1. No abnormalities were found in the child’s cardiopulmonary auscultation and no abnormalities were found in the external genitalia. The vulva was at stage T1 and the pubic hair was at stage P1.

Growth hormone (GH) provocation test: GH peak was < 5.99 ng/ml. Pituitary magnetic resonance imaging (MRI) revealed that the pituitary was small in volume, no obvious abnormalities were found in the morphology and signal. Abdominal color Doppler ultrasound revealed gallbladder stones and no abnormalities were found in the liver, spleen, and pancreas. Initial gonad B ultrasound revealed that no obvious uterine and ovarian echoes were detected in the pelvic cavity. At the late follow-ups, gonad B ultrasound revealed that the child’s ovarian was small in volume.

Chromosome 3 in the chromosomes of the peripheral blood was ring-shaped. Karyotype: 46, XX, r(3) (p26; q29) [30] (Fig. 1, Table I).

No pathogenic point mutation was found in the coding region of the relevant genes. However, by analyzing copy number and related SNPs, it was revealed that there was at least 2 Mb of copies of the heterozygous deletion variants (x1) in the 3p26 region and at least 911.2 kb of copies of the repeat variants (x3) in the 3q29 region (Fig. 2).

DISCUSSION

A ring chromosome is a rare chromosomal structural abnormality (2-5). Since the first case was discovered in 1996, only 13 patients with ring chromosome 3 syndrome have been reported in previous literature (6-8). Moreover, in previous patients, other gene deletions involving

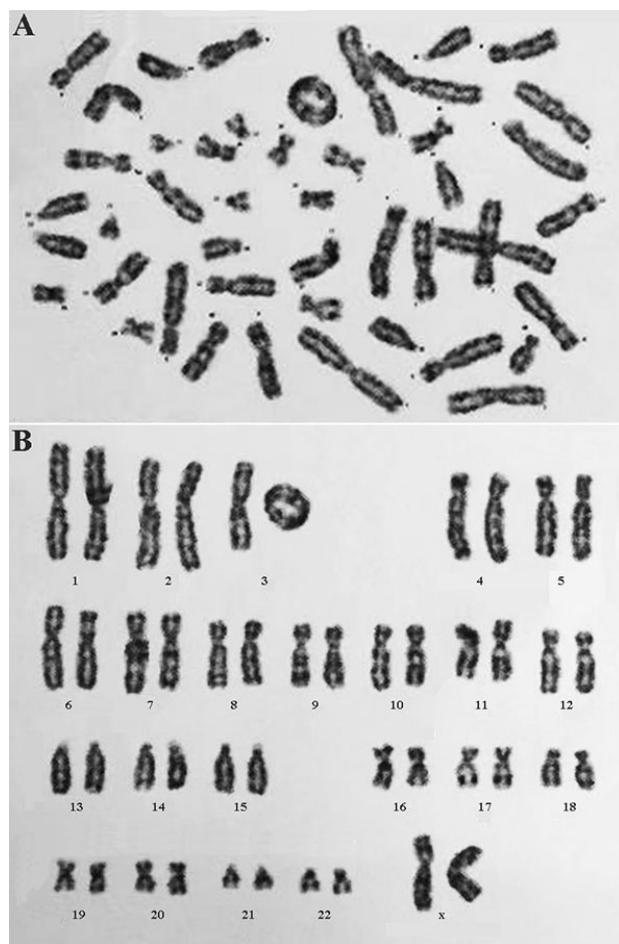
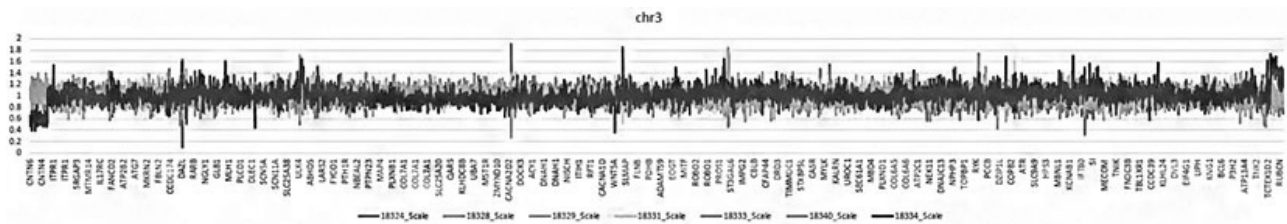


Fig. 1. Analysis of peripheral blood chromosome. A) Original image. B) Karyotype image.

Table I. Gene deletion fragment

Gene	OMIM	Hereditary mode	HG19 location	Transcript	Nucleotide and amino acid changes	Heterozygous state	Population frequency	ACMGvariation classification	Related diseases/literature	ORIGIN
3p26	-	-	chr3:1189693-3221371	-	>2.0Mb deletion	×1	-	Class 1 - pathogenic mutation	Circular chromosome 3 syndrome	New
3q29	-	-	Chr3:19677149-4-197682644	-	>911.2Kb repetition	×3	-	Class 1 - pathogenic mutation	Circular chromosome 3 syndrome	New

**Fig. 2.** High precision exon PLUS detection report.

neurodevelopment and motor development retardation were also reported such as *CHLI*, *CNTN6*, *CNTN4*, *ITPR1*, *GRM7*, and *BHLHE40* (9-12). Previous patients were only detected by cytogenetic techniques, but there was no exact information about the breakpoints and related genes. However, in our study, the results revealed that the breakpoints on chromosome 3 of the patient were located at q29 and p26 and the two broken ends with centromeres were recombined, forming the ring chromosome 3. There was at least 2 Mb of copies of heterozygous deletion variants in chr3: 1189693-3221371 of the 3p26 region, involving the Online Mendelian Inheritance in Man (OMIM) genes such as *TRNT1* and *CRBN*. There were at least 911.2 kb of copies of the repeat variants in chr3: 19677149-197682644 of the 3q29 region, involving OMIM genes such as *RUBCN* and *RPL35A*.

This study reports a rare case of ring chromosome 3 syndrome the major clinical manifestations of which were mental retardation and some special

facial features. To date, patients with the same 3p26 region copy number heterozygous deletion mutation and 3q29 region copy number duplication mutation as this child have not been reported.

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LETTER TO THE EDITOR

THYROID STIMULATING HORMONE RECEPTOR NOVEL MUTATION RESULTS IN FAMILIAL HEREDITARY HEARING LOSSH. WANG¹, L. SUN² and S. ZHOU¹

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Hereditary hearing impairment is the most frequent sensorineural deficit in humans (1). It has been assumed that in developed countries more than 60% of all cases are caused genetically (2). The non-syndromic forms of hearing impairment are usually monogenic. The mode of transmission includes autosomal recessive, autosomal dominant, X-linked and mitochondrial (3). Up to 200 genes are estimated either to be involved in the inner or middle ear development or to play an important role in signal transduction, for example GJB2, GJB6, MYO7A, DIAPH1, PRPS1, POU3F4, MTTS1(4). In the last decades, Next-generation sequencing techniques have significantly increased the possibilities of genetic analysis in complex diseases such as hearing loss (5). Whole exome sequencing has been widely used in research and clinical work because of massive coverage and affordable cost.

Thyroid hormone is critical for auditory development and has well-known actions in the inner ear and middle ear, thus any component included in hypothalamus-pituitary-thyroid axis plays an important role in congenital hearing loss. Thyroid stimulating hormone receptor (TSHR) encodes the protein Thyroid Stimulating Hormone Receptor participates in regulating thyroid hormone secretion and damaging the gene could result in hearing loss,

which has been verified by animal models (6). In the present study, we describe a Chinese family with non-syndromic progressive hereditary hearing impairment showing an autosomal recessive mode of transmission linked to TSHR gene, with a novel mutation, p. Lys565Glu, which might be the cause of the non-syndromic hearing loss (NSHL) phenotype presented by the affected individuals.

MATERIALS AND METHODS*Study subjects*

The proband L01 is a Chinese woman, 23 years old, who had no hearing at birth. Her husband L04 was diagnosed with deafness after administration of over-the-counter antibiotics used to treat infections. Their first daughter L03 was found to have hearing impairment after testing with universal newborn hearing screening. Now she is 2 years old, and has no improvement in hearing. The proband has her second pregnancy at present. These worried parents urgently wished to know whether the fetus is deaf, therefore they came to our institute for genetic counseling with the purpose to decide whether the termination of pregnancy was necessary. The parents signed an informed consent to participate in the genetic study for themselves and their young children. The history-taking included a detailed family history, neurologic and

Key words: familial hereditary hearing loss; novel mutation; whole exome sequencing

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clinical genetic assessment, ultrasonic examination of the thyroid, and semen analysis. Blood samples were taken in ethylenediaminetetraacetic acid (EDTA) anticoagulation vacuum blood collection tube from the index of available family members for DNA extraction and downstream molecular studies. The fetus underwent a Chorionic fine needle puncture test to obtain tissue for the study. The pedigree of the family is described in Fig. 1.

DNA extraction and exome sequencing

Genomic DNA of all participants except the fetus was extracted from whole blood collected in EDTA-K2 tubes with a commercially available DNA isolation kit according to the protocol provided by the manufacturer (QIAGEN, Germany) (7). The fetus chorionic tissue was used for DNA extracting using same technique as mentioned above.

Exome sequencing was performed using Ion Torrent proton machine. Adapter-ligated libraries for the two participants (L01, L03 in the pedigree mentioned above) were built using Ion Ampliseq™ Library Kit 2.0 (Life Technologies, USA) according to the manufacturer's protocol (8). Then, an Ion One Touch 2 machine (Life Technologies, USA) was used to conduct emulsion PCR for barcoded libraries, which yielded a large number of ISPs with clonally amplified DNA fragments. After loading the enriched ISPs in Ion Torrent PI chip, massively parallel semiconductor sequencing was performed on a Ion Proton™ Sequencer (Ion Torrent) using the Ion Proton Sequencing PI Kit (Life Technologies, USA) according to the manufacturer's instructions.

Bioinformatics analysis

All raw signal data were analyzed automatically using the Torrent Suite v 3.6.2 to generate sequence reads, trim adapter sequences, align to the hg19 human reference genome, analyze coverage and call variants. Then, called variants were annotated with Ion Reporter™. Subsequently, we filtered the annotated variants according to the pipeline. These variants were given precedence based on minor allele frequency of <1% in the 1000 Genomes project phase 1 release and the NHLBI Exome Sequencing Project (NHLBI ESP). All synonymous or benign variants were excluded. The final 5 variants were conducted in silicon prediction with online software SIFT and Polyphen. Putative pathogenic

variants were tested in HGMD PRO to see whether they were reported.

Mutation validation

All the sorted out variants were validated by Sanger sequencing performed on ABI 3130XL automated genetic analyzer (Applied Biosystems, Foster City, California, USA). The mutation TSHR p.K565E and COL11A2C.2112_2112delG was further analyzed in 200 normal individuals of the same ethnic background and geographical region by Sanger sequencing using the same method.

RESULTS

Clinical features

The proband L03 was born with blue sclera, which is rarely seen in Chinese. In the subsequent neonatal auditory screening she was found to have a hearing defect. Furthermore, the father L04 and mother L01 in this family were also deaf, the only difference between them being that one was congenital and the other was caused by antibiotics. Their clinical features are shown in Table I.

Sequencing output and coverage

After exome sequencing on the Ion torrent proton, we achieved an average output of 42437417 mapped reads per sample in the NSHL family. Mean depth for the mapped reads is 119. In summary, 14.9G aligned bases were covered at least 4.4 times. The mean uniformity of base coverage is 88.77% in this panel. In summary, we acquired 51436 variants using variants caller plugin, after standard filter we obtained 281 variants shared by all members of the family. Then we searched associated genes in the professional website of deafness. Ultimately, we got five hearing loss-associated genes (COL11A2, TSPEAR, DUOX2, HMX3, TSHR) at 6 loci including 4 INDEL variants, 3 SNVs. Among these variants only TSHR gene was identified and successfully validated by Sanger sequencing from the three samples of the family except the father, who has an INDEL variant in gene COL11A2 (Table II).

Pathogenic mutation identification

By filtering the HGMD database and Clin Var

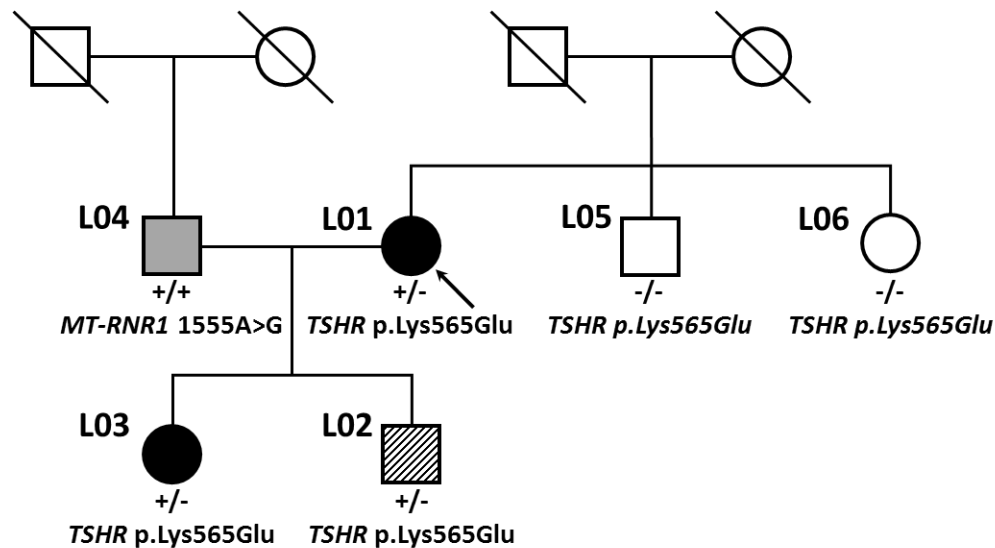


Fig. 1. The pedigree of the hereditary hearing loss family

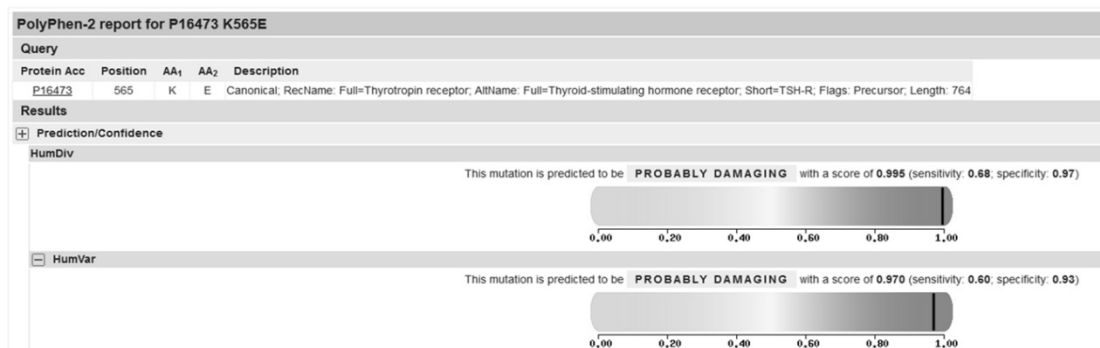


Fig. 2. TSHR variant (*c.1693A>G*, *p.Lys565Glu*) causes a damaging mutation in conserved domain predicted by Polyphen2

database, the identified variant (*c.1693A>G*, *p.Lys565Glu*) in TSHR has not yet been reported. However, online bioinformatics prediction (SIFT AND Polyphen2, see Table II) result implied the TSHR variant is in a conserved domain and disruption of the protein will lead to deleterious consequence (Fig. 4).

Structural modeling

In the PDB database, using the entry “TSHR”, we get the secondary structure feature of the protein. Through the analysis of the three-dimensional model of the patient’s protein, it was found that due to the mutation of amino acids, the energy distribution and torsion Angle of amino acids changed, which

could be an important aspect for protein structure damaging (Fig. 5).

DISCUSSION

Hearing loss can occur at any stage of life. Of these, approximately 1/1000~1/800 newborns are born with severe and extreme hearing defects, most of which are diagnosed as unexplained. With the rapid development of sequencing technology, it is possible to study the molecular mechanism of congenital deafness. This study provides a method for clinicians to effectively map the causes of hearing loss with unknown reasons. Furthermore, we found a novel mutation in reported genes which can build

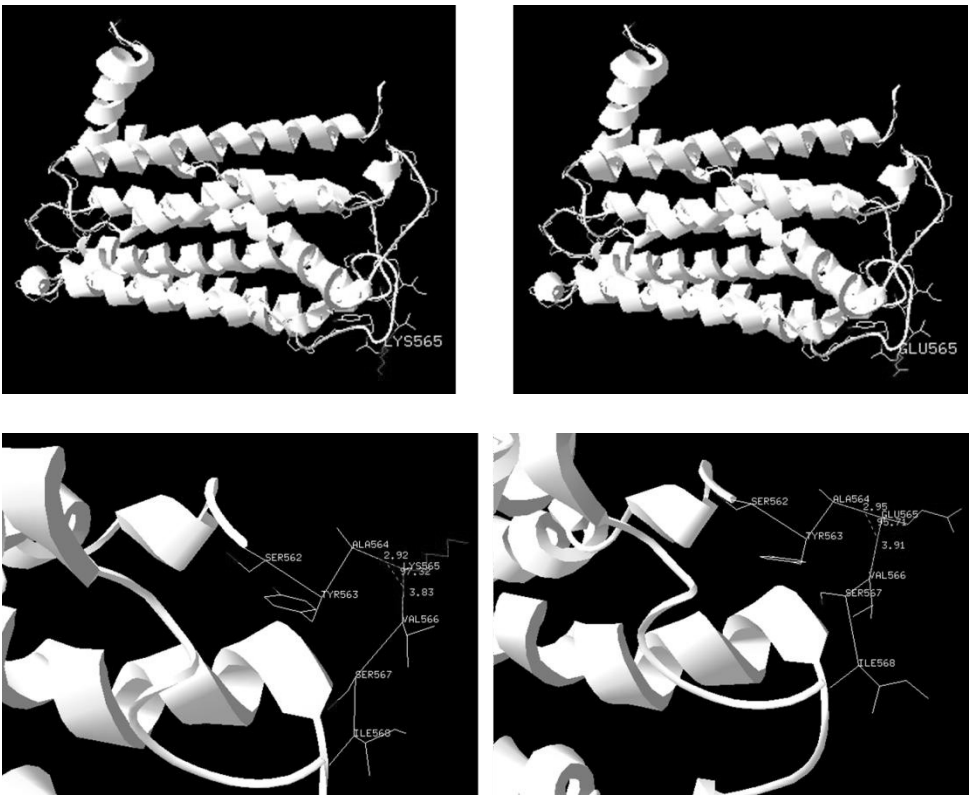


Fig. 3. *TSHR* variant (c.1693A>G, p.Lys565Glu) located in the extracellular domain of the receptor caused the changing in amino acid energy distribution and torsion angle

Table I. *Clinical features of the family members*

Clinical features	Father (L04)	Mother (L01)	Daughter (L03)
Hearing loss	+	+	+
Eye features	-	Blue iris	Blue iris
Thyroid image	NA	NORMAL	NORMAL
Thyroid hormone	NORMAL	NORMAL	NORMAL
TSH	1.46	3.59	4.98
FT3	5.98	4.49	6.75
FRT4	12.85	8.63	14.12
Skin freckles	-	+	-

Table II. Description of the mutation found by Exome sequence and confirmed by Sanger sequencing

Family member ID	gene	cDNA change	Amino acid change	Polyphen-2	SIFT	Affected protein domain	Reference
L04	COL11A2						
L01	TSHR	c.1693A>G	p.Lys565Glu	0.995	0.24	7 transmembrane receptor (rhodopsin family)	Novel
L03	TSHR	c.1693A>G	p.Lys565Glu	0.995	0.24	7 transmembrane receptor (rhodopsin family)	Novel
L02	TSHR	c.1693A>G	p.Lys565Glu	0.995	0.24	7 transmembrane receptor (rhodopsin family)	Novel

the bridge between the phenotype and the genotypes.

Though there was mice model for the TSHR mutation, we did not conduct any functional research for the mutations reported in this article. We could not test the mutated protein at mRNA and protein level for the reason that it is difficult to get the thyroid tissue for next step research. Despite the TSHR mutation being well co-segregated in the reported family, we did not conduct further research on other congenital hearing loss cohort or families. That is, we did not validate this mutation in another unrelated individual.

In conclusion, we have identified a family in which heterozygous mutations in TSHR co-segregated with congenital hearing loss phenotype. This study highlights the influence of thyroid stimulating hormones on bone development in the inner and middle ear, which is a main cause of congenital deafness. We conducted a prenatal genetic diagnosis for the fetus and carried out intervention measures according to the results. Next-generation sequencing offers highly promising techniques for molecular diagnosis, especially in small families with a high probability of disability disorders such as deafness.

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LETTER TO THE EDITOR

ROLE OF PD-L1 IN RADIO-SENSITIVITY OF ESOPHAGEAL SQUAMOUS CELL CARCINOMA

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To the Editor,

In recent years, tumor immunity has become a hotspot of research. Immune escape may be essential for tumor development. It was found that T-cell immunosuppression plays an important role in the recurrence and metastasis of esophageal cancer (1, 2). The co-stimulatory signals of the immune system are divided into positive and negative co-stimulatory signals, which are vital for antigen specific activation of T cells. In addition, the survival and apoptosis of T cells are also affected by positive co-stimulatory signals (3). Programmed death-ligand 1 (PD-L1) is highly expressed in many tumors when negative co-stimulatory signals are produced (4). Moreover, studies have shown that tumor cells can inhibit the immune killing function of T cells by over-expressing PD-L1, thereby leading to immune escape of cancer cells (5, 6). This study investigated the potential of PD-L1 as an independent predictor of ESCC prognosis, so as to provide some theoretical guidance for the clinical treatment.

MATERIALS AND METHODS

Subjects

This study enrolled a total of 369 ESCC patients

whose diagnosis was confirmed by postoperative pathology in Affiliated Hongqi Hospital of Mudanjiang Medical University between January 2013 and July 2018. The clinicopathological data of all patients were collected. After excluding some patients based on the inclusion and exclusion criteria of this study, 198 patients with R0 resected thoracic ESCC were followed-up until September, 2019. Overall, complete follow-up data were obtained from 104 patients. Using an online random number generator, 50 subjects were randomly selected from the 104 patients and included for the final analysis. Subsequently, paraffin-embedded tissue samples were collected from these patients, and the tissue samples from 10 randomly selected patients were used as controls. Prior to this study, informed consent was obtained from all patients or their legal guardians and attached to their medical record. After review, the protocol was approved by the medical ethics committee of the Affiliated Hongqi Hospital of Mudanjiang Medical University.

Inclusion criteria: ESCC patients not previously treated by chemotherapy, anti-tumor therapy or immunotherapy prior to surgery; diagnosis of ESCC confirmed by pre-operative gastroscopic biopsy; no obvious signs of local invasion or distant metastasis; patients with clinical stage I-III ESCC (According to the UICC/AJCC2009 7th edition, supra-clavicular, para-aortic lymph nodes,

Key words: esophageal squamous cell carcinoma; PD-L1; radio sensitivity; prognosis

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and lymph nodes around the esophagus were deemed as regional lymph nodes) undergoing R0 radical resection; diagnosis of ESCC further confirmed by postoperative pathological examination.

Exclusion criteria: patients with incomplete clinicopathological or follow-up data; survival of less than three months after surgery; cervical esophageal cancer or abdominal esophageal cancer; other types of primary cancer or history of uncured malignant tumors; diagnosis of non-squamous cell carcinoma or other types of squamous cell carcinoma by postoperative pathological examination; presence of distant metastasis.

Clinicopathological information of patients: Clinical data, such as gender, age, tumor location, tumor size, depth of invasion, pathological stage, presence of lymph node metastasis, and 5-year survival, were collected from all patients. The expression of PD-L1 in tumor tissues and paracancerous tissues was detected by immunohistochemistry assays. Furthermore, the relationship between the expression of PD-L1 and the above clinicopathological features was analyzed.

Immunohistochemistry

First, paraffin embedded tissue blocks were frozen at -20°C (Haier, China) and then sliced to 3 μm thick tissue sections by a slicer (Leica, Germany). Subsequently, the tissue sections were mounted onto adhesive slides, labeled, dried and then placed in a 70°C incubator (Beijing Fuyilian Medical Equipment Co., Ltd., China) for 2 h. After treatment with xylene (Thermo Fisher, USA) (2 times, 10 min each) $\rightarrow$ 100% alcohol (2 times, 3 min each) $\rightarrow$ 95% alcohol, 80% alcohol, and 70% alcohol (2 min each), the sections were rinsed with running tap water for 6 min. Subsequently, the sections were incubated at room temperature for 2.5 h with primary antibodies. Following 3 min $\times$ 3 times of PBS washing, the sections were incubated for 20 min at room temperature with a polymer adjuvant working solution. After another 3 min $\times$ 3 times of PBS washing, the sections were incubated for 30 min at room temperature with horseradish peroxidase-labeled goat anti-rabbit/mouse IgG secondary antibodies (Wuhan Dr. Bioengineering Co., Ltd., China). In this immunochemistry assay, PBS was used as the negative control.

The overall staining intensity of each slide and its proportion of positive cells were scored: The staining

intensity was scored as follows: 0 (colorless), 1 (light yellow), 2 (brown yellow), and 3 (tan); the proportion of positive cells was scored as follows: 0 ($<5\%$), 1 (5-25%), 2 (26-50%), and 3 ($>50\%$). The entire sliced tissues were observed under a low power microscope and three regions with high PD-L1 expressions were selected. The number of positive cells in these selected regions was counted under a high magnification lens (400x magnification). The final result of each slide was an average of two observations. A score of 0 to 1 was judged as negative, while a score of more than 1 was judged as positive. Sections confirmed with positive PD-L1 expressions were used as positive controls.

Statistics and data analysis

Statistical data analysis was performed using SPSS version 17.0 statistical software. The continuous measurement data were expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$). The count data were expressed in rate (%). The *t*-test, χ^2 test, and Fisher exact probability method were used to compare the categorical variables among different groups. Survival rates were calculated using the Kaplan-Meier method to plot the survival curves. The log-rank method was used to compare the rate of survival among different groups. Multivariate analysis of prognosis was performed using a Cox regression model. The test level was set as $\alpha=0.05$, and $p<0.05$ was considered statistically significant.

RESULTS

Comparative analysis of PD-L1 expressions and general clinicopathological data of patients with thoracic ESCC

The age of the 50 thoracic ESCC patients enrolled in this study was 60.19 ± 5.732 years, and their median age was 60 years. There were 39 males and 11 females, 14 were of Hui nationality and 36 of Han nationality; 25 patients had smoking history, whereas 14 patients had history of alcohol consumption. The average body mass index of all patients was 23.57 ± 2.89 . In addition, 8 patients suffered from underlying diseases, such as hypertension, coronary heart disease, and diabetes. Among different types of surgical anastomosis methods, 24 patients were treated by cervical anastomosis, 20 by thoracic-

apical anastomosis and 6 by sub arch anastomosis. The average size of tumors in these patients was 4.15 ± 1.73 cm. In terms of the degree of tissue differentiation, the tumors in 24 patients were well-differentiated/moderately differentiated, while the tumors in 26 patients were poorly differentiated. Furthermore, 25 patients had T1/T2 depth of tumor infiltration, while 25 patients had T3/T4 depth of tumor infiltration. The number of lymph node dissection was 9.21 ± 5.43 and 25 patients had lymph node metastasis. The postoperative pathological stage was stage I/II in 26 patients and stage III in the other 24 patients; 15 patients received postoperative adjuvant therapy (radiotherapy or chemotherapy). As shown in Table I, the results of statistical analysis showed that the expression level of PD-L1 protein in 50 patients with thoracic ESCC was not significantly correlated with age, gender, tumor location, maximum tumor diameter, tumor length, tumor invasion in adjacent tissues, and clinical stage

and volume of primary tumors ($P > 0.05$). Fig. 1A and B shows the immunohistochemistry results of tumor tissues expressing PD-L1. Fig. 1C and D shows the immunohistochemistry results of PD-L1 protein expressions in ESCC tissues. Fig. 1 C and D show positive and negative expressions of PD-L1 protein in ESCC tissues, respectively. As can be seen from Fig. 1C, The cell membrane and cytoplasm of the tumor tissues were yellowish brown and positive for PD-L1 expressions (Fig. 1). Among adjacent tissue samples collected from 10 ESCC patients, there was 1 case of weak positive expressions of PD-L1. In the negative control, a light yellow or yellowish-brown color was observed, indicating that the sample was free of PD-L1 expressions (Fig. 1D).

Correlation between PD-L1 expressions and the prognosis of patients with thoracic ESCC

Among the 104 patients with complete follow-up data, 50 patients were selected for subsequent analysis.

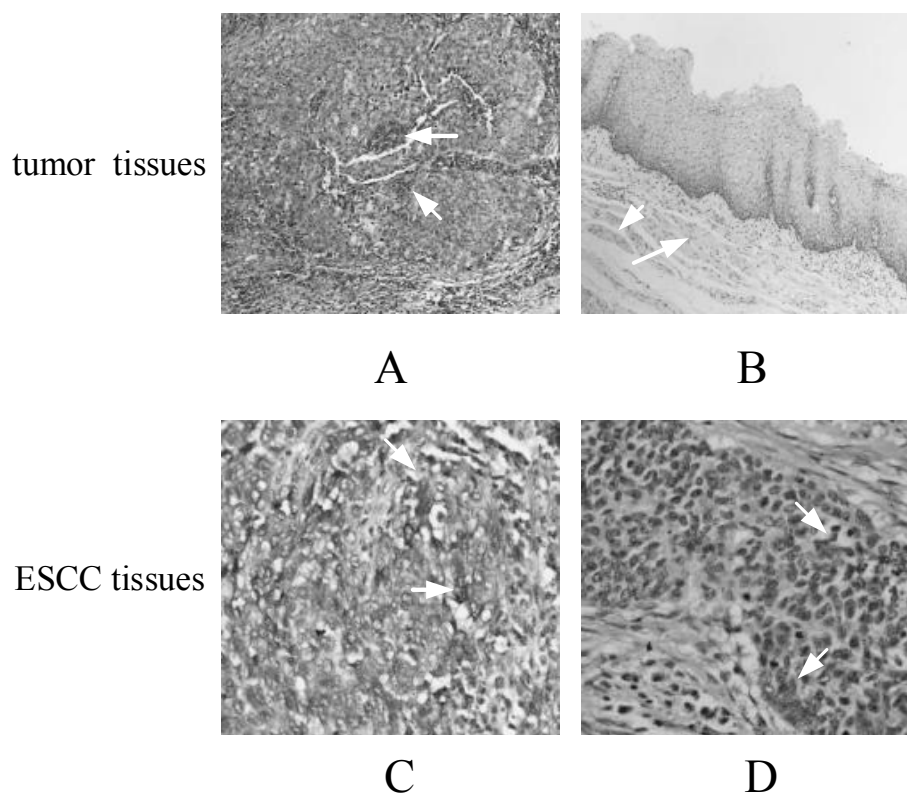


Fig. 1. Immunohistochemistry of tumor tissues and ESCC tissues ($\times 400$). **A)** Cancer tissues; **B)** Paracancerous tissues; **C)** Positive expression of PD-L1; **D)** Negative expression of PD-L1.

Of these 50 patients, 28 showed positive expressions of PD-L1, whereas 22 patients showed negative expressions of PD-L1. The five-year survival rate of patients with positive PD-L1 expressions was 21.43% (6/28), while the five-year survival rate of patients with negative expressions was 45.45% (10/22). Survival curves were plotted using the Kaplan-Meier method to calculate the five-year survival rate. The Log-rank method was used to compare the rate of five-year survival between the two groups and in the group of positive PD-L1 expressions was significantly lower than that in the group of negative PD-L1 expressions ($P < 0.05$) (Fig. 2).

Prognostic risk factors in patients with thoracic ESCC

One-way Log-rank method was used to analyze the prognostic risk factors in patients with thoracic ESCC. The results showed that age, gender, ethnicity,

smoking history, alcohol intake history, body mass index, the presence of underlying diseases (hypertension, coronary heart disease, diabetes), tumor location, method of surgical anastomosis, tumor size, degree of tumor differentiation, number of lymph node dissection and the use of postoperative adjuvant therapy (chemotherapy) were not significant risk factors ($P > 0.05$). However, the depth of tumor infiltration (T1/T2 vs T3/T4), lymph node status, postoperative pathological stage (I/II vs stage III), and PD-L1 expressions (PD-L1 positive vs PD-L1 negative) were significant risk factors ($P < 0.05$). Multivariate analysis also showed that the PD-L1 expression was an independent risk factor for the prognosis of patients with thoracic ESCC (HR=2.845, 95% CI: 1.300–6.226, $P = 0.009$). Furthermore, PD-L1 was highly expressed in cancer tissues of patients with thoracic ESCC but lowly expressed in adjacent normal tissues, as shown in Table I.

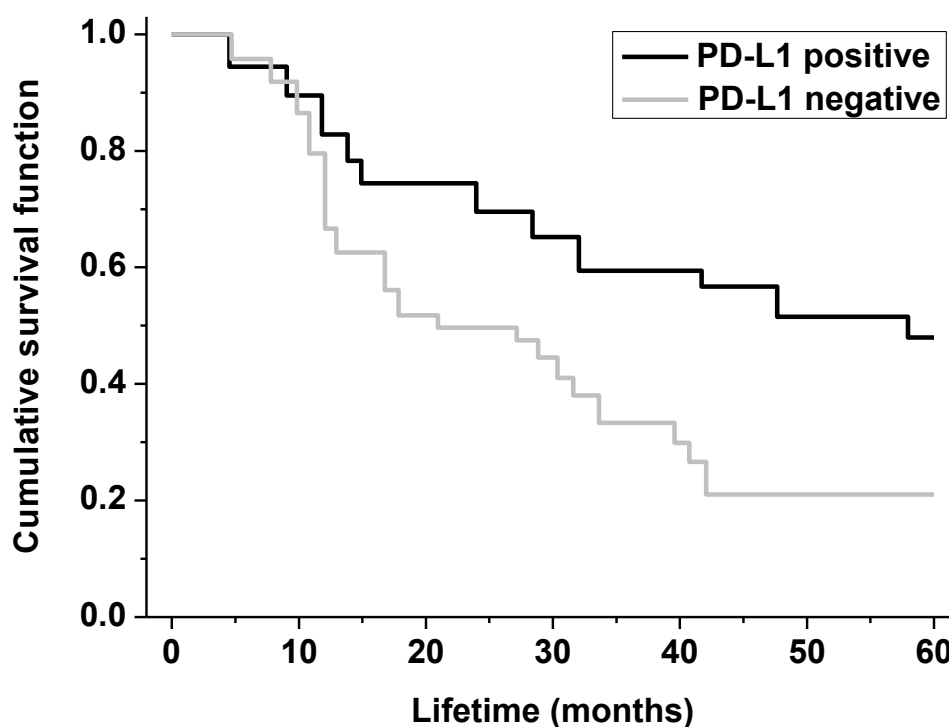


Fig. 2. Survival curves of thoracic ESCC patients with different PD-L1 expression levels.

Table I. *Univariate analysis of factors affecting the prognosis of patients with thoracic ESCC.*

Clinical pathological index	Number of patients	Five-year survival rate (%)	Chi-squared value	P value
Age				
≤60	27	27.5	0.908	0.327
>60	23	40.3		
Gender				
Male	39	34.2	0.005	0.938
Female	11	32.9		
Ethnicity				
Hui Ethnicity	14	25.6	0.069	0.789
Han Ethnicity	36	36.9		
Body mass index				
<18.5	9	12.3	0.635	0.751
18.5-25	31	339.6		
>25	10	32.7		
History of smoking				
Yes	25	27.6	0.352	0.543
No	25	41.3		
History of alcohol intake				
Yes	14	42.8	0.621	0.445
No	36	30.5		
Accompanied by underlying diseases				
Yes	9	25.7	0.153	0.712
No	41	35.9		
Surgical approach				
Neck anastomosis	24	34.3	0.331	0.869
Thoracic anastomosis	20	36.9		
Under arch anastomosis	6	19.8		
Tumor size (cm)				
≤4	29	45.7	3.286	0.068
>4	21	15.2		
Tissue differentiation				
Well-differentiated/ moderately differentiated	24	22.5	3.478	0.063
Poorly differentiated	26	42.6		
Tumor location				
Upper section	5	19.8	2.524	0.291
Middle section	30	30.1		
Lower section	15	46.5		

Infiltration depth					
T1/T2	25	58.2	13.473	0.000	
T3/T4	25	11.4			
Lymph node state					
Negative	24	16.5	5.734	0.019	
Positive	26	49.8			
Postoperative adjuvant therapy					
Yes	16	21.3	2.418	0.124	
No	24	39.6			
Number of lymph node dissection					
<6	14	35.5	2.263	0.325	
June 12	18	44.3			
>12	18	22.7			
Pathological staging					
I/II	26	55.7	12.579	0.000	
III	24	12.3			
PD-L1 expressions					
Positive	28	21.43	4.158	0.039	
Negative	22	45.45			

Multivariate COX analysis of prognostic risk factors in patients with thoracic ESCC

The univariate analysis of prognostic risk factors in patients with thoracic ESCC included four indicators: depth of tumor invasion (T1/T2 vs T3/T4), lymph node status, postoperative pathological stage (stage I/II vs III), and PD-L1 expression (PD-L1 positive vs PD-L1 negative). The results of multivariate COX analysis showed that the depth of invasion, lymph node status, and postoperative pathological stage were not significant risk factors ($P > 0.05$), whereas the expression of PD-L1 (PD-L1 positive vs PD-L1 negative) was a significant risk factor (HR (Exp(B)) = 2.845, 95% CI: 1.300 - 6.226, $P = 0.009$). According to the results of statistical analysis, the five-year survival rate of the patients in the PD-L1 positive group was 21.43% (6/28), whereas the five-year survival rate of the patients in the PD-L1 negative group was 45.45% (10/22). Therefore, there was significant difference in the five-year survival between the PD-L1 positive

group and the PD-L1 negative group ($P < 0.05$), and the five-year survival rate of the patients in the PD-L1 positive group was significantly lower than that of the patients in the PD-L1 negative group. Fig. 3 shows a representative immunohistochemistry image of ESCC cancer tissues and ESCC paracancerous tissues. There was no yellowish or brown staining, therefore the expression of PD-L1 protein was negative.

DISCUSSION

At present, the traditional treatments of esophageal cancer tend to result in a poor prognosis. Immune checkpoint inhibitors were shown to inhibit the immune activity of T cells and the secretion of T cell cytokines (7). Therefore, the binding of T cell antigen receptor (TCR) to the surface histocompatibility complex (MHC) of tumor cells was inhibited by immune checkpoint inhibitors (8, 9), which could be used as an effective therapeutic approach

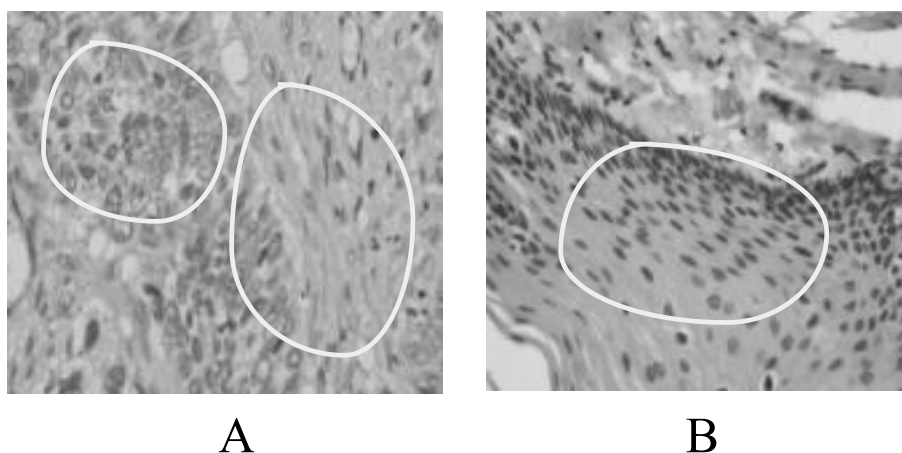


Fig. 3. Negative control of PD-L1 ($\times 400$). **A)** ESCC cancer tissues; **B)** ESCC paracancerous tissue.

for targeted blockade of relevant pathways. The immunohistochemistry results of this study showed that the positive site of PD-L1 staining was mainly located in cell membrane and cytoplasm. Moreover, the positive rate of PD-L1 in 50 patients was 56%, which was also close to the results of relevant research (10, 11). In addition, the overall prognosis of patients receiving adjuvant treatment was worse compared to those without adjuvant treatment. When the number of lymph node dissection was more than 12, the surgical area became broader, causing greater trauma and an increased risk of long-term complications. The 5-year survival rate of patients of stage III (12%) was significantly lower compared to those of stage I / II (56%) ($P < 0.05$), which was consistent with the results of another study (12).

In conclusion, the depth of tumor infiltration, lymph node status, pathological stage, and PD-L1 expressions are important factors affecting the prognosis of thoracic ESCC. However, further studies are needed to determine whether PD-L1 can be used as an indicator for the poor prognosis of ESCC.

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LETTER TO THE EDITOR

ROLE OF INFLAMMATORY FACTORS IN PERIPHERAL BLOOD CELLS OF PATIENTS WITH ISCHEMIC CEREBROVASCULAR DISEASE

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To the Editor,

Ischemic cerebrovascular disease (ICVD) may lead to significant changes in cerebral hemodynamics, resulting in decreased blood perfusion, and eventually causing brain necrosis and loss of function in the area of blood supply (1). Cerebral artery stenosis (CAS) is one of the important factors of ICVD (2).

Tumor necrosis factor- α (TNF- α) is secreted by a variety of cells in the central nervous system, which can change permeability of the inner wall of blood vessels, release free radicals, and lead to restenosis of ischemic cerebral arteries (3). It has been shown that the levels of inflammatory factors, such as TNF- α , interleukin-6 (IL-6) and C-reactive protein (CRP), increase significantly during the onset of cerebral artery stenosis in patients with acute cerebral infarction (4). Moreover, the homocysteine (HCY) level is related to the risk of cerebrovascular disease, and is considered as an independent risk factor of cerebrovascular disease (5). Studies have shown that the fibrinogen (FIB) level is closely related to the viscosity of blood (6, 7). In the early stage, FIB and low density lipoprotein (LDL) can invade the arterial wall through blood, so as to change permeability of endothelium. With the participation of LDL cells, the formation and development of atherosclerotic plaques

are accelerated. This study was designed to detect the level of inflammatory factors in ICVD patients with cerebral artery stenosis and provide a basis for clinical diagnosis, treatment and prognosis of ICVD.

MATERIALS AND METHODS

Study subjects

In this study, 95 ICVD patients who were treated in the First People's Hospital of Jiashan County from February 2017 to May 2019 were enrolled and divided into grade I group (34 cases), grade II group (30 cases), and grade III group (31 cases) according to the degree of cerebral artery stenosis using thrombolysis in myocardial infarction (TIMI) grading system. In addition, 30 healthy subjects who simultaneously underwent physical examination were assigned to the control group. The study protocol was approved by the Ethics Committee of First People's Hospital of Jiashan County and all the patients signed informed consent for their participation.

The eligible patients were examined by magnetic resonance angiography (MRA), Transcranial Doppler (TCD), color Doppler flow imaging (CDFI), computed tomography angiography (CTA) or digital subtraction angiography (DSA) to indicate whether there was vascular stenosis or not.

Key words: ICVD; CAS; inflammatory factors; immune response; inflammatory intervention

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Imaging examination

The degree of carotid artery stenosis was detected by color Doppler ultrasonography (CDUS). From the beginning of the carotid artery, the common carotid artery and internal carotid artery on both sides were checked. According to the characteristics of CDUS, the expansion, stiffness, and plaque scores of common carotid arteries were calculated.

Sample collection and measurement

After admission, blood samples were collected within 72 h of symptom onset. At 7:00 a.m., 6 mL of elbow venous blood were taken under fasting status. Relevant drugs needed to be discontinued 2 days before blood withdrawal. Meanwhile, the fasting patients' capillaries were measured at about 7:00 a.m. The specimens were placed in 10% ethylenediaminetetraacetic acid (EDTA) disodium salt and aprotinin test tubes, and mixed evenly. After centrifugation for 10 min at 3000 rpm, separation and determination of plasma were carried out. An enzymatic method was used to determine blood glucose levels. Simultaneously, a liquid-phase assay was utilized to measure plasma TNF- α concentrations, and an immunoturbidimetric assay was applied to detect lipoprotein levels.

Statistical analysis

SPSS 19.0 software was used to analyze the collected data. Normally distributed data were presented as mean $\pm$ standard deviation (SD). One-way analysis of variance (ANOVA) was used for making comparison between

the groups. Moreover, least significant difference (LSD) was employed to carry out multiple comparisons. Unconditional logistic regression analysis of data was undertaken to screen the factors influencing CAS, and $P < 0.05$ was considered statistically significant.

RESULTS

Distribution of cerebral artery stenosis

In this study, ICVD patients showed different degrees of CAS, of which 40 were cases of simple anterior circulation artery stenosis (22 cases of middle cerebral artery stenosis in anterior circulation and 18 cases of anterior cerebral artery stenosis), accounting for 42.11%; in addition, simple posterior circulation stenosis was noted in 45 cases (14, 12, and 19 cases of posterior middle vertebral artery, posterior cerebral artery stenosis, and basilar artery stenosis, accounting for 31.11%, 26.67%, and 42.22%, respectively), accounting for 47.37%. Furthermore, it was found that 10 cases had stenosis of anterior circulation combined with posterior circulation, accounting for 10.53%.

The intima-media thickness (IMT), stiffness, and plaque scores of groups I, II, and III were significantly higher than those in the control group ($P < 0.05$), and the expansibility was significantly lower than that of the control group ($P < 0.05$). The degree of cerebral artery stenosis in grade I group was notably higher than that in grade II group and grade III group, while the degree in grade II group was markedly higher

Table I. Comparison of CAS among different groups

Group	IMT(mm)	Stiffness (mmHg)	Plaque scores	Expansibility (mmHg ⁻¹ *100)
Grade I group	1.79 $\pm$ 0.74*	817.44 $\pm$ 101.56*	6.02 $\pm$ 0.93*	0.25 $\pm$ 0.07*
Grade II group	1.51 $\pm$ 0.53*&	623.35 $\pm$ 92.43*&	4.13 $\pm$ 0.68*&	0.45 $\pm$ 0.09*&
Grade III group	1.25 $\pm$ 0.42*#&	433.46 $\pm$ 56.45*#&	2.97 $\pm$ 0.61*#&	0.79 $\pm$ 0.12*#&
Control group	0.66 $\pm$ 0.26	211.65 $\pm$ 48.66	1.25 $\pm$ 0.55	1.03 $\pm$ 0.21
F-value	16.356	11.466	10.647	16.168
P-value	0.018	0.035	0.036	0.011

* $P < 0.05$, compared with the control group; # $P < 0.05$, compared with grade I group; & $P < 0.05$, compared with grade II group.

than that in grade III group ($P < 0.05$) (Table I).

There was no notable change in velocity or a slight increase in velocity in grade I stenosis. The peak systolic velocity (PSV) and end-diastolic velocity

(EDV) increased in grade II stenosis, and mosaic-colored blood flow signals appeared in the stenosis (Fig. 1A). In grade III stenosis, the blood flow bundles became thinner, PSV remarkably increased,

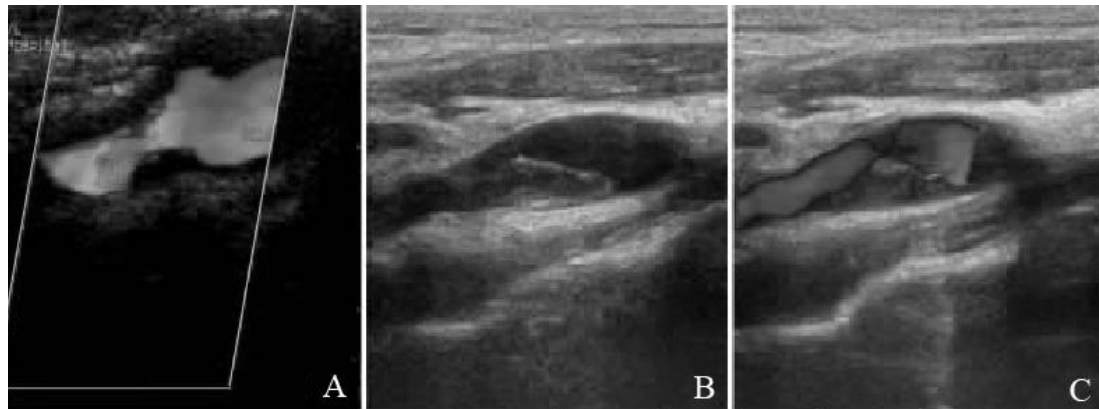


Fig. 1. Ultrasonography of carotid artery stenosis. *A)* The degree of stenosis is 50%. Mosaic-colored blood flow signals can be observed in the posterior segment of the stenosis; *B)* Two-dimensional ultrasound shows soft plaques, resulting in severe stenosis of the lumen; *C)* After stenosis, the blood flow becomes thinner and turbulence forms.

Table II. Analysis of factors influencing CAS in ICVD patients

Index		Experimental group	Control group	T/ χ^2	P-value
Age (years)		61.44±12.41	58.54±9.45	3.74	0.002
Fasting blood glucose (mmol/L)		8.94±3.23	5.03±1.32	10.45	<0.001
Glycosylated hemoglobin (%)		8.13±1.63	5.56±0.73	11.45	<0.001
HCY(umol/L)		22.23±8.32	7.54±1.28	16.44	<0.001
TNF- α		3.48±0.73	2.35±0.37	18.36	<0.001
hs-CRP(mg/L)		10.63±2.38	3.87±1.92	21.49	<0.001
FIB(mg/L)		3.73±1.13	1.33±0.64	16.65	<0.001
Gender (cases)	Male	49	21	2.64	0.147
	Female	46	9		
Hypertension (cases)	Yes	58	3	16.38	<0.001
	No	37	27		
Diabetes mellitus (cases)	Yes	64	4	31.35	<0.001
	No	31	26		
Coronary heart disease (cases)	Yes	54	3	22.43	<0.001
	No	41	27		
History of stroke (cases)	Yes	73	2	28.18	<0.001
	No	22	28		
History of smoking (cases)	Yes	73	6	25.24	<0.001
	No	22	24		

EDV markedly elevated, and proximal resistance to blood flow was also enhanced (Fig. 1B, C).

Analysis of factors influencing CAS in ICVD patients

Table II shows that the incidence of CAS in ICVD patients is closely associated with factors such as age, hypertension, diabetes mellitus, history of stroke, history of smoking, drinking, glycosylated hemoglobin, HCY, TNF- α , hs-CRP, and fibrinogen (FIB) ($P < 0.05$).

Unconditional logistic regression analysis of CAS risk factors in ICVD patients

The degree of ICVD stenosis (0 = control, 1 = grade I stenosis, 2 = grade II stenosis, 3 = grade III stenosis) is variable. In this study, other statistically significant variables (age, hypertension, diabetes mellitus, coronary heart disease (CHD), history of stroke, history of smoking, FBG, RBG, glycosylated hemoglobin, IMT, stiffness, plaque scores, dilatability) were adjusted, and the levels of hs-CRP, TNF- α , HCY, and FIB were included. The results showed that the risk of arterial stenosis increased with the elevation of TNF- α ($P = 0.001$). Other parameters were not changed significantly ($P > 0.05$).

The levels of hs-CRP, TNF- α , HCY, and FIB

In the present study, the levels of hs-CRP, TNF- α , HCY, and FIB in ICVD patients were significantly higher than those in the control group ($P < 0.05$). Moreover, these levels in the grade III group and grade II group were significantly higher than those in

the grade I group ($P < 0.05$). In addition, the severer the stenosis was, the higher the levels of hs-CRP, TNF- α , HCY and FIB were, as presented in Table III.

DISCUSSION

The incidence of ICVD is generally high (8). In this study, all the selected ICVD patients showed CAS of different degrees. The results of this study revealed that the incidence of CAS in ICVD patients was closely associated with age, hypertension, diabetes mellitus, history of stroke, history of smoking, history of drinking, FBG, RBG, glycosylated hemoglobin, IMT, stiffness, plaque scores, dilatation, FIB, and HCY. Hence, effective therapeutic modalities should be explored in order to reduce the incidence of CAS in ICVD patients.

A large number of studies showed that the essence of atherosclerosis was a chronic inflammatory process (9, 10). Inflammatory factors participated in the formation and affected the stability of atherosclerotic plaques. Inflammatory responses, as the initiating factor of ischemic cerebral artery restenosis, could promote lipid deposition, plaque rupture and inflammatory factor accumulation (11). The results of the present study showed that the incidence of CAS was closely associated with TNF- α ($P < 0.05$), thus, inflammatory response can directly affect its occurrence and development. In addition, it was disclosed that the levels of HCY, FIB, and hs-CRP were closely associated with the degree of CAS. The levels of HCY, FIB, and hs-CRP

Table III. Levels of hs-CRP, TNF- α , HCY, and FIB.

Group	G	TNF- α	HCY(umol/L)	FIB(mg/L)
Grade I group	8.85 $\pm$ 1.21*	2.94 $\pm$ 0.15*	17.34 $\pm$ 1.45*	2.63 $\pm$ 0.91*
Grade II group	10.61 $\pm$ 2.13*#	3.61 $\pm$ 0.29*#	21.24 $\pm$ 4.52*#	3.61 $\pm$ 0.63*#
Grade III group	12.03 $\pm$ 2.18*#&	4.05 $\pm$ 0.16*#&	33.42 $\pm$ 7.26*#&	4.92 $\pm$ 0.98*#&
Control group	3.88 $\pm$ 0.63	2.39 $\pm$ 0.13	7.34 $\pm$ 1.55	1.16 $\pm$ 0.22
F value	147.374	584.29	162.25	303.25
P value	<0.001	<0.001	<0.001	<0.001

* $P < 0.05$, compared with the control group; # $P < 0.05$, compared with grade I group; & $P < 0.05$, compared with grade II group.

were positively correlated with CAS, indicating that these levels could predict the severity of CAS.

In conclusion, CAS occurs in ICVD patients after onset of symptoms, and the degree of stenosis is closely associated with inflammatory factors in peripheral blood cells. Inflammatory response can activate the expansion of systemic immune response, leading to plaque rupture or stenosis.

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LETTER TO THE EDITOR

ASSOCIATION BETWEEN ARTERIAL INTIMA RESPONSE BEFORE AND AFTER HIGH FAT LOAD AND PROGRESSION OF ARTERIOSCLEROSIS

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To the Editor,

At present, atherosclerotic cardiovascular disease (ASCVD) ranks first in the disease spectrum. Furthermore, its incidence is increasing, and patients are significantly becoming younger (1). There are different opinions on the pathogenesis of atherosclerosis, which include the following: damage response mechanism, inflammation mechanism and oxidative stress mechanism. Endothelial injury, inflammation, and smooth muscle cell proliferation and migration are key links in the development of atherosclerosis, and are the present research hotspots. Studies have revealed that high-fat diet can cause chronic inflammation, and in turn induce the occurrence and development of vascular plaque (2, 3). Few studies have been conducted on the mechanism of action and dynamic evolution process of related inflammation indicators.

In the present study, a high-fat load was given to experimental rabbits at different stages of atherosclerosis progression, and the changes in plasma monocyte chemokine-1 (MCP-1), adiponectin (APN), platelet-derived growth factor-BB (PDGF-BB) and transforming growth factor β 1 (TGF- β 1)

levels before and after high-fat load were analyzed. The association between arterial intima response and atherosclerosis progression was further explored, in order to elucidate the effect of high-fat load on atherosclerosis progression and plaque stability.

MATERIALS AND METHODS

Establishment of the atherosclerosis animal models

Ten healthy male New Zealand white rabbits (three months old, weighing 2.5-3 kg) were purchased from Shanxi Provincial Animal Husbandry Institute. The experimental models were established using the method described by Kolodgie et al. (4). Briefly, the rabbits were fed with high-fat diet for 12 weeks. The high-fat diet contained 1% cholesterol (Beijing Solarbia Technology Co., Ltd. China), 8% yolk and 91% basic forage. In addition, the high fat-load was given at time 0, and the 4th, 8th and 12th weeks. The high-fat load feed contained 2% cholesterol, 8% yolk and 90% basic forage.

Detection of plasma levels of MCP-1, PDGF-BB, TGF- β 1 and APN

At time 0 and the 4th, 8th and 12th weeks, 3 ml of blood was

Key words: high fat load; atherosclerosis; arterial intima response

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collected from the ear vein using a vacuum anticoagulant tube after 12 hours of fasting, and at 2, 4, 6 and 8 hours after high-fat load. Then, the samples were centrifuged at 1,000 rpm for 15 min using an automatic biochemical analyzer centrifuge (BECKMAN, USA). Serum was separated and then frozen immediately at -80°C until analysis. The serum MCP-1, PDGF-BB, TGF- β 1 and APN levels were determined by enzyme-linked immunosorbent assay (ELISA) (Westang Biotech, Shanghai, China).

Ultrasound examination of the abdominal aorta of experimental animals

At time 0 and the 4th, 8th and 12th weeks, all animals received intramuscular injection of Sumianxin II (0.1-0.2 ml/kg) for general anesthesia, and were placed in a supine position. Then, the transverse and longitudinal sections of the abdominal aorta of these rabbits were detected by color Doppler ultrasound (MyLab 90, Esaote,

Italy) using a vessel probe (frequency, 7-10 MHz), from top to bottom along the midline of the abdomen.

Pathological examination of the abdominal aorta of experimental subjects

At week 12, the experimental animals were sacrificed. The abdominal aorta was separated, routinely stained with hematoxylin and eosin (H&E), and optical microscopy (Olympus BX53, Shanghai Lance Electronics Technology Co., Ltd.) was used to observe the atherosclerosis in the transverse sections of the blood vessels.

Statistical analysis

Data were analyzed using statistical software SPSS 24.0. Measurement data were expressed as mean $\pm$ standard deviation. The dynamic changes of each index were analyzed by two-factor repeated measures. $P < 0.05$ was considered statistically significant.

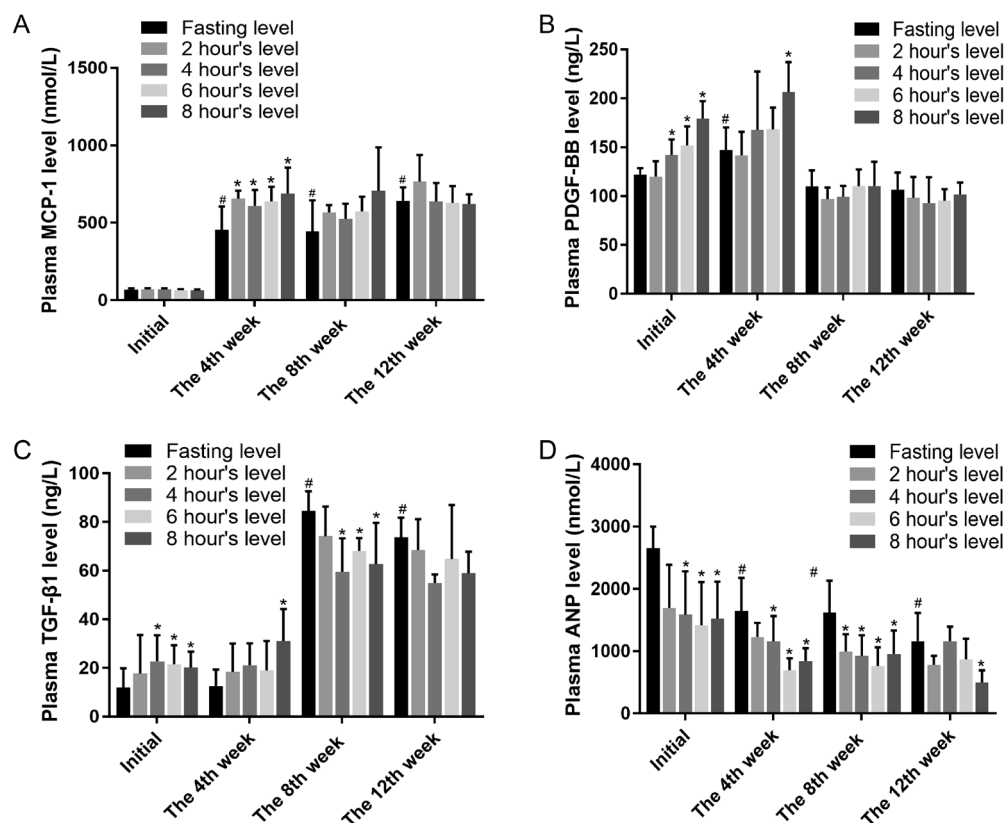


Fig. 1. Changes in plasma MCP-1 (A), PDGF-BB (B), TGF- β 1 (C), and ANP (D) before and after high-fat load in the process of atherosclerosis. * $P < 0.05$ vs fasting level at the same week; # $P < 0.05$ vs initial fasting level at different weeks.

RESULTS

Changes in plasma MCP-1, PDGF-BB, TGF- β 1 and APN before and after high-fat load in the process of atherosclerosis

As shown in Fig. 1A, the fasting MCP-1 level increased, and the highest level appeared at the 12th week ($P<0.05$). Furthermore, the post-load level exhibited an overall upward-downward-upward trend ($P>0.05$) at the 4th week, and this level increased at 2, 4, 6 and 8 hours after high-fat load, when compared with the fasting level ($P<0.05$). The highest level appeared at the 8th hour ($P<0.05$).

As shown in Fig. 1B, the PDGF-BB fasting level

initially increased, and subsequently decreased. The highest level appeared at the 4th week ($P<0.05$). Furthermore, the post-load level first decreased, and subsequently increased ($P>0.05$). Then, at the 4th, 6th and 8th hours after initial load, the level was higher than the fasting level, and the highest level appeared at the 8th hour ($P<0.05$).

As shown in Fig. 1C, the TGF- β 1 fasting level initially increased, and subsequently decreased. The highest level appeared at the 8th week ($P<0.05$). This level increased within four weeks after high-fat load. After the initial load, and at the 4th, 6th and 8th hour, the level was higher than fasting level, and the highest level appeared at the 8th hour ($P<0.05$). Furthermore,

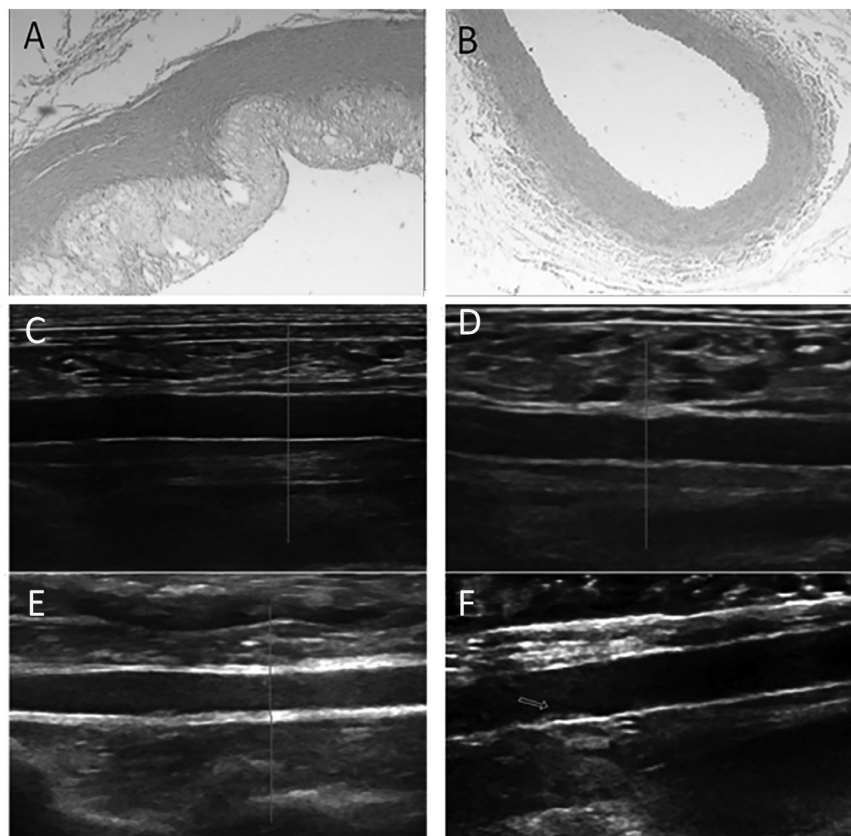


Fig. 2. *A-B*) Results of the pathological examination of the abdominal aorta at different times points (H&E, 100 $\times$): **A**) At the beginning of the experiment, the intima of the blood vessel was continuous and smooth, smooth muscle cells were arranged in an orderly manner, and no foam cells were observed. **B**) At the 12th week of the experiment, the vascular endothelial cells were swelling, the smooth muscle cells were irregularly arranged and significantly proliferated, the intima was destroyed, and a large number of foam cells and an obvious “empty cytoplasm phenomenon” could be observed. *(C-F)* Observation of the abdominal aortic wall by color Doppler ultrasound: **(C)** At the beginning of the experiment, the arterial wall was smooth; **(D)** At week four, the intima-media thickness increased; **(E)** At week eight, the arterial wall was rough and small plaque formed; **(F)** At week 12, the arterial intima-media significantly thickened and plaque formed.

the post-load level increased at the 4th week, these levels were lower than the fasting level at the 4th, 6th and 8th hours, and the highest level appeared at the 4th hour ($P<0.05$).

As shown in Fig. 1D, the APN fasting level decreased, and the highest level appeared at the 12th week ($P<0.05$). At the beginning, and at the 4th and 8th weeks, the post-load level first decreased, and subsequently increased. At the 4th, 6th and 8th hours, the post-load levels were lower than the fasting level ($P<0.05$), and the lowest level appeared at the 6th hour ($P<0.05$). At week 12, the post-load level exhibited a downward-upward-downward trend, and at the 8th hour, this was significantly lower than the fasting level ($P<0.05$).

DISCUSSION

Long-term continuous high-fat diet can cause atherosclerosis and plaque formation (5). Intima injury is the initial event of this process. Among the various theories on the pathogenesis of atherosclerosis, the damage response theory and inflammation theory are dominant. A large amount of anti-inflammatory factors and anti-smooth muscle proliferation factors are largely consumed in the process of atherosclerosis. MCP-1, PDGF-BB, TGF- β 1 and APN are the representatives of these factors.

MCP-1 can induce monocytes and macrophages to enter the intima and form foam cells (6). PDGF-BB has the highest activity in promoting the migration and proliferation of smooth muscle cells (7). TGF- β 1 is a typical anti-inflammatory and anti-foam cell factor, which has a bidirectional regulation in the process of atherosclerosis (8). APN could play an anti-atherosclerosis role by inhibiting the early inflammation and proliferation of vascular smooth muscle cells (9). Previous studies have proved that the levels of the factors mentioned above were increased after continuous high-fat diet (10-12), giving evidence how high-fat diet affects the progression of atherosclerosis. In the present study, MCP-1 level in the fasting state increased at weeks 4, 8 and 12, and the post-load level increased at the 4th week. The PDGF-BB

and TGF- β 1 fasting level initially increased, and subsequently decreased. The post-load level of PDGF-BB initially decreased, and subsequently increased. But the post-load level of TGF- β 1 increased at the 4th week. Moreover, APN fasting level decreased at weeks 4, 8 and 12.

Our results indicated that the self-regulation at the early stage of high-fat diet was strong. However, at the later stage, the anti-inflammatory effect was weakened, and arteriosclerosis became prone to progression. Inflammatory response was significant at 4-8 hours after a single high-fat load

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LETTER TO THE EDITOR

DIAGNOSTIC VALUE OF JOINT DETECTION OF WHITE BLOOD CELLS, BLOOD GLUCOSE, AND SERUM LACTIC ACID FOR HAND-FOOT-AND-MOUTH DISEASEJT. LIU¹, XA. LI², QL. XIE³, HT. YANG², HM. ZHOU⁴ and T. ZHANG¹¹*Department of Infectious Diseases, Jinxiang Hospital Affiliated to Jining Medical College, Jining, China;* ²*Laboratory Science, Jinxiang Hospital Affiliated to Jining Medical College, Jining, China;*³*Departemnt of Neonatology Jinxiang Hospital Affiliated to Jining Medical College, Jining, China;*⁴*Ministry of Nursing, Jinxiang Hospital Affiliated to Jining Medical College, Jining, China**Received June 26, 2019 – Accepted November 8, 2019*

To the Editor,

Hand-foot-and-mouth disease (HFMD) is a common infectious disease among infants and toddlers caused by Coxsackievirus A16 (CA16) and enterovirus 71 (EV71) (1). Most cases of HFMD are not life-threatening, however, some patients with complications have a poor prognosis (2, 3). Currently, no vaccine preventions are available for HFMD, therefore, it is necessary to explore the applicable indicators to monitor its severity and prognosis. During the onset of the disease, the neurology and circulatory systems of pediatric patients are affected. The virus infection contributes to the increased white blood cell levels. At the same time, after the stimulation of different degrees of stress, stress hyperglycemia occurs with the increased secretion of catecholamine, thereby causing serum lactic acid to accumulate (4-6). It has been found that the white blood cell count gradually increases with the progression of HFMD (7). It has been shown that blood glucose and serum lactate levels are associated with death of patients with severe HFMD (8). Thus, white blood cells, blood glucose, and serum lactic acid are important indicators for the disease.

Currently, there are few studies on joint detection and correlation analysis of indicators but most with

only a single indicator analyzed. However, it is of great difficulty to determine a single indicator. Therefore, this study included HFMD patients and healthy children to explore the diagnostic value in joint detection of white blood cells, blood glucose, and serum lactic acid for HFMD, providing a reference for its clinical diagnosis.

MATERIALS AND METHODS*Patients*

A total of 90 children with HFMD who were treated in Jinxiang Hospital Affiliated to Jining Medical College from June 2012 to June 2018 were recruited in the study group; the ratio of male to female was 1.6:1 (55/35); the patients' average age was 22 months. Of these, 67 cases were infected by EV71, and 23 cases were infected by CA16 or other enteroviruses. According to the Guidelines for the Diagnosis and Treatment of HFMD, child patients in the study group were divided into stages I~V. There were 38 patients in stage I, 20 in stage II, 15 in stage III, 4 p in stage IV, and 13 in stage V. The clinical characteristics of patients in each stage are shown in Table I. The average hospital stay was 8.56±5.05 days, and the average intensive care unit (ICU) length

Key words: hand-foot-and-mouth disease; white blood cells; blood glucose; serum lactic acid; joint detection

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of stay was 6.58 ± 4.23 days. Simultaneously, 90 healthy children who passed physical examination in the same period were enrolled in the control group; the ratio of male to female was 1.6:1 (55/35), with an average age of 23 months. There was no significant difference in age, gender ratios, and other reference data between these groups ($P > 0.05$). The study was approved by the Ethics Committee of Jinxiang Hospital Affiliated to Jining Medical College, and all patients and their families were aware of the purpose and significance of the study before signing the informed consent.

White blood cell count

In terms of white blood cell (WBC) count, a total of 1 mL of venous blood samples from children in both groups were added to ethylenediaminetetraacetic acid (EDTA) anticoagulant tubes and were gently agitated. Another tube was taken and added with 0.38 mL of the diluted WBC (Beijing Baiao Laibo Technology Co., Ltd., Beijing, China). The pipette (Eppendorf,

Germany) was applied to another pipette (20 μ L) of venous blood, the residual blood on the surface of the pipette tip was removed, and the tip was inserted into the bottom of the tube with diluted WBC; then, the blood was released and the tip was gently blown several times. Eventually, the test tubes were gently shaken and mixed until the cell suspension had turned completely brown. A total of 6 samples were randomly selected (3 in the study group and 3 in the control group), the DNA was extracted by using the Puregene blood core reagent kit (Qiagen, Germany), and volumes of extracted WBC were detected by using 1.5% agarose gel electrophoresis. The cell suspension was pipetted with a plastic dropper, and a drop of the cell suspension was dripped into white blood cell counting plate (Beijing Dongxun Tiandi Medical Instrument Co., Ltd., Beijing, China), which was kept for a few minutes after the cell was filled; once all the WBC were sunk, an optical microscope (Nikon, Japan) was applied to observe and record the total number of WBC in four corner squares. The number of WBC was obtained after counting and

$$\text{The number of WBCs} = \frac{\text{The number of WBCs in the four corner squares} \times 10}{20} / L$$

Table I. *Clinical characteristics and distributions of child patients in the study group*

Stage	Name of stage	Number of patients	Clinical characteristics
I	The eruptive stage	38	Fever, erythema, or herpes pharyngitis
II	The nervous system involvement stage	20	Drowsiness, susceptibility to fright, nausea, limb trembling, or neck rigidity
III	The earlier cardiopulmonary failure stage	15	Acceleration of heart rate and respiration, coolness extremities, or increase in blood pressure
IV	The cardiopulmonary failure stage	4	Shortness of breath, cyanosis, convulsions, or disturbance of consciousness
V	The recovery stage	13	Recovery of body temperature, recovery of cardiopulmonary functions

calculation. The calculation equation was as follows:

Detection of plasma glucose

The extracted venous blood samples were centrifuged at low speed and placed in a water bath at 37°C (Shanghai Shibei Instrument Equipment Co., Ltd., Shanghai, China) to remove the upper fibrin; then, the serum samples were obtained through centrifugation. The enzyme reagent was prepared by 0.1 mol/l of phosphate buffer (Beijing Baiao Laibo Technology Co., Ltd., Beijing, China), and the phenol solution was prepared by heavy distillation of phenol (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China); the enzyme reagent and the phenol solution were equivalently mixed; then, the glucose (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) was weighed and dissolved in 12 mmol/l benzoic acid solution (Shanghai Qiyi Biotechnology Co., Ltd., Shanghai, China) to formulate 100 mmol/l glucose standard stock solution, which was then diluted in the 5 mmol/l glucose standard solution. A total of 3 test tubes were labeled respectively as the detection tube, the standard tube, and the blank tube, and were added with 3 mL of enzyme-phenol mixed reagent, respectively. Afterwards, the detection tube was added with 20 uL of serum for testing, the standard tube was added with glucose standard solution, and the blank tube was added with the equal amount of distilled water for controlling. Each tube was well-mixed, and then placed in a water bath at 37°C; next, samples of the tubes were placed in a spectrophotometer (Eppendorf, Germany) at a wavelength of 505 nm, the blank tube was zeroed, and the absorbance values of the standard tube and the detection tube were read. The equation of serum

The plasma glucose concentration =

$$\frac{\text{The detected absorbance value}}{\text{The standard absorbance value}} \text{mmol} / \text{L}$$

glucose concentration was as follows:

Detection of serum lactic acid

The venous blood samples in both groups were collected, and the serum was separated (the method was the same as above). Additionally, 20 mmol/l of lactic acid standard solution (Jining Xuli Chemical Co., Ltd., China) was diluted into 5 mmol/l of

working solution, and LD solution (Jining Xuli Chemical Co., Ltd., China) was used to formulate substrate solution. A total of 3 test tubes were labeled respectively as the detection tube, the standard tube, and the blank tube. Each tube was added with 500 uL of substrate solution; the blank tube was added with 10 uL of distilled water for controlling, the standard tube was added with lactic acid working solution, and the detection tube was added with the serum for testing. Each tube was well-mixed and placed into a water bath at 37°C for several minutes, and the reaction was terminated by adding 0.1 mol/l of hydrochloric acid. The wavelength of a spectrophotometer (Eppendorf, Germany) was set to 340 nm, the blank tube was set to zero, and the absorbance values of the standard tubes and the detection tubes were determined. Afterwards, the serum lactic acid content was calculated through the absorbance values, in which the equation was as

The serum lactic acid concentration =

$$\frac{\text{The detected absorbance value}}{\text{The standard absorbance value}} \times 2 \text{mmol} / \text{L}$$

follows:

Statistical analysis

EpiData 3.2 software was applied to analyze the data. If the data were normally distributed, the mean of count data and standard deviation (SD) could be applied to describe the continuous variables, which were expressed as $\bar{x} \pm s$; the *t*-test was applied to compare the difference between the continuous variables; if the data were not in normal distribution, the median and the inter quartile range (IQR) were applied to describe the continuous variables. The accurate *Chi-square* test was used to explain the variables through univariate testing of all categories. $P < 0.05$ was considered statistically significant.

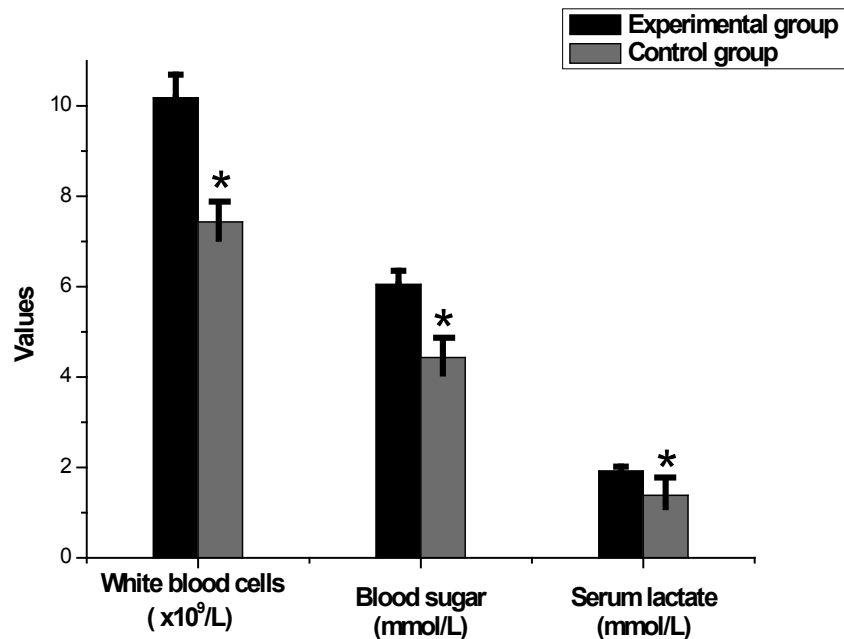
RESULTS

Single detection results of WBC, blood glucose, and serum lactic acid

The detection results of WBC, blood glucose, and serum lactic acid in the study group and the control

Table II. Multivariate analysis results of WBC, blood glucose and serum lactic acid (%)

Experimental parameters	Range	study group (n=90)	Control group (n=90)	P-value
White blood cells ($10^9/L$)	≤ 9.76	3 (3.3)	86 (95.6)	<0.01
	>9.76	87 (96.7)	4 (4.4)	<0.01
Blood glucose (mmol/L)	≤ 5.80	2 (2.2)	87 (96.7)	<0.01
	>5.80	88 (97.8)	3 (3.3)	<0.01
Serum lactic acid (mmol/L)	≤ 1.80	2 (2.2)	88 (97.8)	<0.01
	>1.80	88 (97.8)	2 (2.2)	<0.01

**Fig.1.** Results of single detections of WBC, blood glucose concentration, and serum lactic acid content in both groups ($\bar{x} \pm s$). *: $P < 0.05$, compared with the control group.

group were significantly different. The univariate analysis showed that the number of WBC, blood glucose concentration, and serum lactic acid content in the patients with HFMD were higher than those in healthy children, and the difference was statistically significant ($P < 0.05$) (Fig.1).

The count of WBC in the study group was significantly higher compared with the control

group, which was caused by the viral infection in HFMD. In the pathogenesis, the circulatory systems of child patients were exposed to stress after injuries, leading to the release of granulocytes from the bone marrow. The WBC in peripheral blood proliferated massively and were involved in the immune responses of HFMD. At the same time, it also demonstrated that WBC played an antiviral role

in HFMD. The value of blood glucose in the study group was significantly higher compared with the control group, which resulted from the alteration in blood glucose homeostasis affected by the autonomic nervous system. If the sympathetic nervous system was stimulated, the serum levels of adrenaline and glucagon increased but the level of insulin decreased, causing the deteriorated glucose catabolism. Given the condition of excluding diabetes and other factors, child patients with HFMD were prone to the imbalance of blood glucose regulation, leading to massive accumulations of glucose and high blood glucose. There was a significant difference of serum lactic acid content between the two groups. Lactic acid was an intermediate product in glucose metabolism *in vivo*. As the sympathetic nervous system in child patients with HFMD was excited, the secretion of catecholamines increased, which in turn

caused an accumulation of serum lactic acid.

Positive rates in detection of WBC, blood glucose, and serum lactic acid

In the study group, the positive rates of WBC, blood glucose and serum lactate were 23.3% (21/90), 33.3% (30/90) and 43.3% (39/90), respectively. In the control group, the positive rates of WBC, blood glucose and serum lactate were 2.2% (2/90), 2.2% (2/90) and 4.4% (4/90), respectively. The positive rates of joint detection in the study and control groups were 74.5% (67/90) and 13.3% (12/90), respectively. The positive rates of detection of WBC, blood glucose, and serum lactic acid in the study group and the control group were significantly different. The positive rate of each single detection of children with HFMD was notably increased, and the difference was statistically significant ($P < 0.01$). Compared with the single detection, the sensitivity

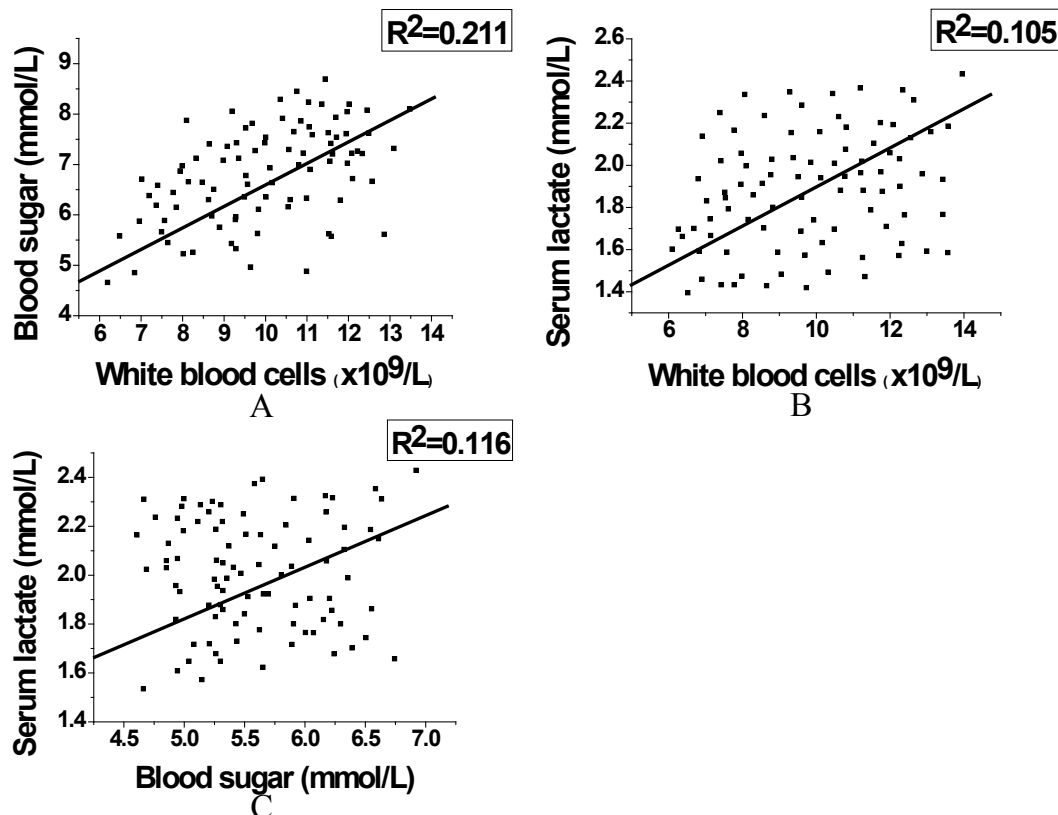


Fig. 2. Scatter diagrams of the relations among white blood cells, blood glucose, and serum lactic acid in the study group. **A)** Scatter diagram of white blood cell count and blood glucose concentration; **B)** Scatter diagram of white blood cell count and serum lactic acid concentration; **C)** Scatter diagram of blood glucose concentration and serum lactic acid concentration)

of the 3-joint-detection was significantly increased, in which the comparative results were statistically significant ($P<0.01$).

In single detection, pediatric patients with HFMD had the highest positive rate of serum lactic acid and the lowest positive rate of white blood cells. It was revealed that HFMD had relatively notable influence on serum lactic acid levels and attenuated effects on WBC count. However, the positive rate of the joint detection of the 3 markers was significantly greater than that of the single detection. The high positive rate of detection of WBC indicated the presence of the disease, while hyperglycemia was more likely to cause vascular diseases, even blindness and disability in severe cases, causing serious damage to heart and brain tissues. Lactic acid was mainly produced by anaerobic respiration; in some pathological conditions, the lactic acid content increased due to tissue hypoxia.

Multivariate risk assessment

The unconditional logistic regression model was used for multivariate analysis of various parameters; the results revealed that WBC, blood glucose, and serum lactic acid levels were closely associated with HFMD. If WBC count exceeded $9.76 \times 10^9/L$, the blood glucose concentration exceeded 5.80 mmol/l, or the serum lactic acid content exceeded 1.80 mmol/l, the risks of HFMD were significantly increased in children, and the difference was statistically significant ($P<0.01$) (Table II).

As shown in Table II, when the WBC, blood glucose, and serum lactic acid levels reached certain thresholds, distribution of the number of cases in the study group and the control group was significantly different. The tested marker levels were mainly below the standard in the control group, while they were higher than the standard in the study group. Therefore, the standard could provide a reference for the detection of HFMD markers and assist the diagnosis of HFMD to a certain extent.

Correlation analysis of WBC, blood glucose, and serum lactic acid

As shown in Fig. 2, the correlation analysis was performed on the WBC, blood glucose, and

serum lactic acid of the child patients in the study group. There was a positive correlation among WBC, blood glucose, and serum lactic acid in child patients with HFMD. In addition, the correlation coefficients (r) between the WBC count with the blood glucose concentration, the WBC count with the serum lactic acid concentration and the blood glucose concentration with the serum lactic acid concentration were 0.478, 0.504 and 0.497 ($P<0.05$), respectively. The increase of blood glucose in children with HFMD was correlated with brain stem injury and stress enhancement in critical conditions. When under stress, the neuroendocrine system was activated with multiple secreted catabolic hormones, which directly stimulated glycogenolysis. Gluconeogenesis was enhanced, and the biological effects of insulin were antagonized in different ways, therefore, blood glucose was significantly increased in a short period. Temporary hyperglycemia could provide sufficient energy for glucose-dependent organs such as brain and heart, which was beneficial. If hyperglycemia continued, increased glycolysis resulted in enhanced lactose production. The high concentration of serum lactic acid would aggravate hypoxia in the tissue and worsen the condition. After treatment, the level of white blood cells in HFMD patients decreased.

DISCUSSION

The count of white blood cells in HFMD patients is often used as an indicator for the disease (9). The results of our research showed that the level of white blood cells in the study group was higher compared with the control group, which was consistent with the research by Zheng et al. (10). In HFMD, the circulatory system is under stress after injury, causing an increase in WBC count. The study of Liu et al. demonstrated that the increased blood glucose level could predict the severity of HFMD. If the blood glucose level increased rapidly, the severity of the disease was increased within 2-4 hours (11). During the onset of the disease, the nervous, circulatory, and respiratory systems of HFMD patients are damaged, the sympathetic nervous system is stimulated, the serum levels of adrenaline and glucagon are

increased, and insulin production is reduced, which leads to impaired glucose catabolism. Therefore, blood glucose levels could be used as a predictor for the development of HFMD. In the present study, the serum lactic acid levels of children in the study group were significantly higher than those in the control group, which was consistent with Cao et al. (12). Due to the sympathetic nervous system in child patients with HFMD being excited, the secretion of catecholamines increased, contributing to the accumulation of lactic acid in the serum. Therefore, serum lactic acid was closely correlated with HFMD.

The results of this study showed that a positive correlation existed between the three indicators above. The increased blood glucose level in children with HFMD was associated with brain stem injury and increased stress in critical conditions. If hyperglycemia continued, glucose anaerobic glycolysis substrate would increase, resulting in the accumulation of lactose. High concentrations of lactic acid could aggravate hypoxia in the tissue, which may worsen the condition. After treatment, the level of WBC in HFMD patients decreased, therefore, the sensitivity of joint detection is more efficient and accurate.

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LETTER TO THE EDITOR

INVOLVEMENT OF THE SUBSTANCE P/NEUROKININ-1 RECEPTOR SYSTEM IN ORAL PAIN AND INFLAMMATION

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To the Editor,

Substance P (SP), a member of the tachykinin family of regulatory peptides, and the neurokinin-1 receptor (NK-1R) are widely distributed by the oral cavity. The dental pulp is densely innervated by sensory, sympathetic and parasympathetic fibers, and terminal fibers containing SP have been observed surrounding blood vessels and associated with odontoblasts. After neuronal excitation, SP is released into the dental pulp and then exerts a vasodilator action and increases plasma extravasation, i.e., SP promotes inflammation. SP is widely distributed throughout the body: the peptide has been located in both central and peripheral nervous systems, in cells not belonging to the nervous system (e.g., immune cells) and in body fluids (blood, cerebrospinal fluid, saliva) (1). The NK-1R binds the undecapeptide with the highest affinity, and for this reason this receptor is also named SP receptor. NK-1R is widely distributed by the body (e.g., central nervous system, peripheral tissues, endothelial and immune cells) (1, 2). SP and NK-1R have been located in the dorsal horn of the spinal cord and in the trigeminal system, and the pain neurotransmitter SP has been localized in ganglion cell bodies and within small myelinated A δ /unmyelinated C fibers. SP, via the

NK-1R, mediates the pain stimulus and, in response to inflammation, nerve fibers containing SP transmit afferent signals to the central nervous system, thus SP contributes to pain transmission (3) (Fig. 1). Therefore, understanding the SP/NK-1R system is crucial for better management of oral pain and inflammation, and the use of NK-1R antagonists for the treatment of these pathologies must be urgently developed.

In peripheral organs, primary afferent neurons are responsible for the neurogenic inflammation in which SP is a key mediator. The oedema is due to an increased vascular permeability and this is mediated by NK-1Rs located in the endothelial cells. SP causes vasodilatation by acting directly on smooth-muscle cells and indirectly by stimulating histamine release from mast cells. In inflammatory processes, SP also contributes to leukocyte recruitment: via the NK-1R, the expression of adhesion molecules was up-regulated in endothelial cells, and an increase in chemokine production or chemotaxis was reported in leukocytes (4). During inflammation, leukocytes can trigger endogenous antinociception through the release of opioid peptides and the activation of opioid receptors located in peripheral sensory neurons (4). Peripheral/systemical, but not intrathecal, NK-1R

Key words: NK-1 receptor antagonist; oral cavity; oral inflammation; oral pain; tachykinin; tooth pulp

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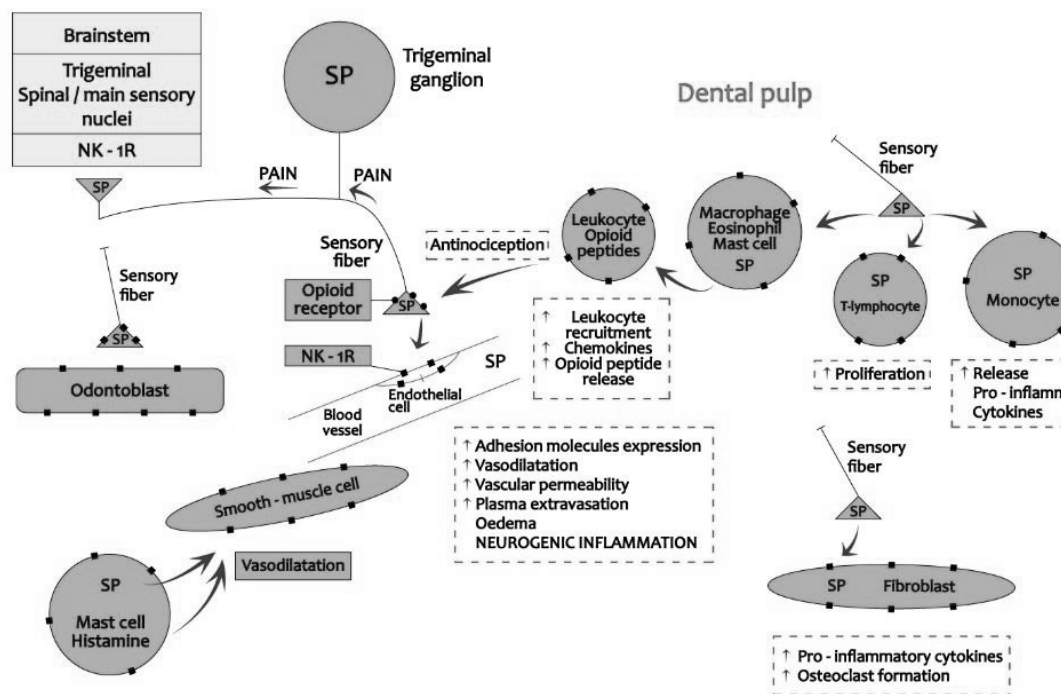


Fig. 1. Possible NK-1R antagonist targets in human dental pulp. The effects (delimited by dashed lines) of substance P (SP) are also shown.

blockade by NK-1R antagonists reduced the stress-induced antinociception and the recruitment of opioid-containing leukocytes (4). The peripheral blockade of the NK-1R did not alter the endothelial expression of intercellular adhesion molecule-1, but decreased the recruitment of macrophages/polymorphonuclear cells. The data suggest that NK-1R antagonists impair the recruitment of opioid-containing leukocytes and stress-induced antinociception, because the endogenous inflammatory pain inhibition depends on the NK-1R-mediated recruitment of opioid-containing leukocytes (4). SP is synthesized by human T-lymphocytes and, after activation with lipopolysaccharides, by dendritic cells/mononuclear phagocytes. SP induces T-lymphocyte proliferation, and most of the immune cells producing SP also express NK-1Rs. SP activates NF- κ B, a transcription factor involved in the control of cytokine expression, and stimulates human peripheral blood monocytes to produce pro-inflammatory cytokines. SP is also synthesized and secreted by monocytes,

macrophages, eosinophils and mast cells (5). SP acts not only as a mediator of the crosstalk between the nervous and the immune systems, but also acts independently in an autocrine/paracrine manner (Fig. 1).

The periodontium is highly innervated by unmyelinated or myelinated sensory fibers containing SP. Tooth movements activate these fibers and then the undecapeptide is released from them, which induces a local inflammatory response (6). In the tooth pulp, SP-containing fibers are widely distributed; these fibers are observed around the blood vessels and are also associated to odontoblasts. The undecapeptide observed in these fibers is synthesized by perikarya located in the trigeminal ganglion and, via a somatofugal axonal transport system, it is transported to nerve terminals (Fig. 1). SP is also released during common dental procedures (e.g., cavity preparation) (Table I).

The level of SP was increased in human dental pulps with asymptomatic inflammation, in patients

affected by granuloma, pulpitis or caries, and in those undergoing standard orthodontic/dental care procedures (7). In fact, it is known: that the tissue level of the peptide was increased in patients with irreversible pulpitis; that the level of SP was also increased in pulps from painful teeth; that the level of the undecapeptide was significantly greater in carious than in sound teeth; and that the level of SP in the human tooth pulp increased with the progression of caries (Table I).

Primary afferent neurons transmit the pain input from the tooth pulp to the brainstem trigeminal main sensory and spinal nuclei (Fig. 1). Dental pain is mediated by neurons expressing SP; the nociceptive stimulus occurs when the undecapeptide is released from nerve terminals in the brainstem (e.g., spinal trigeminal nucleus); in addition, the release of SP in the dental pulp promotes neurogenic inflammation (5) (Fig. 1). Tooth pain can induce a neurogenic inflammatory reaction in the gingiva in association with local elevations of matrix metalloproteinase (MMP)-8 (Table I), which is considered the major tissue destructive protease in gingival crevice fluid (GCF). Tooth pain promotes an increase of MMP-8 and SP levels in GCF, producing simultaneously a local neurogenic inflammation from intrapulpal to surrounding periodontal tissues. It has been also shown that the periradicular inflammation of endodontic origin increased the levels of MMP-8 and SP in GCF (Table I). In experimental animals, the distribution of fibers containing SP has been studied

in peri-implant mucosa around titanium dental implants. These fibers innervate abundantly the peri-implant mucosa. Peri-implant epithelial cells, intraepithelial nerve axons, neutrophils, fibroblasts, nerve fibers in the connective tissue and endothelial cells express NK-1Rs. These morphological data suggest that, in response to neurogenic inflammation (as in healthy gingiva), the SP/NK-1R system is involved in the extravasation of the crevicular fluid, pain transmission and in the endocytosis/migration of neutrophils. In dental pulp, fibroblasts are the most abundant cell type; this cell has been involved in the inflammatory response. SP has been observed in pulpal fibroblasts and the expression of the NK-1R has been also reported in culture of fibroblasts. Stimulation of human dental pulp fibroblasts by SP increased, in a time- and concentration-dependent manner, the levels of pro-inflammatory cytokines and the formation of osteoclasts during orthodontic treatment (Fig. 1). In rat dental pulp, the presence of NK-1Rs has been reported in odontoblasts, nerve terminals associated with the processes of the odontoblasts, endothelial cells of capillaries and post-capillary venules and fibroblasts. In human pulp tissue samples, the highest number of NK-1Rs was found in pulps from teeth having an acute irreversible pulpitis, followed by pulps having an induced inflammation (Table I), whereas the lowest number of these receptors was observed in healthy pulps. Thus, in human pulp, the expression of the NK-1R tissue was significantly increased during neurogenic

Table I. Increased levels OF SP, NK-1R and matrix metalloproteinase (MMP)-8 in human dental pulp and gingival crevicular fluid.

SP (Dental pulp)	Standard orthodontic/dental procedures Asymptomatic inflammation Granuloma Pulpitis Caries Painful tooth
NK-1R (Dental pulp)	Acute irreversible pulpitis Inflammation
SP/MMP-8 (Gingival crevicular fluid)	Tooth pain Periapical inflammation of endodontic origin

inflammation (e.g., acute irreversible pulpitis). In a clinical trial, sixteen healthy premolars were selected from eight patients who were undergoing extraction for orthodontic purposes (8), eight of which were randomly used as controls, and the other eight were assigned to an experimental group (controlled orthodontic intrusive forces applied for 24 h). After this period, teeth were extracted, and pulp samples were obtained. Samples were processed to quantify the expression of SP, calcitonin gene-related peptide, methionine-enkephalin and β -endorphin. After 24 h of the intrusive stimulus, only the level of SP was significantly increased (Table I). This means that, in oral pain and neurogenic inflammation, a correlation between the symptomatic clinical and the increased expression of SP occurs (8). Two studies have been recently performed regarding the effect of a non-steroidal anti-inflammatory drug (ketoprofen) on the gingival and pulpal levels of SP (9, 10). In both cases, ketoprofen did not affect the level of SP.

Thus, many data demonstrate that the SP/NK-1R system is involved in oral pain and inflammation (Fig. 1), suggesting that these pathologies could be treated with NK-1R antagonists. Currently, two types of antagonists are reported: peptide NK-1R antagonists (chemical modifications of SP, in which L-amino acids are replaced by D-amino acids; these compounds can be toxic, show a lower affinity for the NK-1R than natural ligands, are degraded by peptidases and do not cross the blood-brain barrier) and non-peptide NK-1R antagonists (11). Non-peptide NK-1R antagonists (compounds with different chemical composition) are not degraded by peptidases, they are lipid soluble and cross the blood-brain barrier. These antagonists have been used to produce antiemetic, anxiolytic, antidepressive, analgesic, antitumor and anti-inflammatory effects and to treat certain forms of urinary incontinence (11). NK-1R antagonists have been also suggested for the treatment of viral infection and pruritus (1, 11). Moreover, these antagonists did not provoke serious side-effects (even administering 300 mg/day), although drowsiness, vertigo, hiccapping and headaches have been reported (12). In clinical practice only three NK-1R antagonists are currently used for the treatment of vomiting and nausea: aprepitant

(L-754,030, MK-869, Emend), its intravenous administered pro-drug fosaprepitant (L-758,298, MK-0517, Ivemend) and rolapitant (Varubi). The NK-1R antagonist aprepitant was ineffective in human post-operative dental pain when administered at a dose known to be anti-emetic. This was probably due to the low dose administered; in order to obtain an analgesic effect, the dose of NK-1R antagonists must be increased. However, in humans, the NK-1R antagonist CP-99,994 acted as an analgesic in post-operative dental pain. In patients undergoing third molar extraction, the analgesic efficacy of CP-99,994 was compared with ibuprofen. In this clinical trial (double-blind), the initial 60 subjects randomly received, before the oral surgery, one of the following treatments: CP-99,994 (750 μ g/kg infused intravenously over 5 h on a tapering regimen starting 2 h before surgery); ibuprofen (600 mg, oral, 30 min before surgery) or placebo. Compared with placebo, ibuprofen decreased pain from 90 to 240 min postoperatively, whereas CP-99,994 promoted analgesia that was significant at 90 min, but not at subsequent time points. Later, a second study was performed: 18 subjects were randomized to the same regimens starting 30 min before surgery to maximize the amount of CP-99,994 circulating during pain onset. In comparison to placebo, ibuprofen and, to a lesser extent, CP-99,994 blocked pain at 60, 90, and 120 min. The data show that SP is involved in tooth pain and that NK-1R antagonists may be clinically useful. In comparison with animal models, it is true that NK-1R antagonists are rarely analgesic in humans, but this may be explained by poor penetration/specificity of the antagonist and/or the timing/dose of the antagonist treatment.

In summary, in tooth pulp, the SP/NK-1R system mediates dental pain and promotes neurogenic inflammation. For the treatment of these pathologies, the dose of NK-1R antagonists (e.g., aprepitant) must be increased in future human clinical trials.

Conflicts of interest

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LETTER TO THE EDITOR

RISK FACTORS OF PATIENTS WITH INTERSTITIAL LUNG DISEASE COMPLICATED WITH RHEUMATOID ARTHRITIS

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To the Editor,

Interstitial lung disease (ILD) is a heterogeneous disease which is characterized by inflammation and fibrosis of the pulmonary interstitium, alveolar walls, and alveolar space (1). Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease which is featured by chronic symmetric arthritis with multiple organ involvement, and its etiology is still unclear (2). Among the various pulmonary complications of RA, ILD is the most devastating, with its effects ranging from subclinical inflammation/scarring to end-stage pulmonary interstitial fibrosis (3). The pathogenesis of RA-ILD is still unclear. High-resolution computed tomography (HRCT) significantly increased the sensitivity and accuracy of RA-ILD diagnosis compared with single chest radiograms (4). Exertional dyspnea and insidious cough are the main clinical symptoms of RA-ILD (5). Early RA-ILD symptoms are atypical and easy to be missed because the development of respiratory symptoms in most patients with RA and the development of symptoms in subclinical ILD patients over time are unknown prior to the emergence of HRCT imaging evidence for the ILD. Once the ILD is present, symptoms usually progress over time, but the rate of progression varies between individuals and the various pathological types of

ILD. With progression of the disease, there may be a decrease in diffuse function, cyanosis, pulmonary hypertension and other manifestations (6). Therefore, the RA (RA-ILD) with ILD is screened out according to the results of HRCT examination in the research. The risk factors of RA-ILD are analyzed to provide references for the diagnosis, treatment and prognosis of RA-ILD.

MATERIALS AND METHODS*Patients*

Ninety-six RA patients from the Hunan Provincial People's Hospital were selected between November 2017 and March 2019. The subjects had at least 1 joint with swelling and pain, and evidence of synovitis (clinical or ultrasound or MRI). Arthritis caused by other diseases were excluded. Cases with typical RA bony destruction in conventional radiographs were also included. RA combined with ILD (RA-ILD) was finally screened by high-resolution computed tomography (HRCT) according to the ILD diagnostic criteria established by the American Thoracic Society/European Respiratory Association (ATS/ERS) in 2010. The study protocol was approved by the Ethics Committee of Hunan Provincial People's Hospital and all the patients signed informed consent for their participation.

*Key words: interstitial lung disease; rheumatoid arthritis; risk factors; ventilatory dysfunction**Corresponding Author:*

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Exclusion criteria were as follows: Patients with other lung diseases, such as pulmonary infection, tuberculosis, chronic obstructive pulmonary disease, pulmonary heart disease, bronchial asthma, bronchiectasis, sarcoidosis and lung cancer; Patients with pulmonary interstitial diseases caused by inhalation of organic matter excluding pneumoconiosis; Patients with chronic heart, lung, liver, and kidney dysfunction; Patients with long-term use of gold compound, penicillamine, methotrexate and other anti-rheumatic drugs.

The RA patients enrolled were divided into two groups: 50 RA patients without ILD (RA-nILD group) and 46 RA with ILD (RA-ILD group). The 46 patients in the RA-ILD group included 16 males and 30 females with a mean age of 56.3 ± 18.43 years (range, 28-82 years). The average duration of the disease was 9.57 ± 8.93 years (range, 1 month to 38 years). The 50 patients in the RA-nILD group, included 9 males and 41 females with a mean age of 49.54 ± 19.35 years (range, 22-85 years). The average duration of the disease was 8.73 ± 7.53 years (range, 4 months to 33 years).

General information of the patients was collected, including name, gender, age, history of smoking (smoking with ≥ 200 -400 packs/year considered as smoking history), morning stiffness time, number of tender joints when admitted to hospital, number of swollen joints when admitted to hospital, joint function grading, breathing performance, RA disease duration.

Laboratory indicator testing

All laboratory indicators were tested at the laboratory

of the hospital and were the results of their first visit. Erythrocyte sedimentation rate (ESR) (Wei's method), C-reaction protein (CRP), rheumatoid factor (RF), complement C3 and C4, IgA, IgG, IgM were determined by immunoturbidimetry. The anti-cyclic citrulline peptide antibody (anti-CCP antibody) was tested by enzyme-linked immunosorbent assay (ELISA). Fibrinogen and d-dimer were analyzed by CS-5100 automatic coagulation analyzer of Hisen Meikang, Japan. High-resolution computed tomography (HRCT) scan of the lung was used to observe whether it was normal or whether there was ground glass shadow, mesh, honeycomb shadow, consolidation shadow, nodular shadow and interlobular septal thickening or increased lesions.

Pulmonary function test

Patients' lung function was measured using Gansmann Power Cube LF8.5M RC31, which covered carbon monoxide diffusing capacity (DLCO), vital capacity (VC), functional residual capacity (FVC), forced expiratory volume in one second (FEV1), and the ratio of forced expiratory volume in 1 second to forced vital capacity (FEV1/FVC). According to the 2005 ATS/ERS joint guidelines for pulmonary function, ventilatory dysfunction, small airway dysfunction, and diffuse dysfunction can be diagnosed from these data.

Statistical analysis

Data analysis was performed using SPSS 22.0 statistical software and the measurement data were expressed as ($\bar{x} \pm s$). The count data were described by the

Table I. Comparison of risk factors between RA-ILD patients and RA-nILD patients

Indicators	RA-ILD		RA-nILD		χ^2 value	P value
	Number of cases	Proportion (%)	Number of cases	Proportion (%)		
Male	16	34.78	9	18.00	7.018	0.007
Older age (≥ 75 years)	6	13.04	2	4.00	6.433	0.013
History of smoking	12	26.09	5	10.00	5.612	0.024
High titer RF	34	73.91	29	58.00	6.189	0.015
High titer CCP	42	91.30	46	92.00	0.015	0.883

composition ratio. Statistical difference was determined by using two independent sample *t*-tests and Pearson χ^2 test. $P < 0.05$ indicated different difference.

RESULTS

Comparison of risk factors between the two groups

As seen in Table I, the ratios of males, seniors, smoking history, and high titer RF in the RA-ILD

group were significantly higher than those in the RA-nILD group ($P < 0.05$).

Comparison of indicators between the two groups of patients

Table II showed that indicators of joint function such as X-ray staging, erythrocyte sedimentation rate (ESR) and CRP in RA-ILD patients were significantly higher than those in RA-nILD patients

Table II. Comparison of clinical indicators and severity indicators of disease activity in RA-ILD patients and RA-nILD patients

Indicators	RA-ILD	RA-nILD	T value	P value
Morning stiffness time (h)	14.57±13.43	15.43±11.26	-0.213	0.844
Number of tender joints when admitted to hospital	12.25±10.35	11.64±8.52	0.623	0.528
Number of swollen joints when admitted to hospital	8.35±7.52	8.89±7.32	-0.131	0.894
X-ray staging	2.68±0.74	2.17±0.85	2.642	0.004
Joint function grading	2.31±0.73	1.93±0.62	2.902	0.006
ESR (mm/h)	60.17±24.25	49.36±26.52	2.224	0.024
CRP (mg/mL)	49.38±37.26	29.63±21.74	2.642	0.005
RF (IU/mL)	427.13±348.43	466.63±394.43	-0.197	0.834
Anti - CCP antibody (RU/mL)	136.35±94.23	110.34±85.34	2.042	0.039
Complement C3	1.33±1.05	1.14±1.01	1.825	0.075
Complement C4	0.19±0.13	0.23±0.12	-2.153	0.031
Fibrinogen (mg/L)	4.03±1.65	3.79±1.35	0.899	0.382
D-dimer (mg/L)	2.56±2.25	3.35±2.62	-1.387	0.167
IgA(g/L)	3.94±2.07	2.87±1.32	2.243	0.026
IgG(g/L)	17.38±8.73	15.03±9.37	2.123	0.034
IgM(g/L)	1.60±0.77	1.64±0.92	-2.234	0.787

Table III. Comparison of lung function indicators between the two groups

Indicators	RA-ILD group	RA-nILD group	$\chi^2(t)$ value	P value
DLCO (%)	57.43±22.13	82.23±25.23	<0.001	-6.134a
VC (%)	75.87±18.34	90.34±19.13	<0.001	-3.873a
FVC (%)	76.76±18.62	91.45±17.12	<0.001	-4.183a
FEV1.0 (%)	78.97±21.23	98.24±20.32	<0.001	-3.785a
FEV1.0/FVC (%)	100.24±13.24	105.35±11.34	0.459	-0.754a
Ventilatory dysfunction [n (%)]	32(69.57)	6(12.00)	<0.001	18.284
Small airway dysfunction [n (%)]	26(56.52)	11(22.00)	0.135	1.984
Diffuse dysfunction [n (%)]	38(82.61)	12(24.00)	<0.001	16.231

Note: DLCO: carbon monoxide diffusion; VC: vital capacity; FVC: forced vital capacity; FEV1: forced expiratory volume in 1 second; FEV1/FVC: the ratio of forced expiratory volume in 1 second to forced vital capacity; a denotes the *t* value.

($P < 0.05$). The indexes of the RA-ILD group, IgA and IgG were higher than that of the RA-nILD group, and the indexes of RF, complement C4 and IgM were lower than those of the RA-nILD group.

Imaging performance of RA-ILD group

The most common manifestation of pulmonary HRCT in the RA-ILD group was ground-glass opacity. In patients with RA-ILD, ILD classification includes acute interstitial pneumonia (AIP), idiopathic pulmonary fibrosis (IPF), nonspecific interstitial pneumonia (NSIP), cryptogenic organizing pneumonia (COP), respiratory bronchiolitis and interstitial lung disease (RB-ILD), desquamative interstitial pneumonia (DIP), and unclassifiable idiopathic interstitial pneumonia. These are shown in Fig. 1.

Pulmonary function tests in patients with RA-ILD

As seen from Table III, the DLCO, VC, FVC and FEV1 of the RA-ILD group were significantly lower compared to the RA-nILD group ($P < 0.001$). The RA-ILD group had a significantly higher incidence of ventilatory dysfunction and diffuse dysfunction ($P < 0.001$).

DISCUSSION

Numerous studies at home and abroad have confirmed that the occurrence of ILD is closely related to high titer RF (7, 8). The results of this study showed that high titer RF levels in serum of RA-ILD patients were significantly higher than those in RA-nILD patients, indicating that high titer RF was associated with RA-ILD.

Studies have shown conflicting results in the levels of

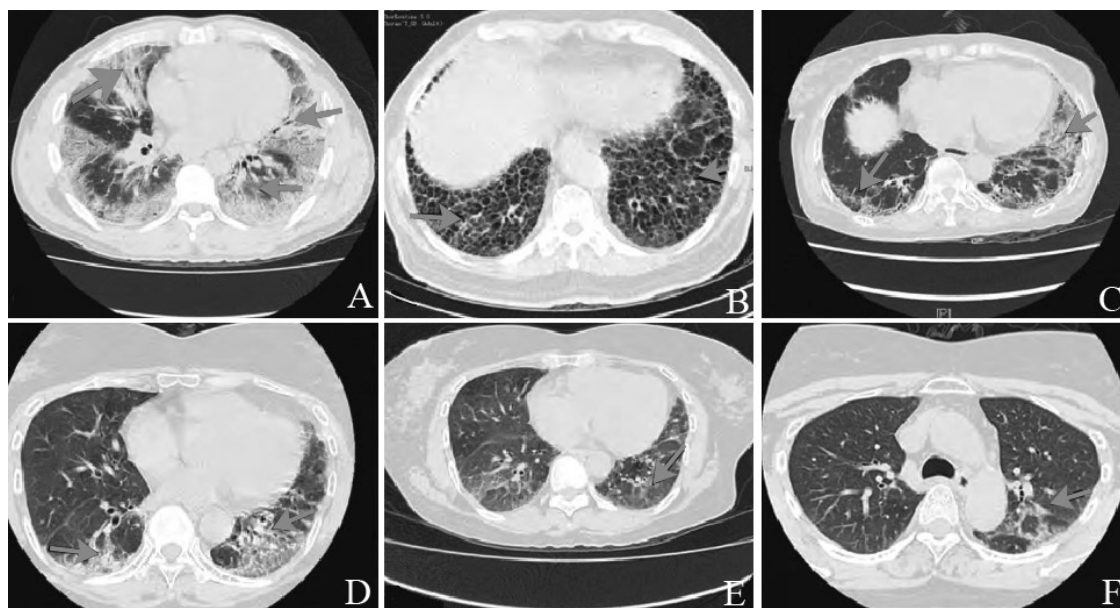


Fig. 1. Lung HRCT image of various RA-ILD. **A)** AIP. CT lung window showed diffuse large slices of consolidation opacity and ground-glass opacity (indicated by the arrow); **B)** IPF. High-resolution CT image of lung window showed extensive cellular opacity changes in both lungs (indicated by the arrow) **C)** NSIP. CT image of lung window showed patchy opacity and ground-glass opacity in the lungs. Some normal pleura was seen (indicated by the arrow); **D)** COP. CT lung window showed multiple patchy consolidation opacity in both lungs, mainly in the lower lung and subpleural area. Air bronchogram (shown by the arrow) was observed; **E)** CT lung window showed that the lung lesions were significantly absorbed before treatment by hormones (as indicated by the arrow); **F)** COP. CT lung window showed a sub-pleural circular shadow in the right lower lung. A slightly lower density of opacity in the lesion showed a typical reversed halo sign (indicated by the arrow).

complement C3 and C4 in patients with RA (9, 10). The results of this study suggest that the level of complement C3 in RA-ILD patients was higher than that in RA-nILD group, while the level of C4 was lower than that in RA-nILD group. This may be related to the following factors: RA's different role in the immune system at different stages of the disease; the use of immunomodulatory drugs and/or hormones in the treatment of RA; and differences in immune abnormalities in RA patients with different levels of activity.

The reticular shadow in HRCT of RA-ILD patients was mainly attributed to the thickening of multiple interlobular septum, and the ground glass changes mainly indicated active alveolitis. Zou et al. (11) pointed out that ground-glass opacity is the most common chest CT manifestation of RA-ILD. This study found that in the RA-LD group, the majority of lung HRCT showed ground glass opacity. This study found that in the RA-LD group, lung HRCT showed ground glass opacity in most patients, which was mainly found in both lower lung and subpleural areas.

Loss of diffuse function in patients with RA with ILD is the earliest and most common pulmonary change. The determination of lung function DLCO mainly reflects the diffuse function, and the decrease of DLCO indicates the decreased diffuse function (12).). In this study, 82.61% of RA-ILD patients had diffuse dysfunction, which was significantly higher compared to the RA-nILD group. At the same time, DLCO, VC, FVC and FEV1 in RA-ILD group were lower than those in RA-nILD group. Therefore, monitoring the lung function of patients with early RA without respiratory symptoms, especially DLCO, is valuable for early detection and diagnosis of ILD.

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LETTER TO THE EDITOR

EFFICACY OF CONTROLLED OVARIAN STIMULATION IN BOTH FOLLICULAR AND LUTEAL PHASES FOR PATIENTS WITH POOR OVARIAN RESPONSE

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To the Editor,

An increasing number of women have received *in vitro* fertilization-embryo transfer (IVF-ET) in advanced maternal age. However, these women are usually faced with higher risk of diminished ovarian reserve and poor ovarian response (POR) during assisted reproduction. As a result, problems such as reduced number of oocytes, poor quality of oocytes and low pregnancy rate abound (1, 2). POR may deteriorate during the course of treatment. Determining how to obtain more oocytes and increase the number of embryos and pregnancy rate are the major concerns of fertility centers (3). In the present study, controlled ovarian stimulation (COS) in both the follicular and luteal phases was used, and its efficacy for POR women was discussed.

MATERIALS AND METHODS

Subjects

Sixty-eight patients with infertility, who received IVF/ICSI-ET at the Fertility Center of the Affiliated Hospital of Southwest Medical University from January 2015 to April 2018, were reviewed. The causes included oviduct infertility, male factor infertility, endometriosis-associated infertility as well as infertility for unknown reasons. The Bologna

criteria were used (4), and the patients included in the study satisfied at least two of the following three criteria: i) age ≥ 40 , or having risk factors for POR and diseases that might reduce follicle number, including chromosomal or genetic abnormalities, pelvic cavity infection, history of ovarian and fallopian tube surgery, and chemotherapy history; ii) POR in previous IVF and a number of oocytes of ≤ 3 using conventional protocols; iii) diminished ovarian reserve, that is, antral follicle count of < 5 or anti-Müllerian hormone (AMH) < 0.5 ng/ml.

All patients provided a signed informed consent, and were considered eligible according to China's family planning policy. Cases of infertility caused by women's endocrine and metabolic abnormalities, genital abnormality and autoimmune diseases were excluded.

Controlled ovarian stimulation

The 68 patients were randomly divided into two groups, A (33 subjects) and B (35 subjects). For group A, ultra-short protocol plus luteal phase ovarian stimulation was used. For group B, mild stimulation protocol in the follicular phase plus luteal phase ovarian stimulation was used. The ultra-short protocol comprised of an intramuscular injection of dipherelin (Ipsen Pharmaceutical Co., Ltd.) from the

Key words: in vitro-fertilization-embryo transfer; poor ovarian response; luteal phase ovarian stimulation

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second to the fourth day of the menstrual cycle plus 225 u/d of urofollitropin (Livzon Pharmaceutical) from the third day of the menstrual cycle. The mild stimulation protocol comprised of 50 mg/d of clomiphene citrate oral (Cyprus) from the third day of the menstrual cycle plus an intramuscular injection of 150 IU/d of menotrophin (HMG, Livzon Pharmaceutical). For both protocols, the dosage was adjusted according to follicular development and hormone levels. A subcutaneous injection of 250 ug of Ovidrel (Merck Sharp) was performed when transvaginal ultrasonography indicated a follicular diameter of ≥ 17 mm, in order to induce follicular maturity. Oocytes were retrieved under the guidance of transvaginal ultrasonography 34-36 h after the injection. Patients with ≥ 3 small follicles in the bilateral ovaries after oocyte retrieval provided a signed informed consent for luteal phase ovarian stimulation. COS continued from the second day after oocyte retrieval using 10 mg of medroxyprogesterone acetate + HMG at 225 u/d. Follicular development was monitored for 4-5 days. The dosage was adjusted, and another subcutaneous injection of 250 ug of Ovidrel was given to induce follicular maturity when transvaginal ultrasonography indicated a follicular diameter of ≥ 17 mm. The oocytes were retrieved again under the guidance of transvaginal ultrasonography after 34-36 h. The oocytes obtained by the two retrievals were used for artificial fertilization at 4-6 h after the retrieval. The fertilization of the ovum was observed at 16-18 h after oocyte retrieval, and the embryos were frozen at 72 h after oocyte retrieval.

Embryo transfer

The evaluation was performed on the second day of the third menstrual cycle after oocyte retrieval. Patients with two or more embryos received frozen embryo transfer (FET), while patients with only one embryo entered the second COS cycle. Endometrial preparation for FET was conventionally performed, which included the natural cycle and hormone replacement cycle. After the embryo transfer, dydrogesterone at 20 mg/d + progesterone injection at 60 mg/d was given for luteal support. Serum hCG level was measured at 14 days after the

transfer. Patients with >3 mIU/ml were considered as biochemical pregnancy, and received further luteal support. Transvaginal ultrasonography was performed at 28 days after the transfer, and the presence of gestational sacs indicated clinical pregnancy.

Observation indicators

The observed indicators were the number of oocytes, number of MII oocytes, number of good-quality embryos on the third day, rate of clinical pregnancy, and rate of embryo implantation. The rate of embryo implantation was the ratio of gestational sacs found by transvaginal ultrasonography at 28 days after FET to the total number of transferred embryos.

Statistical process

All statistical analyses were performed using the SPSS 19.0 software. Measurements were presented as $\bar{x} \pm$ standard deviation (SD). Independent-samples *t*-test was performed for intergroup comparisons. Counts were presented in percentage (%) and analyzed using the χ^2 or Fisher's exact test. $P < 0.05$ was considered statistically significant.

RESULTS

Baseline information

A total of 68 patients received COS in the follicular phase plus luteal phase ovarian stimulation. Among these patients, 33 cases were assigned to group A and 35 cases were assigned to group B. Furthermore, of these 68 patients, 14.71% (10/68) had ≤ 3 oocytes retrieved by conventional protocol during the previous IVF cycle, and 35.29% (24/68) were ≥ 40 years old. The age of all patients ranged from 31-43 years (9.01 ± 2.25 years), and the average duration of infertility was 7.03 ± 4.95 years (1-20 years). Patients in both groups did not significantly differ in terms of age, duration of infertility, and AMH. Furthermore, there were no significant differences in the number of oocytes retrieved by the luteal phase ovarian stimulation, number of MII oocytes, number of good-quality embryos on the third day, rate of oocyte retrieval failure, and rate of having no transferable embryos (Table I).

Table I. Baseline characteristics and effects of luteal phase ovarian stimulation in the two groups

Indicator	Group A	Group B	t/ χ^2	P
Baseline characteristics				
Number of oocyte retrieval cycles	33	35		
Age (year)	38.94±2.54	39.09±1.98	-0.27	0.79
Duration of infertility (year)	6.15±4.34	7.86±5.40	-1.43	0.16
Body mass index	23.43±2.80	22.51±2.54	1.42	0.16
Baseline antral follicle count	4.58±0.83	4.66±1.08	-0.35	0.73
Baseline E ₂ (pg/ml)	41.04±12.17	45.92±12.64	-1.62	0.11
Baseline FSH(IU/L)	12.11±2.61	11.95±2.17	0.27	0.79
AMH	1.11±0.52	1.09±0.40	0.24	0.81
Luteal phase ovarian stimulation				
Number of oocytes retrieved (n)	2.48±0.94	2.57±0.98	-0.37	0.71
Number of MII oocytes (n)	2.15±0.94	2.01±0.87	-0.22	0.83
Number of good-quality embryos on the third day (n)	1.24±0.79	1.03±0.75	1.15	0.26
Rate of MII oocytes (%)	86.59(71 /82)	87.83(77 /90)	0.04	0.85
Rate of good-quality embryos (%)	53.33(32/60)	59.32(35/59)	0.43	0.51
Rate of oocyte retrieval failure (%)	6.06(2/33)	5.71(2/35)	0.00	0.95
Rate of having no transferable embryos (%)	21.21(7/33)	25.71(9/35)	0.19	0.66

Table II. Comparison of effects of ovarian stimulation in different phases

Indicator	Ovarian stimulation in follicular phase	ovarian stimulation in luteal phase	Two oocyte retrieval within one cycle
Group A			
Number of oocytes retrieved (n)	2.15±0.91	2.48±0.94	4.64±1.62 ^{*#}
Number of MII oocytes (n)	1.76±0.83	2.15±0.94	3.91±1.53 ^{*#}
Number of good-quality embryos on the third day (n)	1.36±0.78	1.24±0.79	2.61±1.41 ^{*#}
MI I oocyte ratio (%)	81.70(58/71)	86.59(71/82)	84.31(129 /153)
Rate of good-quality embryos (%)	65.31(32/49)	53.33(32/60)	58.72(64/109)
Rate of oocyte retrieval failure (%)	6.06(2/33)	6.06(2/33)	0.00(0/33)
Rate of having no transferable embryos (%)	18.18(6/33)	21.21(7/33)	12.12(4/33)
Group B			
Number of oocytes retrieved (n)	1.86±0.77	2.57±0.98 [*]	4.43±1.46 [*]
Number of MII oocytes (n)	1.63±0.73	2.20±0.87 [*]	3.83±1.25 [*]
Number of good-quality embryos on the third day (n)	0.86±0.60	1.03±0.75	1.89±1.18 [*]
MI I oocyte ratio (%)	87.69(57/65)	85.56(77 /90)	86.45(134/155)
Rate of good-quality embryos (%)	61.22(30/49)	61.02(36/59)	61.11(66/108)
Rate of oocyte retrieval failure (%)	11.43(4/35)	5.71(2/35)	0.00(0/35)
Rate of having no transferable embryos (%)	25.71(9/35)	25.71(9/35)	14.29(5/35)

^{*} $P < 0.05$ compared with ovarian stimulation in the follicular phase; [#] $P < 0.05$ compared with luteal phase ovarian stimulation, $P < 0.05$

Comparison of the effect of ovarian stimulation in different phases

A comparison was performed between the different protocols. For group A, the average number of oocytes, number of MII oocytes and number of good-quality embryos on the third day did not significantly differ between COS in the follicular phase and luteal phase ovarian stimulation ($P>0.05$). For group B, the average number of oocytes and number of MII oocytes were 1.86 ± 0.77 and 1.63 ± 0.73 , respectively, by COS in the follicular phase, while those by luteal phase ovarian stimulation were 2.57 ± 0.98 and 2.20 ± 0.87 , respectively. There was a significant increase with the latter, when compared with the former ($P<0.05$). However, there were no significant differences in the number of good-quality embryos on the third day, MII oocyte ratio or rate of good-quality embryos between COS in the follicular phase and luteal phase ovarian stimulation. The results of these two ovarian stimulations in the same cycle were combined for each group. It was found that for both groups, the total number of oocytes retrieved, total number of MII oocytes and number of good-quality embryos on the third day after two oocyte retrievals were significantly higher than that of the oocyte retrieval in either the follicular phase or luteal phase ($P<0.05$). However, no significant differences were found in MII oocyte

ratio and rate of good-quality embryos (Table II). No oocytes were retrieved in the follicular phase for two patients in group B. However, among these patients, three and two oocytes were retrieved in the luteal phase, respectively. Furthermore, no oocytes were retrieved in the follicular phase for four patients in group A. However, among these patients, 1, 3, 2 and 1 oocytes were retrieved in the luteal phase, respectively. Thus, no oocytes were retrieved in six patients in the follicular phase (ultra-short protocol in two patients and mild stimulation protocol in four patients), while 15 patients had no transferable embryos (ultra-short protocol in six patients and mild stimulation protocol in nine patients). Furthermore, four patients from the two groups had no oocytes retrieved by luteal phase ovarian stimulation, and 15 patients had no transferable embryos. However, through two oocyte retrievals within one cycle, oocytes were retrieved from all patients, while nine patients had no transferable embryos.

Comparison of effects of different protocols in the follicular phase plus luteal phase ovarian stimulation

The number of good-quality embryos on the third day was 2.61 ± 1.41 and 1.89 ± 1.18 using the ultra-short protocol or mild stimulation protocol in the follicular phase plus luteal phase ovarian stimulation, respectively, and the difference was statistically

Table III. Comparison of effects of different protocols in the follicular phase plus luteal phase ovarian stimulation

Indicator	Ultra-short protocol plus luteal phase ovarian stimulation	Mild stimulation protocol plus luteal phase ovarian stimulation	t/χ^2	P
Number of cycles	33	35		
Total number of oocytes retrieved (n)	4.64 ± 1.62	4.43 ± 1.46	0.56	0.58
Total number of MII oocytes (n)	3.91 ± 1.53	3.83 ± 1.25	0.24	0.81
Total number of good-quality embryos on the third day (n)	2.61 ± 1.41	1.89 ± 1.18	2.29	0.03
MI I oocyte ratio (%)	84.31(129/153)	86.45(134/155)	0.28	0.60
Rate of good-quality embryos (%)	58.72(64/109)	61.11(66/108)	0.13	0.72
Rate of oocyte retrieval failure (%)	0.00(0/33)	0.00(0/35)		1.00
Rate of having no transferable embryos (%)	12.12(4/33)	14.29(5/35)	0.07	0.79

significant. However, these two protocols did not have significant differences in terms of the total number of oocytes retrieved, total number of MII oocytes, and MII oocyte ratio (Table III).

Results of the frozen embryo transfer

To date, 30 patients received FET, and eight achieved clinical pregnancy, with a pregnancy rate of 26.67%. There were three abortion cases, and the abortion rate was 37.5%. Furthermore, four patients gave live birth, and one patient was still in pregnancy at the time of the study. The embryo implantation rate was 13.33% (8/60).

DISCUSSION

POR patients showed low follicle count in ovaries, poor follicle quality, and low sensitivity to ovulation-promoting drugs during IVF-ET. Ultra-short protocol, mild stimulation protocol and oocyte retrieval in the natural cycle are commonly used for POR patients in IVF-ET (5-7). Consistent with previous data (8), this study showed that, of the total number of oocytes retrieved, MII oocytes and good-quality embryos on the third day were significantly higher compared with those either in the follicular or luteal phase. COS in the follicular phase plus luteal phase ovarian stimulation increases the success rate of oocyte retrieval and the rate of having transferable embryos (9). Results showed that the number of oocytes retrieved in the luteal phase, number of MII oocytes and good-quality embryos on the third day were not significantly different between the two groups, indicating the protocol had little impact on the subsequent luteal phase ovarian stimulation. The average number of oocytes and MII oocytes was increased with luteal phase ovarian stimulation compared to mild stimulation protocol. However, no significant differences were observed in the number of oocytes retrieved, MII oocytes, and good-quality embryos between the two groups. The average number of good-quality embryos was significantly different between the ultra-short protocol and mild stimulation protocol plus luteal phase ovarian stimulation, indicating that more good-quality embryos may be retrieved by ultra-short protocol

plus luteal phase ovarian stimulation. Oocytes retrieved by luteal phase ovarian stimulation may have the same development potential as those retrieved in the follicular phase (10, 11). The present study revealed that the values of clinical pregnancy rate after embryo transplantation, the abortion rate, and the embryo implantation rate was in agreement with the study conducted by Wu et al. (12)

In conclusion, COS in the follicular phase plus luteal phase ovarian stimulation provided two chances of oocyte retrieval during one menstrual cycle (13), which maximizes the number of oocytes available within a short period of time, allows more embryos to be prepared, and increases the success rate of IVF-ET for POR patients.

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LETTER TO THE EDITOR

EFFECTS OF ISOFLURANE ANESTHESIA ON EXPRESSION OF GSK-3 β AND INDUCTION OF COGNITIVE IMPAIRMENT IN AGED RATSH. XU^{1*}, CH. LI^{2*}, DQ. XU³, WH. ZHANG⁴ and XC. GAO⁵

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To the Editor,

Postoperative cognitive dysfunction (POCD) is a common complication of anesthesia, which mainly occurs in the elderly. POCD seriously affects the quality of life of patients. With the improvement of medical technology, the influence of surgery and anesthesia on the cognitive function of the elderly patients has attracted attention. At present, the pathogenesis of POCD has not been fully elucidated. Most scholars think that it is related to neurodegeneration, apoptosis and neuroinflammation (1-3).

Isoflurane is widely used in various operations as an inhalation anesthetic. It has been shown that isoflurane induced POCD is closely related to apoptosis of hippocampal neurons (4). GSK-3 β (glycogen synthase kinase 3 β), as a serine/threonine protein kinase, can be degraded by phosphorylation of β -catenin and target ubiquitination, and then sent to proteasome for digestion, resulting in apoptosis of hippocampal neurons (5). In recent years, it has been shown that GSK-3 β and β -catenin play important roles in the regulation of neurogenesis in hippocampus

(6). The activation of β -catenin-related pathway can promote the neurogenesis of hippocampal cells, but the increase of non-phosphorylated β -catenin leads to overproliferation of neural stem cells (7).

To sum up, there are few studies on the mechanism of isoflurane toxic effects on the hippocampus of the aged brain, and it is of great significance to explore the specific mechanism of isoflurane-induced POCD for clinical treatment. Therefore, in this study, the effects of isoflurane anesthesia on the GSK-3 β expression and cognitive function in the elderly rats were explored, providing new ideas for isoflurane anesthesia.

MATERIALS AND METHODS*Animals*

Forty healthy male SD (Sprague-Dawley) rats (aged 18-20 months and weighing 600-800g) were purchased from Kaixue Biotechnology (Shanghai) Co., Ltd., China. The experimental animals were fed regularly, and allowed free access to food and water. The circadian rhythm was correctly maintained. The ambient temperature was

Key words: isoflurane; GSK-3 β ; cognitive impairment; neural stem cells in dentate gyrus of hippocampus

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15-20°C and humidity was 45-65%. All procedures of the animal experiments conformed to the International Regulations on the Protection and Management of Animals, and the protocol was approved by the ethics committee of Rizhao Central Hospital.

The rats were randomly divided into four groups (10 rats in each group): blank control group (group A); 0.5% isoflurane group (group B); 1% isoflurane group (group C); and 1.5% isoflurane group (group D). Rats in group A inhaled a mixture of 30% oxygen and 70% nitrogen for 4 h. Rats in groups B, C and D inhaled 0.5% isoflurane, 1% isoflurane and 1.5% isoflurane, respectively, for 4 h under the same oxygen inhalation conditions as those in group A.

Five rats were randomly selected from groups A, B, C and D for immunohistochemical staining of BrdU labeled cells. Before the onset of anesthesia, 5-bromodeoxyuridine (BrdU, Shanghai Yuanmu Biotechnology Co., Ltd., China) was injected intraperitoneally at a dose of 200mg/kg. The remaining 5 rats in each group were used for the protein immunoblotting test.

Establishment of isoflurane anesthesia models in rats

The rats were placed in a transparent anesthesia chamber maintained at a constant temperature of 37°C. Both sides of the box were provided with openings connecting the intake and outlet passages. One of the holes of the intake passage was connected to an anesthetic ventilator (Excel 210, Ohmeda, Finland). The inhalation concentration of the volatile tank was set to change the concentration of isoflurane in the anesthesia box. A gas monitor (Datex, Ohmeda, Finland) was connected to one side of the outlet channel to monitor the concentration of oxygen, carbon dioxide and isoflurane in the outflow gas. Rats in group A inhaled 30% oxygen for 4 h. Under the same oxygen inhalation conditions, rats in groups B, C and D inhaled isoflurane at concentrations of 0.5%, 1% and 1.5%, respectively, for 4 h. During the experiment, attention was paid to observe the breathing frequency and skin color of rats in order to maintain uniform breathing and ruddy skin color as well as to avoid hypoxic asphyxia.

Morris water maze test for cognitive function in rats

The Morris water maze test was used to examine the cognitive function of all rats. The animals were all placed in the Morris water maze (Shanghai Xinruan Information

Technology Co., Ltd., China) for 120 seconds to swim freely. Starting from the second day, the rats were trained to find a platform under the water surface. They were trained 5 times every morning and every afternoon, respectively, with an interval of 1 minute. The swimming trajectory of rats was collected by an automatic image surveillance system (Shanghai Xinruan Information Technology Co., Ltd., China) with the speed and time of finding the platform (escape latency) recorded. If the rats failed to find the platform within 120 seconds, help would be given. The escape latency was set as 120 seconds. After 3 consecutive days of training, the rats were removed from the platform and placed in the Morris water maze. The number of times they crossed the original platform within 120 seconds was recorded.

Sampling and preparation of specimens

After anesthesia, the rats were fixed on a foam board to expose their hearts. A needle connecting saline was inserted into the left ventricle. After slightly enlarging the heart, the right atrium was cut to drain the blood and perfusion fluid. Phosphate buffer solution (PBS) with pH of 7.4 at 0.1M (Xiamen Research Biotechnology Co., Ltd., China) was used for cardiac perfusion. After the effluent became clear of blood color, the rat was perfused with 20 mL of polyformaldehyde (4%, Wuhan Chujiang Haoyu Chemical Technology Development Co., Ltd., China). The brain of the rat was then immersed in 4% polyformaldehyde after craniotomy. Hematoxylin eosin staining was used to stain the specimens.

Detection of neural stem cell proliferation in the dentate gyrus of hippocampus by BrdU labeled cell immunohistochemical staining

After conventional dewaxing and baking, the slices were put into 2 mol/L hydrochloric acid (Xi'an Chuangyuan Chemical Co., Ltd., China) and incubated for 15 min (37°C). They were then transferred into borate buffer (0.1mol/L, pH 8.5, Shanghai Xin Yu Biotech Co., Ltd., China) for 10 min. PBS was then used for flushing. The slices were placed in sealing liquid and left to dry for 1 h at room temperature. BrdU-anti-dilution solution (dilution ratio, 1:500) was dripped and incubated in the wet box of immunohistochemistry (4°C, Beijing Bioeasy Technology Co., Ltd., China). After 24 h, it was heated for 30 min at 37°C, washed with PBS, and incubated in

3% hydrogen peroxide (Beijing Think-Far Technology Co., Ltd, China) for 10 min. PBS was used for rinsing, and polymerase assistant (Shanghai Lifefeng Biotech Co., Ltd., China) was added to react for 20 min. After rinsing with PBS, the sheep anti-rabbit/mouse IgG polymer labeled with horseradish peroxidase (Shanghai YuanMu Biological Technology Co., Ltd., China) was dripped and incubated at room temperature for another 30 min. DAB (Diaminobenzidine, Jin Ruibo Xiamen Biotechnology Co., Ltd., China) was added to stain the specimen and the results were observed under a microscope; 5% hematoxylin and ethanol hydrochloride were used for re-staining and differentiation. After dehydration, the slices were sealed. Under an inverted phase contrast microscope (Leica, Germany), the proliferation of neural stem cells in dentate gyrus of hippocampus was observed. Moreover, BrdU positive cells were counted.

BCA protein concentration kit for protein concentration measurement of samples

The rats were sacrificed according to the procedures mentioned above. Craniotomy was performed to harvest the brain. The hippocampal tissue was then dissected and transferred into the EP (Eppendorf) tube. Cytoplasmic protein extract and protease inhibitor were mixed evenly at a ratio of 20:1, and benzyl sulfonyl fluoride (Shanghai Zhenyu Biotechnology Co., Ltd., China) was added to make a final concentration of 1 mM. 300 mL of the mixture was added into the hippocampus tissue before being crushed with a mechanical grinding rod. An ultrasonic crusher (Guangzhou Koster Scientific Instrument Co., Ltd., China) was used to ensure three cycles of complete mixing. The homogenate was then incubated on ice for 10 min, shaken for 10 s every 3 min and then put into a cryogenic centrifuge for 10 min (4°C, 12000 rpm). The supernatant was extracted and placed in a precooled EP tube. It was divided into two parts. One part was used for determination of protein concentrations. The other part was mixed evenly by adding 5×loading buffer (Beijing Noble Rydr Technology Co., Ltd., China) in a ratio of 4:1 before being placed in a dry thermostat for 5 min and stored at -80°C. Different concentrations of BCA standards were then prepared in PBS and added to a marked 96-well plate. 200 µL of BCA working solution was then added to each well, and after thoroughly mixing, was placed in an incubator (37°C) for 30 min and then left to cool. An

automatic microplate reader (Molecular Devices, USA) measured absorbance at a wavelength of 562 nm.

Western blot for detection of β -catenin and P-GSK-3 β protein expressions in rat hippocampus

10% of the separation gel and 4% of the concentration gel were prepared, sampled and subjected to electrophoresis. PVDF (polyvinylidene fluoride) film was used in a Bio-Rad standard wet membrane conversion device. At a constant current of 180mA, the film was transferred for 1.5 h. The PVDF membrane was then removed and dyed with Lichunhong dye solution (Wuhan Purity Biotechnology Co., Ltd., China). After selecting the protein region, it was washed with double steaming water, and sealed in a shaking bed for 60 min with a sealing liquid (5% skimmed milk, room temperature). TBST (Tris-Buffered Saline and Tween 20, Shanghai Youyu Biotechnology Co., Ltd., China) was used to rinse for three cycles of 5 min. Primary antibody dilution (1:1000 dilution ratio) included rabbit anti- β -catenin antibody (Shanghai Guyan Biotechnology Co., Ltd., China), rabbit anti-P-GSK-3 β antibody (Ser9, Wuhan Huamei Bioengineering Co., Ltd., China), and mouse anti- β -actin antibody (Shanghai Zhenyu Biotechnology Co., Ltd., China) were added. After being maintained at 4°C overnight, TBST was used to rinse 3 times for 5 min each cycle. Both the β -catenin and P-GSK-3 β were added with the horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody dilution (1:3000 dilution), while the β -actin strip was added with a horseradish peroxidase-labeled goat anti-mouse IgG secondary antibody dilution (1:3000 dilution). After 1 h, TBST was used to rinse 3 times for 5 min each cycle. In the darkroom, the color was developed with electrochemiluminescence (ECL) working fluid (Nanjing enjing Biotechnology Co., Ltd., China). Odyssey dual-color infrared fluorescence imaging system (LI-COR, USA) was used to scan and the gray value of protein bands was measured.

Statistical analysis

SPSS21.0 statistical software was used for data processing and analysis. Quantitative data that confirmed to normal distribution were described as mean $\pm$ standard deviation. Independent samples *t*-test or rank-sum test was used to compare the two groups. The *Chi*-squared test was used to compare the rates of different groups.

RESULTS

Water maze experiments

On the first day of training, compared with group A, the escape latencies of rats in group B, C and D were prolonged although there was no significant difference ($P > 0.05$). On the 2nd and 3rd days of training, compared with group A, the escape latency of group B rats was not significantly different, while the latencies of group C and D rats were significantly prolonged ($*P < 0.05$). On the fourth day after removing

the platform, the number of rats in group C and D crossing the original platform within 120 seconds was significantly reduced ($*P < 0.05$) (Table I).

Immunohistochemical staining of BrdU labeled cells

The number of BrdU-positive cells and the expression of BrdU-positive protein in rat hippocampus are shown in Fig. 1 and Fig. 2A. The number of BrdU positive cells in the dentate gyrus of the hippocampus in group B decreased ($P > 0.05$). The number of BrdU positive cells in the dentate

Table I. Results of the Morris water maze test for cognitive function in rats

Group	1d (s)	2d (s)	3d (s)	4d (s)
Group A	37.6±5.2	25.6±3.5	12.7±1.7	10.9±1.3
Group B	39.8±6.7 [#]	28.4±4.1 [#]	16.6±2.1 [#]	9.5±1.1 [#]
Group C	48.3±7.3 [#]	37.6±4.8 [*]	27.8±2.5 [*]	6.1±0.5 [*]
Group D	58.9±8.5 [#]	46.1±5.7 [*]	35.7±3.6 [*]	2.2±0.2 [*]

Compared with the rats in group A, [#] $P > 0.05$.

Compared with the rats in group A, ^{*} $P < 0.05$

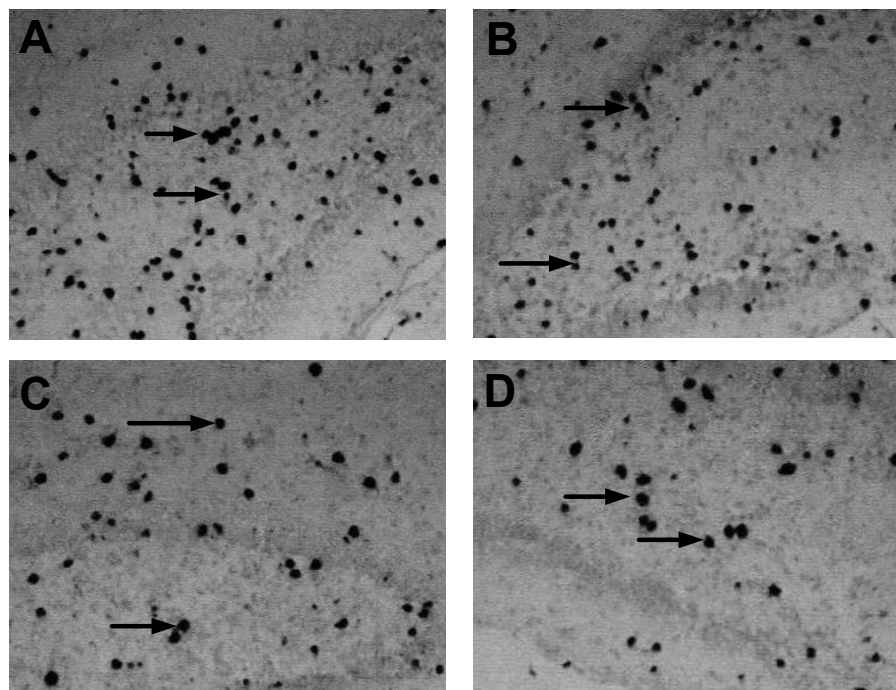


Fig. 1. Observation on the colonization of neural stem cells from the hippocampal dentate gyrus in four groups of rats under the microscope. **A)** blank control group, $\times 100$; **B)** 0.5% isoflurane group, $\times 100$; **C)** 1% isoflurane group, $\times 100$; **D)** 1.5% isoflurane group, $\times 100$; the arrow marks BrdU positive cells.

gyrus of the hippocampus in groups C and D dropped significantly lower than that in group A ($P < 0.05$).

β -catenin protein expressions

In the Western blot test, the expression of the β -catenin protein is shown in Fig. 2B. There was no significant difference in the expression of the β -catenin protein between group B and group A ($P > 0.05$), but the expression of β -catenin protein in group C and group D was significantly lower than that in group A ($P < 0.05$). The expression of β -catenin protein is shown in Fig. 3.

P-GSK-3 β protein expressions

In the Western blot test, the expression of P-GSK-3 β protein in the hippocampus of rats in group B was similar to that in group A ($P > 0.05$), but that in groups C and D was significantly lower ($P < 0.05$). The expression of P-GSK-3 β protein is shown in Fig. 2C and Fig. 3.

DISCUSSION

In this study, immunohistochemical staining of BrdU labeled cells was used to detect the proliferation

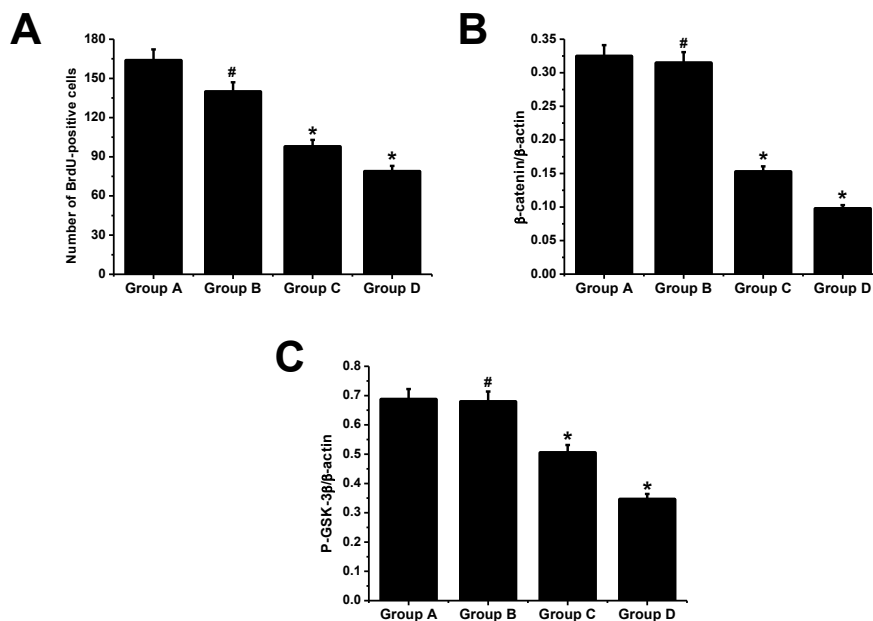


Fig. 2. Number of BrdU positive cells and protein expression results in rat hippocampus. **A)** Number of BrdU positive cells in the hippocampal dentate gyrus of four groups of rats; **B)** expression of the β -catenin protein in the hippocampus of four groups of rats; **C)** P-GSK-3 in the hippocampus of four groups of rats. Changes in the expression of prion protein.

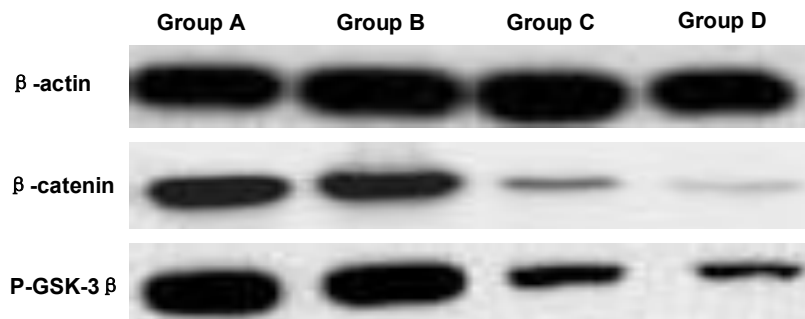


Fig. 3. Protein expressions of β -catenin and P-GSK-3 β in rat hippocampus (β -actin is the internal parameter index of antibody)

of neural stem cells in hippocampus. Compared with group A, the number of BrdU positive cells in group B decreased, while that in group C and group D decreased significantly, indicating that isoflurane could inhibit the proliferation of neural stem cells, and this effect was related to the concentration of isoflurane. Compared with group A, the escape latency of group C and group D was significantly greater, suggesting that 1% and 1.5% isoflurane could induce cognitive dysfunction.

The important components of Wnt signal were β -catenin and GSK-3 β . GSK-3 β could phosphorylate and degrade β -catenin through proteasome pathway (8, 9). The results showed that 1% and 1.5% isoflurane could reduce the expression of β -catenin and P-GSK-3 β at protein levels in hippocampus. It was speculated that isoflurane could promote the depolymerization of β -catenin and inhibit the proliferation of neural stem cells by down regulating the phosphorylation level of GSK-3 β . The inhibition effect of high concentration isoflurane was more obvious. Bai et al. showed that fluoxetine, an antidepressant, could promote the proliferation of hippocampal neural stem cells by activating the GSK-3 β / β -catenin signaling pathway, which was consistent with the results of the present study, indicating that GSK-3 β / β -catenin signaling pathway played an important role in the regulation of hippocampal neural stem cell proliferation (10).

In conclusion, high concentrations (1% and 1.5%) of isoflurane could inhibit the proliferation of rat hippocampal neural stem cells and induce cognitive dysfunction, while a low concentration (0.5%) of isoflurane had no obvious effect, which may be related to the phosphorylation of GSK-3 β and the down-regulation of β -catenin expressions at protein levels.

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LETTER TO THE EDITOR

LIPID PEROXIDATION AND TOTAL OXIDANT/ANTIOXIDANT STATUS IN
COLORECTAL CANCER PATIENTSK. JANION¹, E. SZCZEPAŃSKA², E. NOWAKOWSKA-ZAJDEL^{1,3}, K. WALKIEWICZ³
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To the Editor,

Colorectal cancer (CRC) is one of the most common malignancies in the world. Its prevalence is projected to increase by 60% by the year 2030. At present, over 2/3 of all CRC cases are diagnosed in countries with a high or very high human development index. However, CRC is becoming increasingly more prevalent in countries of a low income. The risk of the disease increases with age, but the prevalence in young patients has increased in recent years. It is suggested that this increased prevalence is associated with a change of health-related behaviour, encompassing incorrect diet, containing a high content of highly processed foods, and sedentary lifestyle (1).

The issue of oxidative stress in the course of various diseases, including CRC, has recently become a point of great interest. A shift of the balance between oxidative and antioxidant agents to the benefit of the former is observed when free radicals are produced in excessive and uncontrolled amounts. They are highly reactive compounds that may oxidase cellular

macroparticles, such as proteins, DNA and lipids (2). Oxidative stress is a common factor between inflammatory bowel diseases and CRC because of activation of the inflammatory cascade (cytokines, tumour necrosis factor- α) and tissue damage (3).

Lipid peroxidation is a complex and multi-step process that, consequently, leads to cell membrane damage. Due to a range of reactions, the membrane properties of fluidity and permeability are lost. The final products of lipid peroxidation, such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), are strongly mutagenic and toxic compounds. MDA may disintegrate to acetaldehyde (AA) that has cytotoxic and carcinogenic properties (4, 5). Both MDA and AA may link with DNA, thereby generating reactive oxygen species (ROS), which may, in turn, cause errors during copying genetic information, thus leading to genetic mutations (5).

Despite certain instability, serum MDA concentrations are believed to be an important oxidative stress marker. Total oxidant status (TOS) is used for estimating the general oxidation status

Key words: colorectal cancer; oxidative stress; lipid peroxidation products

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while total antioxidant status (TAS) is used for measuring the general antioxidant status of the body. The aforementioned parameters may aid in the assessment of the intensity of oxidative stress and the function of repair systems that protect the body against ROS, including free radicals (5).

The goal of this study was to evaluate the selected oxidative stress markers in patients with CRC depending on some clinical features (sex, age, body mass index, comorbidities and clinical stage of cancer).

MATERIALS AND METHODS

The study was conducted on a group of 75 patients (31 women and 44 men) with CRC at any clinical stage. At the time of the study, the patients were hospitalized in the Department of Clinical Oncology of the Provincial

Specialist Hospital No 4 in Bytom, Poland. The characteristics of all the patients are presented in Table I.

Patients with history of another cancer (apart from squamous cell skin cancer) or with systemic diseases requiring long-term immunosuppression, diagnosed infection (tuberculosis, hepatitis B or C, HIV or AIDS) or cancer-associated cachexia were excluded from the investigation.

During hospitalization, serum samples (3cm³) were collected from all patients. Patient blood samples were collected before the initiation of systemic therapy. The tests were performed on blood collected in the morning from fasting patients (approximately 8 to 10 h after the last meal). Blood was immediately centrifuged and serum was stored frozen at -80°C until analysis.

The following information was extracted from patient medical records: CRC stage according to the TNM

Table I. Clinical characteristics of the study group including patient gender.

Clinical features		Sex		Total (n=75)
		Female (n=31)	Male (n=44)	
Age [years]		69.00 (61.00-75.00)	65.00 (57.50-69.50)	67.00 (59.00-73.00)
Body weight [kg]		66.95 (SD±10.40)	77.59 (SD±12.37)	73.19 (SD±9.98)
BMI [kg/m ²]		26.94 (SD±4.87)	26.09 (SD±3.75)	26.44 (SD±4.24)
Interpretation of BMI (according to WHO)	Normal body weight	13 (41.94%)	20 (45.45%)	33 (44.00%)
	Overweight	9 (29.03%)	17 (38.64%)	26 (34.67%)
	Obesity	9 (29.03%)	7 (15.91%)	16 (21.33%)
Cardiovascular diseases	Yes	22 (70.97%)	22 (50.00%)	44 (58.67%)
	No	9 (29.03%)	22 (50.00%)	31 (41.33%)
Type 2 diabetes mellitus	Yes	4 (12.90%)	4 (9.09%)	8 (10.67%)
	No	27 (81.10%)	40 (90.91%)	67 (89.33%)
TNM	I	1 (3.23%)	3 (6.82%)	4 (5.33%)
	II	9 (29.03%)	9 (20.45%)	18 (24.00%)
	III	9 (29.03%)	17 (38.64%)	26 (34.67%)
	IV	12 (38.71%)	15 (34.09%)	27 (36.00%)
Grading	Gx	7 (22.58%)	7 (15.92%)	14 (18.67%)
	G1	3 (9.68%)	5 (11.36%)	8 (10.67%)
	G2	17 (54.84%)	26 (59.08%)	43 (57.33%)
	G3	4 (12.90%)	6 (13.64%)	10 (13.33%)

Table II. Descriptive statistics of selected oxidative stress markers assayed from the serum.

Clinical features		TOS [$\mu\text{mol/L}$]	P	TAS [$\mu\text{mol/L}$]	p	MDA [ng/mL]	p
Sex	Female (n=31)	889.86 (± 376.09)	0.300	247.22 (233.33- 324.42)	0.776	2053.33 (1118.94- 3398.18)	0.851
	Male (n=44)	792.18 (± 410.05)		253.20 (234.06- 290.43)		2473.81 (1296.52- 3208.23)	
Age	< 60 years (n=20)	827.06 (± 323.14)	0.942	260.47 (237.47- 317.41)	0.715	1363.10 (930.99- 2797.54)	0.047
	$\geq$ 60 years (n=55)	834.55 (± 423.00)		250.82 (232.53- 310.66)		2494.71 (1345.52- 3332.79)	
Cardiovascular diseases	No (n=31)	713.40 (± 419.67)	0.028	243.75 (225.85- 290.49)	0.910	2494.71 (1202.38- 3332.79)	0.113
	Yes (n=44)	916.50 (± 361.30)		253.20 (236.66- 321.35)		2136.23 (1145.91- 32328.30)	
Type 2 diabetes mellitus	No (n=67)	829.04 (± 401.14)	0.826	250.82 (233.33- 294.23)	0.537	2085.58 (1118.94- 3207.38)	0.027
	Yes (n=8)	861.97 (± 381.57)		264.51 (235.73- 355.54)		3238.30 (2708.54- 4213.93)	
Interpretation of BMI	Normal body weight (n=33)	882.78 (± 436.47)	0.759	262.31 (242.94- 290.49)	0.949	2452.91 (875.55- 3207.38)	0.434
	Overweight (n=26)	736.43 (± 339.00)		248.29 (231.32- 318.27)		2022.15 (1311.65- 3207.38)	
	Obesity (n=16)	885.16 (± 392.44)		250.82 (233.96- 294.57)		2909.98 (1610.51- 3431.81)	
TNM	I and II (n=22)	710.18 (± 403.01)	0.248	255.17 (224.65- 337.11)	0.918	2209.51 (1067.92- 3207.38)	0.306
	III (n=26)	864.78 (± 416.59)		251.16 (233.33- 290.36)		2095.05 (1247.52- 2922.36)	
	IV (n=27)	901.23 (± 362.36)		251.49 (235.60- 290.49)		2848.22 (1159.57- 3679.28)	
Total (n=75)		832.55 (± 396.73)	-	251.49 (233.33- 310.66)	-	2372.30 (1159.57- 3269.21)	-

Classification of Malignant Tumours, in accordance with the guidelines of the American Joint Committee on Cancer (8th Edition Cancer Staging Manual 2015); histological malignancy grade; comorbidities (cardiovascular diseases, type 2 diabetes mellitus). Oxidative stress markers were determined by measuring TOS and TAS, and MDA concentrations. MDA (ELISA Kit Cloud-Clone Corp., cat. No CEA597Ge, Houston, TX, USA) was assayed using enzyme-linked immunosorbent assay (ELISA), whereas TOS and TAS were determined using the following reagent kits: PerOx (Immunodiagnostik, cat. No KC 5100, Bensheim, Germany) and ImAnOx (Immunodiagnostik, cat. No KC 5200, Bensheim, Germany), in accordance with the methodology provided by the manufacturer. The absorbance readings were registered at waves of 450 nm and calibrated according to the standard curve for MDA concentration in ng/mL, for TOS and TAS levels in $\mu\text{mol/L}$. All samples were run in duplicate.

Statistical analysis

The data were then compiled and analysed in Microsoft Excel 2010, and statistical calculations were performed in Statistica 13.3 (TIBCO Statistica™, StatSoft, Kraków, Poland). The Shapiro-Wilk W test and normality diagrams were applied to check whether quantitative variables meet the normal distribution. Quantitative variables were presented as arithmetic means with standard deviation (SD) for a near-to-normal distribution or median values with interquartile ranges (from P25 to P75) for a non-normal distribution. The quantitative variables with distribution nearing the normal distribution were compared with the use of the Student's *t*-test (for two samples), and the quantitative variables with a non-normal distribution were analysed with a non-parametric Mann-Whitney U test (for two samples) or Kruskal-Wallis ANOVA (for multiple samples). A two-sided test with a Bonferroni correction was used for a post hoc analysis. The relationships between the tested variables were analysed using the Spearman's correlation coefficient. For all analyses, the value of $p < 0.05$ was considered statistically significant.

The study was approved by the Ethics Committee of the Medical University of Silesia in Katowice, Poland (Approval No KNW/0022/KB1/43/17 dated 30 May 2017). Patient participation was voluntary.

All patients gave written informed consent to participate in the project.

RESULTS

The selected oxidative stress markers were tested taking into account sex, age, comorbidities, i.e. cardiovascular diseases (CVD), type 2 diabetes mellitus (T2DM), body mass index (BMI) and clinical stage in accordance with the TNM Classification of Malignant Tumours (Table II).

The analyses demonstrated statistically significant differences in the MDA concentration (Mann-Whitney U test; $p = 0.047$) between patients < 60 years and patients ≥ 60 years. However, there were no differences in the serum TOS level (Student's *t*-test; $p = 0.942$) and TAS level (Mann-Whitney test; $p = 0.715$) and patients from these age groups (Table II).

It was observed that mean TOS level was higher in patients with CVD in comparison patients without CVD and this difference was statistically significant (Student's *t*-test; $p = 0.028$). There were no differences in the TAS level (Mann-Whitney test; $p = 0.910$) and MDA concentration (Mann-Whitney U test; $p = 0.113$) between patients with CVD and patients without CVD in the study group. There were differences in the MDA concentration (Mann-Whitney U test; $p = 0.027$) between patients with T2DM and without T2DM, but there were no differences in the TOS level (Student's *t*-test; $p = 0.826$) and the TAS level (Mann-Whitney test; $p = 0.537$) between patients from these groups (Table II).

Moreover, there were no differences in the TOS, TAS and MDA concentration and other clinical feature, i.e. sex, BMI and clinical stage in the TNM Classification of Malignant Tumours (Table II).

DISCUSSION

One of the more important risk factors for CRC includes improper diet (poor in wholemeal products and raw vegetables and fruit, but rich in highly processed foods) and sedentary lifestyle, which is conducive to obesity (6). Fat accumulation in the body, especially visceral fat, is the basic feature of metabolic syndrome, which is the primary risk factor for CVD

and T2DM (7). A positive relationship between obesity and its metabolic consequences is well-known, but mechanisms underlying this process are complex and have not been fully identified. The present study revealed significantly higher TOS levels in patients with a positive history of CVD and significantly higher MDA concentrations in patients with concomitant T2DM compared to patients without these diseases. Moreover, many other authors have noted worse function of the antioxidant barrier in patients with metabolic conditions due to overproduction of free radicals (8, 9). A common finding of various studies was an increased MDA level in patients with T2DM compared to individuals without this disease (10).

Due to the ageing process, the antioxidant barrier of the body may deteriorate, and lipid peroxidation may become more enhanced. The available data provide inconsistent information in this matter. However, various observations note an increased concentration of oxidative stress markers in elderly people compared to matching younger age groups. The present study revealed statistically significant differences between MDA levels in patients under 60 years of age compared to those aged 60 years and over. Studies of other authors also confirm enhanced lipid peroxidation with age. Fasna et al. evaluated lipid peroxidation levels in 150 randomly selected healthy volunteers and noted a marked increase in serum MDA levels ($p < 0.001$) in older individuals (60–90 years) compared to controls (20–30 years) (11). These observations are also supported by measurements performed by Junqueira et al. They demonstrated that the lipid peroxidation level in individuals over 50 years of age is higher by 20–40% compared to controls (20–29 years). Furthermore, the authors observed a strong positive correlation between age and the concentration of lipid peroxidation products ($p < 0.01$; $r = 0.972$) (12).

To conclude, substantial evidence suggests that oxidative stress takes part in the mechanism of CRC. However, it is a product of various factors, including comorbidities, ageing and environmental factors. The answer to a question about the degree to which these factors determine the oxidative stress level is still an open issue. The presented results should be treated with caution. The sample size and the lack of environmental factors in the analysis, e.g. diet, dietary supplements,

ethylene alcohol consumption and tobacco smoking, are the limitations of this study. That is why future research should include larger group of CRC patients and a control group. In addition, environmental factors should be considered in the future.

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LETTER TO THE EDITOR

**EXTERNAL APICAL ROOT RESORPTION AMONG THE SAUDI POPULATION:
A PROSPECTIVE RADIOGRAPHIC STUDY OF MAXILLARY AND MANDIBULAR
INCISORS DURING ORTHODONTIC TREATMENT**I. ALSHAHRANI¹, M. AJMAL², T. ALAM², M. LUQMAN²,
M.A. KAMRAN¹ and H. AL MOHIY³*¹Department of Paediatric Dentistry and Orthodontic Sciences, King Khalid University College of Dentistry, Abha, Saudi Arabia; ²Department of Diagnostic Sciences, King Khalid University College of Dentistry, Abha, Saudi Arabia; ³Department of Radiologic Sciences, College of Applied Sciences, King Khalid University College of Dentistry, Abha, Saudi Arabia**Received December 28, 2019 – Accepted February 6, 2020*

To the Editor,

Resorption of roots is an inevitable and relatively common side effect of orthodontic tooth movement. The term ‘orthodontically-induced inflammatory root resorption’ (OIIRR) is mainly used in orthodontics to differentiate from other causes of root resorption in permanent teeth (1). External apical root resorption (EARR) is an undesirable sequel that can significantly compromise the success of orthodontic treatment. It is usually common in the apical region because of the concentration of orthodontic forces and an increased accumulation of stress at the root apex (2). It is believed to occur as a response to altered alignment of periodontal fibers at the apical end due to the presence of cellular cementum at the apex (with patent vasculature), rendering the periapical cementum more friable and susceptible to trauma and concomitant vascular stasis (3, 4). Although clinically inconsequential, a relatively small percentage of patients express EARR severe enough to cause undesirable and irreversible damage to the roots (5). Orthopantomogram (OPG) and periapical views are the most widely used diagnostic technique. A two-dimensional image underrates the actual amount of root resorption as compared to computerized

tomography (6). Despite limitations, the periapical paralleling technique is the most preferred because it has the most favorable benefit to risk ratio in detecting the degree of apical root material loss. It provides the most appropriate information with the least irradiation when used for teeth that are most likely to exhibit blunting of roots: maxillary and mandibular incisors. Also, distortion and superimposition errors are less as compared to the OPG or the lateral head film. Therefore, the purpose of this study was to measure the amount of EARR of maxillary and mandibular incisor teeth and to evaluate its clinical significance during 9 months of active orthodontic treatment.

MATERIALS AND METHODS

The study was conducted in the Department of Orthodontics and Dentofacial Orthopaedics, King Khalid University, Abha, Saudi Arabia (SRC/ETH/2018-19/024). The study sample consisted of a total of 320 teeth from 40 patients, recruited by convenience sampling, and divided into two groups with mean age 15.6 (range: 14-18) years. The patients undergoing treatment in the Department of Orthodontics were randomly selected and only

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those cases of Angle Class I malocclusion with upper and lower anterior crowding were divided into two groups: Group 1 (n=20) extraction cases, and Group 2 (n=20) non-extraction cases. All patients' parents or guardians were required to sign informed consent.

Cases with a previous history of orthodontic or endodontic treatment involving incisors, cases with bimaxillary protrusion, impacted maxillary canines, traumatic injuries, crown or root fractures, genetic or developmental anomalies of teeth, severely dilacerated roots, congenitally missing laterals, incomplete root formation at the start of treatment, maxillary incisors with caries and/or periodontal disease and patients with systemic disorders, hormonal imbalance were duly excluded from the study.

All the patients were treated with pre-coated bracket pre-adjusted appliances (Roth brackets) with 0.022" bracket slots and edgewise mechanics were

used during the treatment. The archwire used initially was 0.013" Nickel-Titanium followed by 0.014 Ni-Ti, 0.018 Ni-Ti, and 0.016x 0.022 Ni-Ti wires. All the cases were treated by the same clinician to avoid inter-personnel errors.

Periapical radiographs of both maxillary and mandibular incisors (right and left central and lateral incisors) were obtained at the following time-points: at the beginning of treatment (T0), 3 months (T1), 6 months (T2), and 9 months (T3) after the treatment, by using Dentsply Rinn XCP paralleling devices. Single rooted teeth were selected to avoid errors with multiple roots and differential resorption. Radiographic examination was conducted carefully to ensure good quality images for interpretation and determination of root length. Radiographs were analyzed by two observers using CS Imaging software 7.0.3 under 100% magnifications on an LCD monitor. EARR was measured and calculated in the following manner. Mesial and distal edge points (a, b, c, d, e, and f) were identified and marked at the region of incisal edge, cemento enamel junction and apical foramen respectively on both the pre-treatment and post-treatment periapical radiographs. Horizontal lines (a-b, c-d, and e-f) were drawn to connect the edge points and the central point on these lines were connected vertically. The distance between the horizontal lines (a-b and c-d) was measured as "crown height" (A) and the distance between the horizontal lines (c-d and e-f) was measured as the "root length" (B) (Fig. 1).

In the present study, the "root length" of each tooth was used to measure apical root resorption in millimeters (mm) using CS imaging software to a precision of 0.1 mm. The crown length (unaffected by EARR) was used as a reference to correct potential differences in geometric projection in subsequent radiographs. The averages of the pre-treatment and post-treatment crown lengths were computed by the following formula:

$Cx = (C1+C2)/2$, where Cx= average crown length, C1= pre-treatment crown length and C2= post-treatment crown length.

Following the calculation of the average crown length, the following formula was used to adjust the pre-treatment and post-treatment root lengths:

$R1 \text{ (adjusted)} = R1 \times (Cx/C1)$ where R1= Average root length.

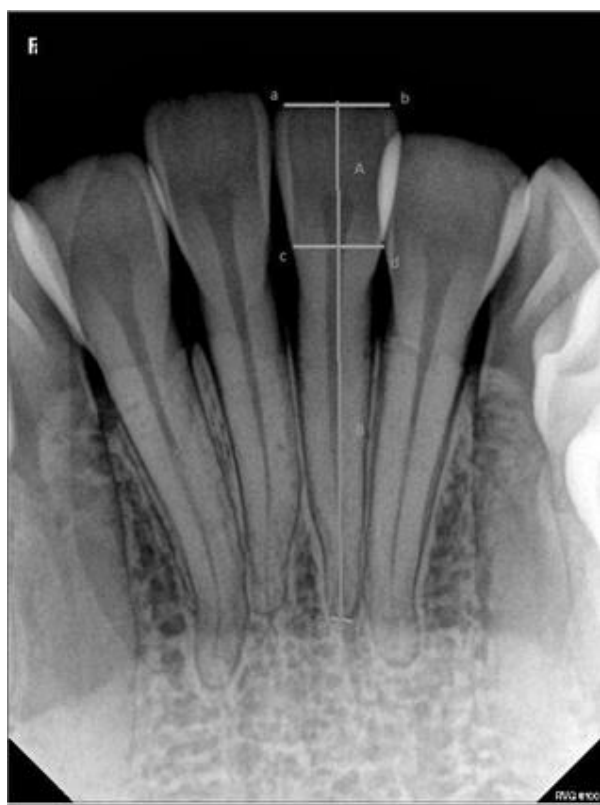


Fig. 1. Mesial and distal edge points *a)* Mesial incisal edge point; *b)* distal incisal edge point; *c)* mesial cemento enamel junction point; *d)* distal cemento enamel junction point; *e)* mesial edge point of the apical foramen; *f)* distal edge point of the apical foramen; *A)* crown height; *B)* root length; *C)* (A+B) total tooth length.

The resultant difference between the second (post-treatment) and first (pre-treatment) root length measurements were quantified as EARR. Root length assessment was carried out for a total of 320 radiographs by 2 examiners.

Statistical analysis

Data were analyzed using SPSS software (VERSION

20) and mixed-method ANOVA was used to analyze the data, and an interclass correlation coefficient analysis was carried out to assess the inter-examiner reliability. The independent variable in this analysis was time and dependent variable was the treatment (non-extraction and extraction) and its influence on root resorption. Mauchly's sphericity test was performed to validate the repeated measures analysis of variance.

Table I. Mean root length of maxillary and mandibular incisors at different time intervals.

(A) Maxillary incisors					
Group-1 (Extraction)	T0 (mean±SD)	T1 (mean±SD)	T2 (mean±SD)	T3 (mean±SD)	P-value
Tooth #11	16.56±0.27	16.09±0.56	15.77±0.69	15.73±0.66	0.00
Tooth #12	16.56±0.27	16.06±0.56	15.71±0.71	15.68±0.68	0.00
Tooth #21	16.46±0.24	16.16±0.44	15.60±0.60	15.45±0.45	0.00
Tooth #22	17.11±0.34	16.35±0.37	16.00±0.43	15.90±0.41	0.00
Group-2 (Non-extraction)					
Tooth #11	16.66±0.42	15.76±0.43	15.36±0.69	15.32±0.49	0.00*
Tooth #12	16.77±0.63	15.89±0.59	15.57±0.61	15.42±0.59	0.00*
Tooth #21	16.77±0.54	15.80±0.52	15.36±0.66	15.30±0.59	0.00*
Tooth #22	17.25±0.59	16.43±0.68	15.95±0.75	15.88±0.76	0.00*
(B) Mandibular incisors					
Group-1 (Extraction)					
Tooth #31	14.56±0.20	14.09±0.56	13.77±0.69	13.73±0.66	0.00*
Tooth #32	15.11±0.34	14.38±0.39	14.04±0.47	13.94±0.46	0.00*
Tooth #41	14.64±0.26	14.10±0.57	13.79±0.69	13.75±0.67	0.00*
Tooth #42	15.11±0.34	14.35±0.37	14.00±0.43	13.90±0.41	0.00*
Group-2 (Non-extraction)					
Tooth #31	14.76±0.60	13.85±0.70	13.47±0.77	13.43±0.77	0.00*
Tooth #32	15.28±0.70	14.48±0.88	13.91±0.70	13.85±0.67	0.00*
Tooth #41	14.80±0.76	14.04±0.98	13.49±0.77	13.44±0.75	0.00*
Tooth #42	15.29±0.74	14.53±0.93	13.92±0.67	13.83±0.59	0.00*

NS: Non-significant; * Statistically significant at $p < 0.05$

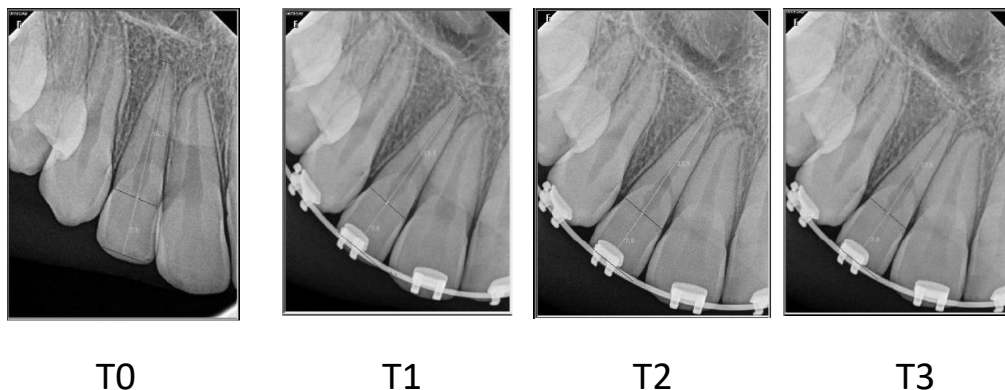


Fig. 2. Periapical radiographs of a patient showing the progressive resorption in maxillary lateral incisor.

RESULTS

A total of 320 teeth from 40 patients were divided into two groups of 20 cases each. The mean age of the subjects was 14-18 years and consisted of 20 females and 20 male patients. In the present study, there was progressive resorption of roots with the time that was significant for all the eight incisors.

In the maxillary central incisor teeth (11 and 21) there was significant resorption of roots observed in both the treatment groups with time (Mauchy's test of Sphericity<0.005, within-subjects effect <0.005, *F*- Value 268.625 for 11 and *F*- Value 155.457 for 21). In the maxillary lateral incisor teeth (12 and 22) there was significant resorption of roots observed in both the treatment groups with time (Fig. 2) (Mauchy's test of Sphericity<0.005, within-subjects effect <0.005, *F*- Value 268.625 for 12 and *F*- Value 168.503 for 22) (Table I).

In the mandibular central incisor teeth (31 and 41) with time there was significant resorption of roots observed in both the treatment groups (Mauchy's test of Sphericity<0.005, within-subjects' effect <0.005, *F*- Value 178.707 for 31 and *F*- Value 182.708 for 41). In the Mandibular lateral incisor teeth (32 and 42) with time there was significant resorption of roots observed in both the treatment groups (Mauchy's test of Sphericity<0.005, within-subjects effect <0.005, *F*- Value 282.105 for 32 and *F*- Value 150.907 for 42) (Table II).

Comparison of root resorption between treatment intervals (T0, T1, T2, and T3), showed that the amount of resorption was statistically significant

for all maxillary and mandibular incisors except treatment interval T2-T3 for right maxillary lateral incisors (12) and mandibular central incisors (31 and 41), which was non-significant..

A comparison of mean root resorption among the groups based on extraction or non-extraction of cases revealed overall root resorption in both the groups. However, the extent of root resorption was relatively lesser in Group 1 but was statistically insignificant. The inter-examiner reliability and correlation between the two examiners was 97.5%.

DISCUSSION

The average apical root resorption in maxillary incisors, especially the maxillary lateral incisors, is found to be consistently more than any other analyzed tooth, followed by mandibular incisors and mandibular first molars (7, 8). In this study, there was a progressive resorption of roots over time that was significant for all the eight incisors and the extent of resorption was relatively more for lateral incisors, particularly the mandibular lateral incisors. It is reasoned that maxillary incisors are most subjected to orthodontic treatment either due to esthetic or functional reasons and the conical shape of these roots makes them susceptible to the resorption (9). Studies have also reported a consistent association between anatomical factors commonly seen in maxillary lateral incisors such as greater tooth length, narrow, pointed and deviated roots to exhibit more root resorption during orthodontic treatment (10).

Tooth extraction is a known risk factor for

Table II. Maxillary and mandibular root resorption time trend analysis.

	T0-T1		T0-T2		T0-T3		T1-T2		T1-T3		T2-T3	
	Mean±SD	P-value	Mean±SD	P-value	Mean±SD	P-value	Mean±SD	P-value	Mean±SD	P-value	Mean±SD	P-value
Maxillary teeth												
Tooth #11	0.77±0.52	0.00*	1.23±0.69	0.00*	1.30±0.71	0.00*	0.77±0.52	0.00*	0.46±0.39	0.00*	0.70±0.14	0.00*
Tooth #12	0.68±0.67	0.00*	0.68±0.67	0.00*	1.07±0.78	0.00*	0.38±0.43	0.00*	0.43±0.42	0.00*	0.45±0.18	NS
Tooth #21	0.72±0.74	0.00*	0.60±0.67	0.00*	1.02±0.77	0.00*	0.48±0.42	0.00*	0.46±0.42	0.00*	0.67±0.14	0.00*
Tooth #22	0.79±0.81	0.00*	1.20±0.90	0.00*	1.29±0.88	0.00*	0.41±0.30	0.00*	0.30±0.32	0.00*	0.82±0.20	0.01*
Mandibular teeth												
Tooth #31	0.69±0.05	0.00*	1.04±0.76	0.00*	1.08±0.74	0.00*	0.35±0.37	0.00*	0.39±0.03	0.00*	0.04±0.01	NS
Tooth #32	0.76±0.05	0.00*	1.22±0.06	0.00*	1.29±0.06	0.00*	0.45±0.03	0.00*	0.53±0.04	0.00*	0.07±0.16	0.00*
Tooth #41	0.64±0.63	0.00*	1.08±0.07	0.00*	1.12±0.06	0.00*	0.43±0.04	0.00*	0.47±0.04	0.00*	0.04±0.01	NS
Tooth #42	0.77±0.08	0.00*	1.24±0.09	0.00*	1.33±0.09	0.00*	0.46±0.04	0.00*	0.56±0.05	0.00*	0.09±0.02	0.00*

NS: Non-significant; * Statistically significant at $p < 0.05$

EARR during orthodontic tooth movement because extraction cases require longer treatment time and are associated with increased and/or extensive movements and retraction of the apex. In the present study, overall root resorption was appreciated in both extraction and non-extraction cases in respect to both time and time intervals. However, the extent of root resorption was relatively lesser in Group 1 but was statistically insignificant. This is in sharp contrast to various studies that have noted more severe EARR among extraction cases (11, 12). This contrasting finding could be due to mechanisms that are likely to be independent of known factors causing EARR or due to genetic causes.

Our study demonstrated EARR in almost all the teeth expressed as root shortening. This does not mean 100% root resorption in our study because none of them demonstrated EARR more than 2 mm, as we sought to identify change in root length as expressed only as a slight change in apical contour with/without actual root shortening as a result of resorption, whereas few authors consider EARR only when root resorption of more than 4mm or 1/3 of the original root length occurs, which essentially means that any EARR of less than 4 mm could be a transient change involving remodeling of roots during active tooth movement.

It should be noted that root resorption associated with orthodontic treatment ceases with the termination of active treatment and usually does not affect the functional capacity or periodontal integrity of the teeth. Considering the consistent occurrence of EARR, progress radiographs obtained every 3-months during treatment is of great clinical significance in identifying EARR early in the process so that the orthodontist takes necessary precaution to reduce the extent of resorption.

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LETTER TO THE EDITOR

EFFECTS OF HIGH CONCENTRATION INSULIN ON THE EXPRESSION OF GLUCOSE TRANSPORTERS IN RENAL TUBULAR EPITHELIAL CELLS

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To the Editor,

The kidney plays an important role in the process of glucose metabolism in the body. Under physiological conditions, the kidney filters about 180 g of glucose through the glomerulus every day, but eventually less than 0.5 g is excreted from the urine (1). Renal reabsorption of glucose depends on two types of glucose transporters, sodium-dependent glucose transporters (SGLTs) (2), and glucose transporters (GLUTs) (3). After glomerular filtration, up to 90% glucose is actively transported by SGLT2 in the proximal tubules S1 and S2 and facilitates diffusion and reabsorption of GLUT2 along the concentration gradient on the basement membrane (4). The remaining 10% of glucose is reabsorbed through the active transport of SGLT1 in the proximal tubule S3 segment and the facilitation and diffusion of carrier GLUT1 on the basement membrane (5). It has been found that the blood glucose level of patients with type 2 diabetes becomes higher, due to the increased expression of SGLT2 receptor in renal tubules. Before the increase of blood glucose, whether the metabolic disorder characterized by insulin resistance/hyperinsulinemia

affects the structure and function of renal tubules and participates in the occurrence and development of diabetic nephropathy remains to be further investigated. Therefore, in this study, the changes of glucose reabsorption by renal tubular epithelial cells under different insulin concentrations and at different intervention time points were observed.

MATERIALS AND METHODS

Cell culture

Human renal tubular epithelial cell line (HK-2) and adherent cells were used in this study. The cryopreserved HK-2 cells were taken out for cell recovery. Cells were observed under an inverted microscope. When the cell growth was in the logarithmic phase and confluency reached 70-80%, cell passage was carried out. Microscopically, the cells grew well and regularly. When about 80% of the bottom was covered, the cells were frozen. The cells were frozen at 4°C for 30 min, -20°C for 2 h and -80°C overnight. 50 uL of cell suspension was absorbed by a pipette and dropped from the edge of the cover slide into the gap between the cell counter plate and the cover slide. The cells were counted under an inverted microscope.

*Key words: insulin; glucose transporter; renal tubular epithelial cells; protein**Corresponding Author:*

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MTT assay

Cell suspension was prepared and HK-2 cells were seeded into 96-well plates (4×10^3 /well). When the cells covered about 80-90% of the well plate area, different concentrations of insulin (0, 1, 5, 10, 50, 10^2 , 10^3 ng/mL) were added. Six compound wells were set for each concentration. After 24 h of treatment, MTT solution was added and the well plate was transferred into a cell culture box to avoid light. After 4 h of reaction, blue crystals could be seen. After careful absorption of the supernatant, 150 μ L DMSO was added to each well. After fully shaking for 10 min to dissolve the crystals, the enzyme label was used to read the OD value and it was repeated three times.

Fluorescence quantitative polymerase chain reaction (FQ-PCR)

TRIzol method was used to extract total RNA from HK-2 cells and the extracted RNA was dissolved in RNase-free water. The reverse transcription reaction was used to obtain the DNA, and the content of the RNA was determined by SYBR Mixture RT-PCR kit.

Primer sequences were as follows: SGLT1, 5'-CAAGATCCGGAAGGGTGCAT-3' (forward) and 5'-TGTAGTCAAAGAGCTGCCCCG-3' (reverse). SGLT2, 5'-GGTCATTGCCGCGTATTTCC-3' (forward) and 5'-ATGTTGCTGGCGAACAGAGA-3' (reverse). GLUT1, 5'-GGACACTATCCCGATCTGCC-3' (forward) and 5'-GCACGTGCTTTCACACCTTT-3' (reverse).

GLUT2, 5'-TGCAGCCAATACCAGAAACCA-3' (forward) and 5'-CCGATGGTTCTGCCCTTTTGG-3' (reverse). Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was used as the internal reference. The reaction conditions were set as follows: pre-denaturation at 94°C for 3 min, denaturation at 94°C for 15 s, annealing at 60°C for 15 s and elongation at 72°C for 15 s, with a total of 40 cycles. The relative expression = $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct$ (target gene) - Ct (internal reference gene). The Ct value was the number of cycles experienced by the fluorescent signal in each reactor tube when it reached the threshold.

Western blot

Western blot was used to detect the protein expression, and GAPDH was used as the internal reference to calculate the gray ratio of protein bands for semi-quantitative analysis. Cell suspension was prepared, total protein was extracted by RIPA lysate, and the protein concentration

was determined by BCA protein quantitative kit (Beijing Kangwei Century Biotechnology Co., Ltd.). SDS sample buffer was added and denatured at 100°C. SDS-PAGE electrophoresis was performed, and protein was transferred to a PVDF membrane. The PVDF membrane was incubated with a primary antibody for 2 h after blocking at room temperature for 2 h and kept overnight at 4°C. Then, the PVDF membrane was incubated in secondary antibody at room temperature for 1 h. ECL was used for color developing. Bio-Rad gel image imaging system and Quality one software were used for scanning analysis. The experiment was repeated 5 times, and the mean value of the results was recorded.

Statistical analysis

SPSS 22.0 statistical software was used for data analysis. Normality test and homogeneity test of variance were used for measuring data. Normal distribution data were expressed as ($\bar{x} \pm s$). Independent sample *t*-test was used for comparison between the two groups, univariate analysis of variance was used for comparison between multiple groups, and SNK-q test was used for comparison between two groups. The test level was $\alpha=0.05$, and $P<0.05$ indicated significant difference.

RESULTS

Cell morphology

HK-2 cells were observed to grow adherently under an inverted microscope. The cell morphology was cobblestone-like or paving stone-like. The cell margin was round and blunt. The transparency and refraction were good under the microscope. The cell-to-cell junction was tight, and the cell growth was good, therefore further experiments could be carried out (Fig. 1).

Effects of the insulin concentration on cell growth

After 24-hour insulin treatment at 0, 1, 5, 10, 50, 10^2 , 10^3 ng/mL, respectively, the number and activity of cells increased with the increased insulin concentrations (OD values of cell viability: 0ng/ml: 0.93 ± 0.41 ; 1ng/mL: 0.96 ± 0.47 ; 5ng/mL: 1.05 ± 0.66 ; 10ng/mL: 1.17 ± 0.38 ; 50ng/mL: 1.26 ± 0.65 ; 100ng/mL: 1.17 ± 0.58 ; 1000ng/mL: 1.05 ± 0.61 ; respectively) and reached the maximum at 50 ng/

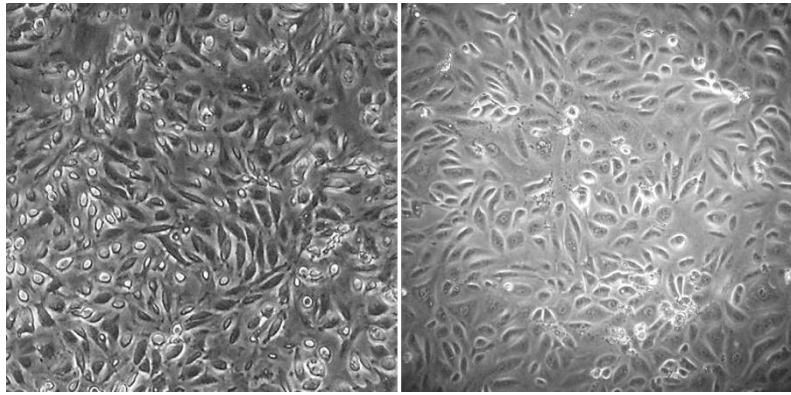


Fig. 1. Cell morphology of HK-2

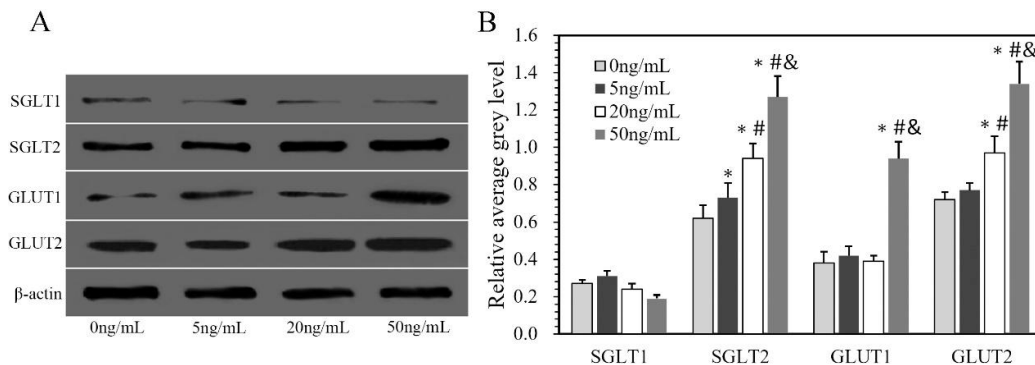


Fig.2. The expression of various proteins after 24 h of treatment with different concentrations of insulin. **A)** Western blot test chart; **B)** statistical chart of relative gray value. Note: compared with 0ng/ml, * $P < 0.05$; compared with 5ng/ml, # $P < 0.05$; compared with 20ng/ml, & $P < 0.05$; Note: Compared with 0ng/mL, * $P < 0.05$; Compared with 5ng/mL, # $P < 0.05$; Compared with 20ng/mL, & $P < 0.05$.

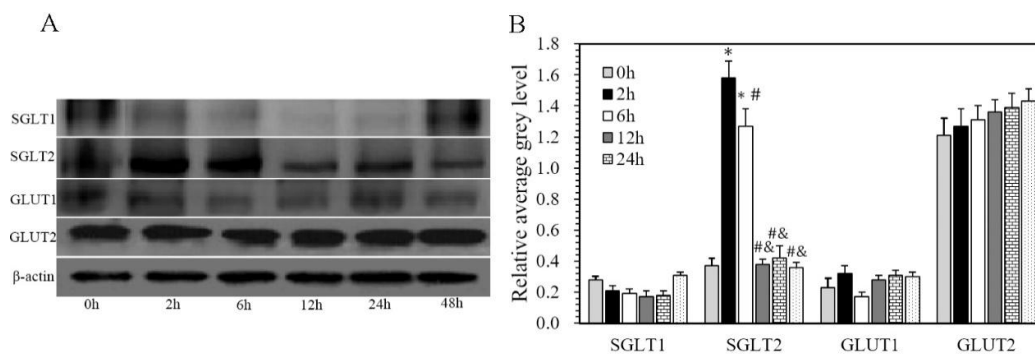


Fig. 3. Protein expressions in hyperinsulinemic environment after treatment at different time points. **A)** Western blot test chart; **B)** statistical chart of relative gray value) Note: Compared with 0h, * $P < 0.05$; Compared with 2h, # $P < 0.05$; Compared with 6h, & $P < 0.05$.

mL. Subsequently, the number and viability of cells decreased. Therefore, combined with the results of MTT and 0-5 ng/mL physiological concentration range, the concentration of insulin was determined to be 0 ng/mL (control group), 5 ng/mL (low dose group), 20 ng/mL (medium dose group) and 50 ng/mL (high dose group), a total of four groups.

Effects of insulin concentration on glucose transporter at the mRNA level in HK-2 cells

The expression of SGLT1 receptor in HK-2 cells after 24 h of insulin treatment showed no significant difference among the four groups ($P > 0.05$). SGLT2 increased significantly with the higher concentration ($P < 0.05$). The basic expression of GLUT1 was low. The expression of mRNA increased with the higher insulin concentration and increased significantly at 50 ng/mL compared with the control group ($P < 0.05$). The basic expression of GLUT2 was high, and the expression of GLUT2 mRNA increased significantly with the elevated insulin concentration ($P < 0.05$). The data are shown in Table I.

Effects of treatment time of insulin on glucose transporter expression at the mRNA level

In hyperinsulinemic environment, the SGLT1 receptor expression in HK-2 cells was highest at 6 h after treatment, which was significantly different from that at 0 h ($P < 0.05$). There was no significant difference at the remaining treatment time. The expression of SGLT2 receptor reached the highest level at 6 h ($P < 0.05$), and then decreased gradually with time, but was still higher than that at 0 h. The expression of GLUT1 receptor reached the highest level at 6 h ($P < 0.05$), and then decreased with time. The expression of GLUT2 receptor reached its peak at 6 h ($P < 0.05$), and then decreased gradually with the prolongation of treatment time, but was still higher than that at 0 h. The data are shown in Table II.

Effects of insulin concentration on the glucose transporter expression at the protein level in HK-2 cells

HK-2 cells were treated with different concentrations of insulin for 24 h. The total protein was extracted, and the protein expression was

Table I. *Glucose transporter mRNA expression*

Insulin treatment concentration (ng/mL)	0	5	20	50	P value
SGLT1	0.73±0.27	0.98±0.41	1.27±0.33	0.96±0.25	>0.05
SGLT2	1.34±0.24	4.12±1.02	7.84±0.93	18.15±3.19	<0.05
GLUT1	0.57±0.23	0.94±0.44	1.35±0.15	2.17±0.53	<0.05
GLUT2	5.82±2.01	104.28±43.28	206.19±60.01	355.92±81.29	<0.05

Table II. *Changes of glucose transporter mRNA expression under different treatment times of insulin*

Treatment time (h)	0	2	6	12	24	48	P value
SGLT1	1.05±0.31	2.18±0.68	5.34±0.64	1.87±0.74	1.67±0.67	1.78±0.73	<0.05
SGLT2	0.85±0.14	2.62±0.36	3.25±0.42	1.91±0.25	1.53±0.33	1.38±0.26	<0.05
GLUT1	0.87±0.08	1.28±0.21	3.21±0.35	1.72±0.23	1.63±0.15	1.46±0.35	<0.05
GLUT2	2.02±0.44	8.18±1.02	41.28±4.23	32.19±6.03	19.29±3.35	7.11±0.83	<0.05

detected by Western blot (Fig. 2). HK-2 cells were treated with different concentrations of insulin, and there was no significant difference in the expression of SGLT1 receptor between the four groups ($P > 0.05$), suggesting that the change of insulin concentrations did not affect the expression of SGLT1 receptor. The expression of SGLT2 receptor protein increased with the elevated insulin concentration ($P < 0.05$), suggesting that insulin could up-regulate the expression of SGLT2 receptor protein in a concentration-dependent manner. The expression of GLUT1 receptor protein increased in the high concentration group ($P < 0.05$), but there was no significant difference in the other three groups. The basic expression of GLUT2 was higher and increased with the raised insulin concentration ($P < 0.05$), suggesting that insulin could up-regulate the expression of GLUT2 receptor in a concentration-dependent manner.

Effects of treatment time of insulin on Glucose transporter levels at the protein level in HK-2 cells

HK-2 cells were treated with 50 ng/mL insulin for 0 h, 2 h, 6 h, 12 h, 24 h and 48 h, respectively. Total cell proteins were extracted, and Western blot was used to detect the expression of each receptor protein (Fig. 3). There was no significant difference in the expression of SGLT1 receptor between different treatment times of insulin ($P > 0.05$). The expression of SGLT2 receptor protein increased significantly after 2 and 6 h of treatment compared with the control group ($P < 0.05$), and then prolonged with the treatment time. The expression of SGLT2 receptor protein decreased gradually. The expression of GLUT1 was less in the basic state, and the expression of GLUT1 was increased at different times after insulin treatment compared with the control group, but there was no significant difference ($P > 0.05$). The basic expression of GLUT2 was high, and with the prolongation of insulin treatment time, the expression of GLUT2 receptor protein increased gradually ($P > 0.05$).

DISCUSSION

The glucose transport capacity of SGLT1 and

SGLT2 receptors depends on their expression level (7, 8). Some studies have shown that the expression or activity of SGLTs remained unchanged or even decreased in diabetes mellitus (9, 10). The expression of GLUT1 decreased while the expression of SGLT2 and GLUT2 increased in renal tubules of STZ diabetic rats, but the expression of SGLT1 did not change significantly (11). Therefore, the changes of glucose transport system in diabetes mellitus are still controversial and need to be further studied.

In this study, it was found that insulin up-regulated the expression of SGLT2 and GLUT2 in a concentration-dependent manner. High concentrations of insulin could up-regulate the expression of glucose transporters SGLT2, GLUT1 and GLUT2. In addition, it was found that high concentrations of insulin significantly up-regulated the expression of SGLT1, SGLT2, GLUT1 and GLUT2 in a short period. High concentrations of insulin could promote the expression of glucose transporter mRNA. Western blot showed that SGLT2 and GLUT2 were the main receptors mediating glucose reabsorption in renal tubular epithelial cells. The effect of high concentrations of insulin on glucose transporter and glucose reabsorption was significant in a short time, and then gradually weakened with time.

In conclusion, it was found that insulin could promote renal glucose reabsorption by promoting the expression of glucose transporters in renal tubular epithelial cells.

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LETTER TO THE EDITOR

LOWER VITAMIN D AND SEX HORMONE BINDING GLOBULIN LEVELS AND HIGHER PROGESTERONE, CORTISOL AND t-PA LEVELS IN EARLY SECOND TRIMESTER ARE ASSOCIATED WITH HIGHER RISK OF DEVELOPING GESTATIONAL DIABETES MELLITUS

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To the Editor,

Pregnancy has been associated with a diabetogenic environment, characterized by hyperinsulinemia and hyperglycemia (1). Gestational diabetes mellitus (GDM) is a disorder with variable severity with onset or first recognition of glucose intolerance during pregnancy (2). It affects approximately 1–14% of all pregnancies and its incidence is increasing steadily. GDM patients are diagnosed during the second or third trimester of pregnancy. GDM is a serious complication of the pregnancy which can elevate the risks of various maternal-fetal disorders, including premature delivery, shoulder dystocia or birth injury, macrosomia and preeclampsia (3).

Currently GDM diagnosis is made in the early third trimester, possibly exposing the infant for a significant period of time to intrauterine metabolic alterations and epigenetic programming. Identification of early biomarkers in pregnant women who later develop GDM may lead to a better understanding of GDM pathogenesis. The combination of biomarkers and risk factors into a predictive model can add to GDM early prediction,

evoke effective prevention strategies and ultimately reduce GDM-related complications (4).

Deficiency of vitamin D is common throughout pregnancy and contributes to glycaemic abnormal control (5). Recent evidence suggests that, in addition to its classical roles in calcium and bone metabolism, vitamin D is involved in glucose homeostasis (6). Sex hormone binding globulin (SHBG) is a liver-based glycoprotein linked to circulating estradiol and testosterone. Insulin suppresses secretion of estradiol and testosterone, and SHBG levels are inversely linked to insulin levels and insulin resistance. Placental hormones, such as human placental lactogen, progesterone, cortisol, growth hormone and prolactin, are the major factors contributing to GDM (7). The present study was, therefore, designed in an attempt to identify the early pregnancy hormonal markers of placentation for screening of GDM.

MATERIALS AND METHODS

Study subjects

Three hundred pregnant women, 176 with positive and

Key words: gestational diabetes mellitus (GDM); tissue plasminogen activator (t-PA); blood serum; sex hormone binding globulin (SHBG); cortisol

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124 with negative familial background of type 2 diabetes mellitus (T2DM) or GDM, were selected from different hospitals of Lahore, Pakistan, at their first antenatal visit during the early second trimester. Informed consent, clearly mentioning the purpose of the study, was signed by the patients. All of the women were screened for GDM both at early and late stages of their pregnancy. During the early care visits of gestation, participants were asked about age, history of previous diabetes, family history of type 2 diabetes mellitus, and smoking habit. Clinical parameters, including body mass index (BMI), age, systolic and diastolic blood pressure (SBP& DBP), gravida, parity and metabolic parameters such as HbA1c, FSG, were taken into account. Table I shows the average values of the clinical characteristics of the participants.

Inclusion criteria were based on positive or negative family history of GDM and T2DM, maternal age between 18 and 40, glycated hemoglobin (HbA1c) of less than 6.5% and without hypertension, renal and cardiac diseases, and current medical treatments which could affect patient hormonal concentration, lipid profile, hepatic and renal function tests.

Subjects with prior history of GDM, T2DM multiple pregnancies, ectopic pregnancy, hypertensive disorders, history of smoking/alcohol abuse, assisted reproductive technology treatment, fetal congenital irregularities and any other confounding pathologies (hyper- or hypothyroidism), polycystic ovarian syndrome, glycated haemoglobin greater than 6.5%, renal or hepatic failure, uncontrolled endocrine or any other metabolic disorder that may influence glucose regulation were excluded. Fasting glycemia was determined and women with fasting plasma glucose (FPG) > 110 mg/dl were deemed to have undiagnosed pregestational diabetes mellitus and were therefore excluded from the study.

Ethical approval for the study was obtained from the Institutional Ethics Review Board of Punjab University.

Blood sampling

Cases were followed-up until the development of GDM that was diagnosed if any of the glucose levels was equal to or greater than 5.1 mmol/l (fasting), 10.0 mmol/l (1 h post-load), or 8.5 mmol/l (2 h post-load). Blood was collected by venipuncture from all of the subjects after overnight fasting, at 14 to 18 weeks of pregnancy. After the determination of fasting glycemia and routine pathology

testing, 5cc blood was drawn from all of the subjects and serum was separated by centrifugation. Samples were brought to room temperature before analyses. Vitamin D 25(OH)D, SHBG, progesterone, cortisol concentration and t-PA were determined using commercially available ELISA kits (DiaMetra, Italy).

Statistical analysis

Statistical analysis was carried out by one-way ANOVA and PROC GLM in SAS software to compare the results in comparable groups. P values (<0.05) were considered as statistically significant.

RESULTS

All the subjects were followed-up until the development of GDM. The subjects who developed GDM were termed as the “GDM” group (n=58). Subjects who did not develop GDM or any other complication during follow-up were treated as the “control” group (n=100). Blood samples for both groups were collected after overnight fasting at predefined intervals; early second trimester, early third trimester (for control group) and at the onset of GDM development (for GDM group).

Comparison between groups

The results showed statistically significant ($P \leq 0.05$) decrease in vitamin D concentration in the GDM group compared to the control group in the early second trimester and early third trimester. Similarly, mean serum SHBG concentration for the GDM group was found to be significantly lower ($p < 0.0001$) than controls at both early second and third trimesters (Table II). Only 58 of the 300 patients developed GDM, therefore Table I includes only GDM developed cases, and 100 of the 300 subjects who did not develop GDM were taken as a control group.

Statistically significant ($P \leq 0.05$) increase in progesterone level was observed in the GDM group compared to the control group. Similarly, the GDM group showed significant increase in t-PA, not only in early second trimester, but also in the early third trimester in comparison with controls. Mean cortisol concentration was significantly higher in the GDM

Table I. Average (means $\pm$ SEM) demographic variables of control group and GDM at early third trimester (24-28th weeks of gestation).

Parameters	Control group n=40	GDM group n=50	t- values	p-values	% difference controls vs GDM patients
Age (years)	28.38 ^a $\pm$ 0.64	29.12 ^a $\pm$ 0.97	-0.641	0.5231	4% $\uparrow$
BMI (kg/m ²)	27.63 ^b $\pm$ 0.23	29.43 ^a $\pm$ 0.23	-5.490	< .0001***	7% $\uparrow$
HbA1c (%)	4.97 ^b $\pm$ 0.10	7.61 ^a $\pm$ 0.14	-15.474	< .0001***	53% $\uparrow$
FSG (mg/dl)	88.80 ^b $\pm$ 0.55	119.32 ^a $\pm$ 5.78	-30.899	< .0001***	33% $\uparrow$
1-hr SG (mg/dl)	139.90 ^b $\pm$ 0.76	182.52 ^a $\pm$ 0.86	-37.064	< .0001***	30% $\uparrow$
2-hr SG (mg/dl)	124.70 ^b $\pm$ 4.18	161.6 ^a $\pm$ 5.75	-8.672	< .0001***	29% $\uparrow$
Gravida (Number)	1.48 ^b $\pm$ 0.17	2.65 ^a $\pm$ 0.10	-54.797	< .0001***	79% $\uparrow$
Parity (Number)	0.56 ^b $\pm$ 0.26	1.08 ^a $\pm$ 0.48	-19.115	< .0001***	92% $\uparrow$
SBP (mmHg)	115.72 ^a $\pm$ 0.66	116.93 ^a $\pm$ 0.57	-1.385	0.1698	1% $\uparrow$
DBP(mmHg)	73.29 ^a $\pm$ 0.31	74.54 ^a $\pm$ 0.30	-2.901	0.0047	2% $\uparrow$

^{a,b} indicate significant differences at $p < 0.05$; Order of significance is as: $a > b$; BMI: Body mass index; HbA1c: glycosylated hemoglobin type A1c; FSG: Fasting serum glucose; 1-hr SG: one hour serum glucose; 2-hr SG: two hour serum glucose; *** : $P < 0.001$

Table II. Overall comparison of hormonal profile in GDM group (n=58) and control group (n=100) at early 2nd trimester (14-18 weeks) and early 3rd trimester (24-28 weeks) of gestation.

Parameters	Early Second Trimester		Early Third Trimester		P-value
	T2C	T2GDM	T3C	T3GDM	
Vitamin D (ng/mL)	46.72 $\pm$ 0.94 ^a	40.49 $\pm$ 1.34 ^b	31.96 $\pm$ 0.11 ^c	23.66 $\pm$ 1.06 ^d	<0.0001
SHBG (nmol/L)	210.39 $\pm$ 6.00 ^a	166.84 $\pm$ 6.75 ^b	139.87 $\pm$ 0.26 ^c	105.65 $\pm$ 3.56 ^d	<0.0001
Progesterone(ng/mL)	51.92 $\pm$ 0.68 ^d	61.73 $\pm$ 1.03 ^c	73.19 $\pm$ 0.64 ^b	89.52 $\pm$ 0.93 ^a	<0.0001
Cortisol (nmol/L)	207.53 $\pm$ 5.79 ^d	233.79 $\pm$ 10.05 ^c	299.82 $\pm$ 0.49 ^b	340.57 $\pm$ 10.02 ^a	<0.0001
t-PA(ng/mL)	5.71 $\pm$ 0.10 ^d	6.87 $\pm$ 0.16 ^c	8.33 $\pm$ 0.16 ^b	9.96 $\pm$ 0.29 ^a	<0.0001

^{a,b,c,d} indicate significant differences at $p < 0.05$; Order of significance is as: $a > b > c > d$; T2C: 2nd trimester control; T3C: 3rd trimester control; T2GDM: 2nd trimester GDM; T3GDM: 3rd trimester GDM

Table III. An overall comparison of hormonal profile in negative family history group (n=124) and positive family history group (n= 176) during early 2nd trimester (14-18 weeks) and early 3rd trimester (24-28 weeks) of gestation.

Parameters	Early Second Trimester		Early Third Trimester	
	Negative History	Positive History	Negative History	Positive History
Vitamin D(ng/mL)	43.44 $\pm$ 0.79 ^a	39.92 $\pm$ 0.99 ^b	30.32 $\pm$ 0.47 ^c	22.18 $\pm$ 0.31 ^d
SHBG(nmol/L)	208.86 $\pm$ 6.01 ^a	167.44 $\pm$ 5.05 ^b	114.07 $\pm$ 2.08 ^c	103.84 $\pm$ 2.15 ^c
Progesterone(ng/mL)	52.23 $\pm$ 0.63 ^d	59.77 $\pm$ 0.53 ^c	69.35 $\pm$ 0.73 ^b	81.11 $\pm$ 0.74 ^a
Cortisol(nmol/L)	206.15 $\pm$ 4.44 ^d	224.05 $\pm$ 6.39 ^c	288.35 $\pm$ 4.39 ^b	319.02 $\pm$ 6.92 ^a
t-PA(ng/mL)	5.32 $\pm$ 0.13 ^d	6.67 $\pm$ 0.09 ^c	7.92 $\pm$ 0.15 ^b	9.58 $\pm$ 0.15 ^a

^{a,b,c,d} indicate significant differences at $p < 0.05$; Order of significance is as: $a > b > c > d$

group when compared with controls in the early second trimester and early third trimester. All subjects showed reduced levels of vitamin D and SHBG, and elevated levels of progesterone, t-PA and cortisol in the early third trimester, but the variations were more pronounced in the GDM group as compared to the control group.

All the subjects were further categorized on the basis of positive and negative family history of GDM or T2DM. Among 300 pregnant women, the percentage of GDM pathogenesis in females with positive family history was found to be 32%, while it was 14% for those with negative family history. Hence, all the GDM subjects were further divided into two groups on the basis of positive/negative family history of GDM/T2DM. A comparison was made between these two groups to access the extent of variation. Strikingly, the changes were more pronounced in the group with positive family history. Significant increase in progesterone, cortisol and t-PA, but significant decrease in vitamin D and SHBG were observed in those with family history of GDM as compared to those without such history, both in early second and third trimesters. The results demonstrate that patients with positive family history of GDM or T2DM are more prone to develop GDM compared to those with negative family history (Table III).

DISCUSSION

During pregnancy, women with GDM are at higher risk for maternal and fetal complications (8). Vitamin D deficiency during early pregnancy can affect the metabolism of glucose in a number of ways: pancreatic cells express hydroxylase; the active vitamin D form binds to the pancreatic cell vitamin D receptor; the human insulin gene promoter contains the vitamin D response element (9). There is also evidence of the role of vitamin D in maintaining the tolerance of glucose by affecting the secretion and sensitivity of insulin (10).

Tissue plasminogen activator (t-PA) reflects endothelial activation, but its circulating concentrations also reflect hepatic fat content, with reduced t-PA associated with weight loss. It has a remarkably strong link with insulin resistance in

females with and without polycystic ovary syndrome (PCOS) (11). Significantly increased mean values of progesterone and cortisol in GDM subjects compared to the controls suggest that in pregnancy complicated with glucose intolerance there are further elevations in the levels of placental hormones which lead to insulin resistance and eventually GDM. The relationship between insulin and SHBG is well established since decreased levels of SHBG are linked with type 2 diabetes development (12). It is concluded that early 2nd trimester non-fasting SHBG could possibly be the best marker to assess subsequent GDM. We excluded all other factors, which could otherwise contribute to the above-mentioned alterations. Moreover, during analysis, the GDM subjects who were found to possess some other clinical complications other than GDM were also excluded. We therefore conclude that the above-mentioned alterations in pregnant women were attributed solely to GDM. Further studies are needed to assess current indicators of GDM. If levels of these studied early biomarkers can be controlled via therapeutic measures at early stages of pregnancy, the onset of said disease can be delayed or prevented in later stages of pregnancy.

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LETTER TO THE EDITOR

INFLUENCE OF PREBIOTIC ADMINISTRATION ON INFLAMMATORY AND GLYCAEMIC MARKERS IN TYPE-2 DIABETES

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To the Editor,

Prebiotics are often administered with the daily diet: they have been widely investigated in recent years, and several researchers have assessed that they can favorably affect health and cell aging. Prebiotics seem to actively enhance several antioxidant pathways that overall improve the immune homeostasis, also increasing the suppression of chronic inflammation and the prevention of insulin resistance (1).

Prebiotics may promote changes in the bacterial composition and/or in the main activities of the gastrointestinal tract; moreover, they have been shown to improve the gut environment and its microbiome, often closely associated with the onset and severity of type-2 diabetes mellitus (T2DM). Furthermore, prebiotics can modulate the production of haemoglobin-A1c (HbA1c), thus modulating the glycaemic values detected just after lunch. Finally, prebiotics have been associated with a slight reduction of the main inflammatory markers, in T2DM patients (1,2).

Growing evidence links prebiotics to better control of glycemia, thanks to their action on the gut microbiota (3). Both animal and human studies have linked gut microbiota to metabolic dysregulation. Patients with

diabetes have a different gut microbiota, in comparison to healthy individuals (3-5), characterized by a high Gram-negative and Gram-positive bacterial ratio, and a low presence of bifidobacteria, which is a microbial population able to give several health benefits (5). Changes in gut microbiota composition, and their impact on gut permeability and glycaemic control, have not been well investigated; in particular, the mechanisms working in humans are still quite unknown (6).

There are currently several strategies for reducing the glycaemic markers in humans: prebiotics are a potentially novel, economic, and safe treatment for T2DM, able to improve the control of T2DM by modulating gut microbiota, gut permeability and local inflammation (7). The aim of this pilot study was to evaluate the influence of prebiotic administration on inflammatory and glycaemic markers in patients affected by type-2 diabetes.

MATERIALS AND METHODS

Twenty patients with body mass index (BMI) 25–40kg/m², 40–65 years of age, reporting a high risk of developing T2DM were enrolled in our study. All participants

Key words: type-2 diabetes; evidence-based medicine; prebiotics; inflammation

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followed a standardized diet (55% carbohydrates, 30% fats, 15% proteins) isocaloric, for 7 weeks. Prebiotic foods (e.g., yogurt) and/or superfoods were not allowed. Written and verbal informed consent was also obtained from each participant.

A randomized double-blinded, placebo-controlled, parallel-groups experimental design was carried out. In the first stage, the participants were allocated into two groups: a) Group using a supplementation with Hyperbiotics Prebiotic Powder (1 scoop/day), or b) Group using a supplementation with 10g/day of a placebo (maltodextrin; 4kcal/g) for 7 weeks.

Measurements of key outcomes, such as body weight or glycaemic markers, were performed at baseline, and at the following time-points.

All measurements were taken during the time-lapse 7:00 to 11:00 a.m. Blood samples were collected at baseline, and after 7 weeks. The samples were stored at -80°C .

The product tested in the experimental group was Hyperbiotics Prebiotic Powder, a soluble dietary formula containing organic fibers from acacia, Jerusalem artichoke and green banana flour (Zuvii™), with non-GMO ingredients, free of gluten, sugar, wheat, psyllium, and dairy.

Statistical analysis

Outcome measures of the exploratory study were

analysed with a *t*-test for paired samples for pre-post differences with time as the factor using Statistical Package for Social Sciences (SPSS for Windows, Version 11.5, Chicago, Ill) software, to detect significant differences between pre-test and post-test scores.

RESULTS

In this study, 20 patients were selected, and randomly enrolled into a treated group (4 females and 6 males) and a placebo Group (3 females and 7 males).

Blood glucose and HbA1c analysis.

The results showed that the administration of prebiotics may significantly induce a better glucose tolerance, mainly demonstrated by a fast and significative reduction of glycaemic markers and glycated hemoglobin (Fig. 1). In fact, in the treated group there was a decrease of 4% in HbA1c values, and a reduction of 2% of glycemia. On the other hand, in the placebo group the variations did not exceed 0.5% in the two time-points.

Analysis of inflammatory markers

In the both the control group and the treated group, it was seen that the serum concentration

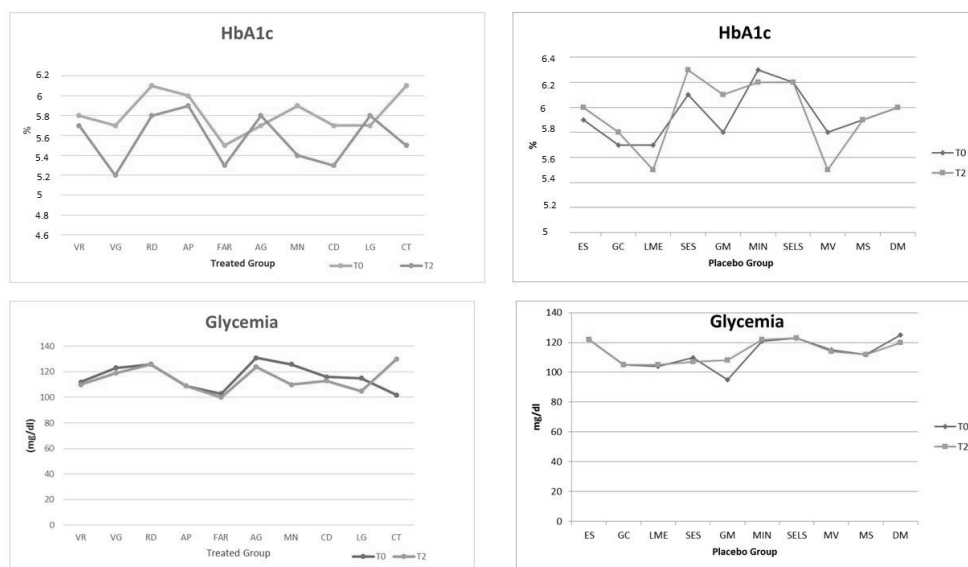


Fig. 1. HbA1c and glycemia levels in treated/placebo groups.

of the main pro-inflammatory markers remains almost unchanged. IL-6 and TNF- α parameters remain almost the same (Fig. 2): these data suggest that prebiotics added in a normal alimentary diet administered to diabetic patients do not positively impact the inflammatory state, but seem to slightly

improve such parameter over time. After careful evaluations of Fig. 2, it can be seen how the levels of IL-6 decrease 3% in the treated group; on the contrary, in the placebo group they remain constant. Additionally, in the treated group, the TNF- α values appear to be substantially constant.

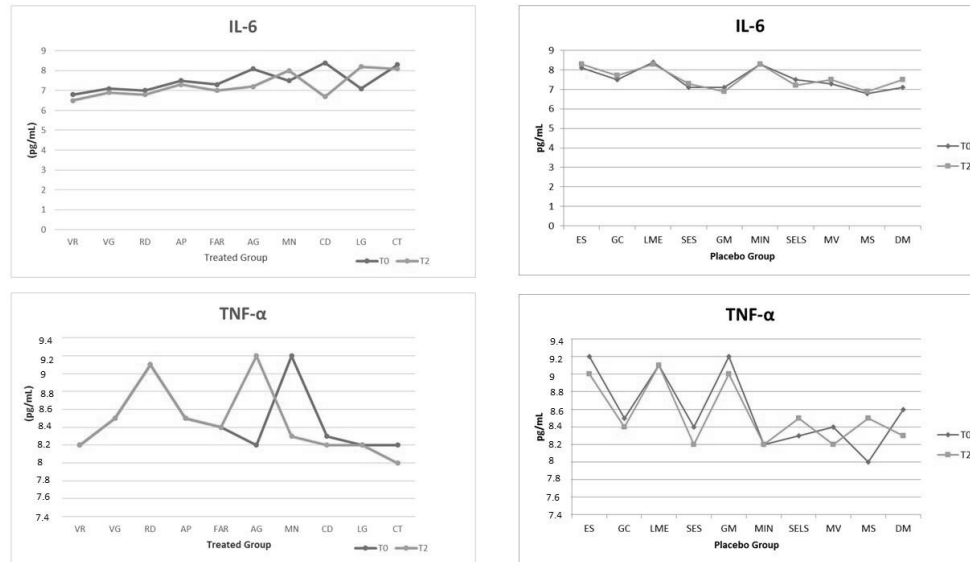


Fig. 2. IL-6 and TNF- α levels in treated/placebo groups.

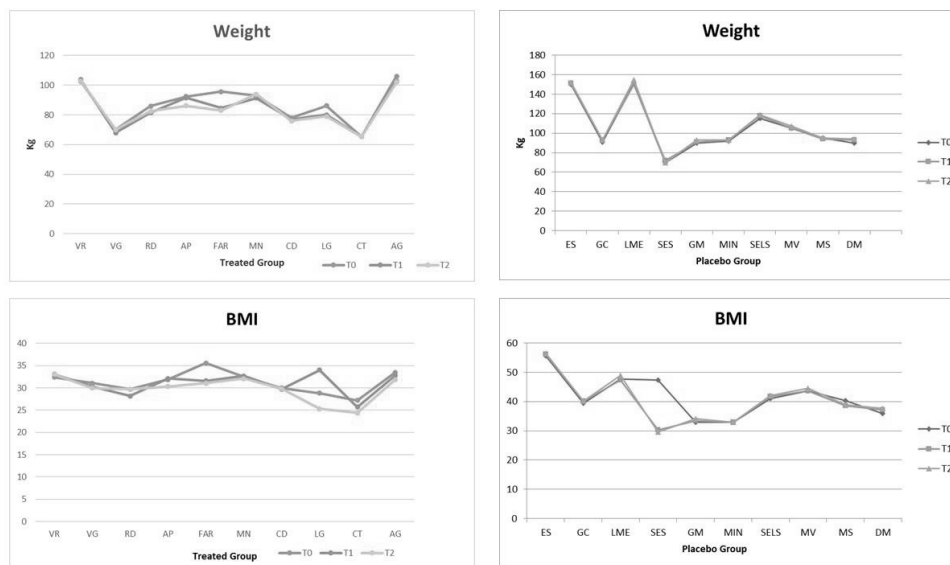


Fig. 3. Weight and BMI values in treated/placebo groups.

Bioimpedance analysis

T2DM patients enrolled in the prebiotics group achieved a better weight reduction. The analysis of bioimpedance data revealed that patients within the treated group showed a weight reduction of about 4%, while the placebo group seemed to maintain the same weight (Fig. 3). On the other hand, the body mass index (BMI) of treated subjects remained unchanged, just like the placebo group.

DISCUSSION

Patients affected by diabetes mellitus have impaired neutrophil and macrophage activity, thus leading towards an acute inflammatory state. Prebiotics may improve blood sugar levels in patients with type 2 diabetes. A deep and systematic evaluation of clinical trials reported in the scientific literature has clearly revealed that the use of prebiotics can have anti-inflammatory and anti-diabetic effects (8).

It is hypothesized that the anti-inflammatory properties of some prebiotic strains may act by reducing inflammation, through the modulation of cytokine levels and other inflammatory mediators, as reported in some in vitro and in vivo studies (9).

One recent clinical study found that the daily assumption of *L. reuteri* enriches the human intestinal microbiota and modifies the intestinal production of locally active factors that increase insulin secretion. This study was based on a daily administration of *L. reuteri* strain (ATCC SD-5865) in tablets (10). A randomized double-blind placebo-controlled study compared the secretion of insulin and proglucagon-derived P peptides and the function of pancreatic beta cells in glucose-stimulated non-diabetic participants (11). However, the consumption of prebiotics did not lead to any significant clinical results, although it was possible to understand that the consumption of prebiotic-enriched bread by diabetic patients had positive effects on the insulin metabolism (12).

In conclusion, it could be possible to improve glycaemic management in T2D, and to decrease the inflammatory cytokines in prediabetic subjects, if selected prebiotics are administered, within a safe and healthy diet. Numerous other studies on this matter showed that prebiotics undoubtedly give benefit to

patients with type 2 diabetes mellitus. This is only a pilot study, therefore we still need to understand the molecular mechanisms involved in the clinical and biological interaction between prebiotics and diabetes.

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LETTER TO THE EDITOR

COULD THE “WATCHFUL WAITING” APPROACH IN A PATIENT WITH GAS GANGRENE SAVE THE LOWER LIMB?

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To the Editor,

Gas gangrene is an unusual complication of traumatic injuries associated with high rates of mortality and morbidity. The organisms responsible for this condition can be classified into two groups: Clostridium-related and Non-Clostridium related (1-2). The most common bacteria isolated from gas gangrene tissues are *C. perfringens*, *C. bifermentans*, *C. tertium*, *Enterococcus*, *Staphylococcus*, *Enterobacter*, *Klebsiella*, *Bacteroides* species (3-4).

Non-Clostridium-related gas gangrene is less frequent than that Clostridium-related and requires different clinical and surgical management as it represents a mixed infection with high mortality rates. In traumatic orthopedic patients, the post-operative outcome relates to different factors, including type of fracture, age, soft tissue damage and pathogen involvement (5). Amputation is considered the most common and resolute treatment.

Case report

A 29-year-old patient was brought to the Emergency Room of “Policlinico Gemelli” after a motorcycle accident. X-ray examinations showed a subdural frontal hematoma, a plurifragmentary left distal humerus fracture, plurifragmentary left ulnar-radius fractures, left head femur posterior luxation associated with superior and posterior acetabular wall

fracture (Figs. 1, 2a,b), and exposed left tibial-fibular distal metaphysis fracture. A large wound from the anterior of the leg, passing by the popliteal fossa with articular knee exposition, was also present.

The emergency treatment was an external fixation of the leg with a tibial-metatarsal bridge Hoffmann 3, followed by an accurate debridement of the soft tissue lacerations, and elbow external fixation. The hip fracture-luxation was not treated at that time.

After the worsening of the patient's state of consciousness, he was transferred to the Intensive Care Unit. About 5 days later, a second orthopedic evaluation was requested by the Intensive Care equipe because of further aggravation of the clinical situation: the leg wound was infected and was complicated with gas gangrene, which evolved to septic shock; the cardiocirculatory function was supported by elevated levels of exogenous catecholamines. The only possible treatment was apparently to disarticulate the left hip joint, because of the acetabular fracture. In the operating room, the choice between disarticulation and simple amputation, which would have allowed to preserve the hip joint, was discussed. This last option would have permitted the patient, in a proximal future, to install a prosthetic leg and walk again. On the other hand, the risk was that incomplete asportation of infected tissues could lead to the persistence of the septic shock state.

*Key words: gas gangrene; lower limb amputation**Corresponding Author:*

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Fig. 1. X-ray (AP projection) showing the left femur posterior luxation associated with superior and posterior acetabular wall fracture.



a



b

Fig. 2. *a, b*) 3D TC reconstruction highlighting the skeletal situation.

The collegial decision was to proceed with a simple amputation at the III middle of the left femur, with a very accurate curettage and asportation of the infected-necrotic tissues. The microbiological swab showed the presence of three different bacterial species: *Klebsiella oxytoca*, *Enterobacter cloacae*, and *Enterobacter aerogenes*. Antibiotic therapy with amoxicillin/clavulanic acid, clindamycin,

meropenem, linezolid, cefepime was performed. In the following weeks, the septic situation was solved and there was even a neurological improvement.

Meanwhile, the VAC therapy to the distal stump was started, to treat a dehiscent suture before performing another little surgical treatment of resizing and revision. After about 2 months from the accident, with the stability of clinical conditions,



Fig. 3. Postoperative X-ray showing the acetabular osteosynthesis and total hip arthroplasty with LIMA MINIMA.

the ORIF of the acetabular fracture with plate and screw was performed, using cortical spongiosum samples removed from the femoral head, to fill the acetabular bone loss. Then a total hip arthroplasty with LIMA MINIMA prosthesis was carried out (Fig. 3). The decision to utilize this kind of prosthesis was motivated by the small residual femoral bone after the amputation.

The patient gave his informed consent to the processing of his personal data for this study.

DISCUSSION

Gas gangrene is an unusual but fatal complication of injuries in traumatology, and often the most effective treatment is amputation or disarticulation to have a wide margin of healthy soft tissues. The persistence of this kind of infection after any type of treatment (pharmacological and surgical) is frequent and frequently fatal. In this case, considering the young age of the patient (29 years), the decision to perform this unusual treatment allowed to preserve the his ability to walk, by implanting a lower limb prosthesis. This decision risked not to solve the infective condition, however an accurate surgical treatment and targeted antibiotic therapy permitted this to be less demolitive.

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LETTER TO THE EDITOR

SEGMENTAL MANDIBULAR BONE RECONSTRUCTION THROUGH THE USE OF A SPECIAL OSTEOSYNTHESIS PLATE WITH IMPLANTS COVERED WITH A MODULAR ENDOPROTETIC SCAFFOLD OF POLY (ϵ -CAPROLACTONE) COATED WITH HYDROXYAPATITE

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To the Editor,

The mandibular bone provides the skeletal support for the dental elements, assuming a fundamental role regarding function (chewing and phonation) and aesthetic (facial profile). Reconstruction of mandibular defects due to traumas, cancer resections, malformations and osteonecrosis, represents an interesting challenge for clinicians (1, 2). At present, the gold standard for mandibular reconstruction is the use of osseous free flaps, such as fibular free flap or iliac crest free flap. Nevertheless, this type of reconstruction has some tridimensional limits, not being able to replace exactly the architectonic form of the original mandible. Moreover, this technique requires a long operating time, demanding post-operative care, an increase of hospitalization and an important morbidity of the donor site. For these reasons, many authors have discussed different types or methods of mandibular reconstruction, such as the use of reconstructive plate, bone grafts, or biological bone (1-5). To improve and facilitate mandibular replacement, different researchers have tried to find alternative methods for bone reconstruction, such as heterotopic bone induction, modular titan

endoprosthesis through engineered models, and ϵ -polycaprolactone scaffold (6-8).

The aim of the present study is to find an alternative method for mandibular reconstruction, using an innovative system composed of an ergonomic titanium plate, with dental implants connected, covered in ϵ -polycaprolactone scaffold, while ensuring a good osteointegration, replacing the exact morphology of mandible, improving the degree of mastication, and satisfying the aesthetic outcome. The use of the ϵ -polycaprolactone scaffold could allow for a lower operative burden compared with conventional techniques by avoiding creation of a secondary bone defect.

MATERIALS AND METHODS

The proposed project for mandibular reconstruction was designed by the Authors to improve the field of mandibular reconstruction options. A biodegradable endoprosthesis modular scaffold was designed. This scaffold constituted poly ϵ -caprolactone (PCL) which is a bio-absorbable polymer, resistant, durable, stable and bio-available (9, 10). The realization of the PCL scaffold

Key words: poly (ϵ -caprolactone); hydroxiapatite; mandible reconstruction; tissutal engineer; bone morphogenetic protein -2; osteosynthesis; plate dental implants

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was obtained using prototype additive manufacturing and Selective Laser Sintering technology (SLS). A dedicated software monitored the porosity, the structure, and the mechanical resistance of the scaffold. The osteoinductive property of the scaffold was stimulated by the addition of simulated body fluids (mSBF), similar to human plasma, but enriched with calcium and phosphate. mSBF induces the release of growth factors (VEGF e rhBMP-2), moreover, the scaffold is activated by the introduction of stromal multipotent mesenchymal cells from bone marrow (BMSC) produced with cell cultures, or isolated from bone marrow or adipose tissue.

The design phase of the ϵ -PCL scaffold is made up of different stages. Firstly, we defined the correct mandibular resection, then we projected and realized the ϵ -PCL scaffold and the surgical time.

Phase 1

Three-dimensional computed tomography (3D-CT) scanning was performed, and computer-aided design techniques were used to produce an ideal virtual replacement for the mandibular defect. ϵ -PCL scaffold was created using SLS technology (EOS P110), after the creation of a 3D TC model (slice <1mm) in STL file. The printer employs a powdered mix of polycaprolactone and hydroxyapatite 4%.

Using a custom-made technique, an exact stereolithographic model reproducing the mandible was obtained. This led to identifying the mandibular segment for resection and what would have to be reconstructed.

Phase 2

During this phase we projected and created the entire system of reconstruction, formed by a surgical plate (Patent U.S.A-Europa-United States Patent No.: US 6,613,055 B2 Di Emidio (45) Date of Patent: September 2, 2003) upholstered in ϵ PCL scaffold.

The plate has two lateral ports with five holes predisposed for positioning the screws to fix the entire scaffold. Between those two portions, the middle part of the plate is characterized by a tridimensional conformation and pliability; this central portion is composed of a series of spheres interconnected by cylindrical segments. The smooth shape of these components avoids the traumatic action of the plates, enabling the best fitting of the plate system.

The spheres have microholes (0.02 mm Ø) on their surface, obtained with the *auxilium* of a laser. On the top of each sphere we connect a modified dental implant, which could be of different lengths (Fig. 2). These implants have different features: a threaded shank with a microhole at the extremity; inside the implant there is a tubular channel that creates a connection with the internal part (Fig. 3A). When the modified implant is connected with the sphere, an internal via of communication is created between the implant and the plate. This system permits to insert, using a syringe, therapeutic substances, such as Recombinant human bone morphogenetic protein (PMR), in the superior extremity of the dental implant, after removal of the screw top, that go through the implant and arrive at the spheric part of the plate, leading to the diffusion of these substances in the entire PLC scaffold (Fig. 3B-C). After the complete osteointegration of the entire system, it is possible to perform the prosthetic rehabilitation.

Phase 3

The reconstructive system, composed by titanium plate and ϵ -PCL scaffold, was sterilized. Afterwards, the resection was inserted and fixed on human cadaver (Fig. 4). The scaffold was activated using injection of plasma and mSBF; this injection could be repeated during the post-operative weeks by just removing the screw cup from the top of the implants.

RESULTS

The entire reconstructive system, composed of an osteosynthesis plate with implants, a modular endoprotetic scaffold of poly (ϵ -caprolactone) coated with hydroxyapatite, guarantees a precise reconstruction of the bone defects. The system has optimal mechanical properties, porosity of the scaffold is in a range between 37% and 55%, the mechanical compression module is in the range between 52-68-MPa, the maximum resistance is in a range between 2.0- 3.2-Mpa. The fusion degrees for processing PCL is 60°C. The PCL has a molecular weight of 50.000 kDa and a particle size distribution in a range between 10–100 lm. Mechanical analysis confirm a fracture point with a strength equal to 60 N. These characteristic are similar to the biomechanic of human bone, having a good bearing capacity.

Micro-CT analysis shows a homogenous structure and an interconnection of all pores. The print time is 180 min.

The poly (ϵ -caprolactone) scaffold added with growth substance (rhBMP-2 and BMSC) has a capacity of osteoinduction, which demonstrated that

the bone regeneration was obtained in 6 months.

DISCUSSION

PCL scaffolds are largely used for bone and cartilage reconstruction . The possibility to print and shape

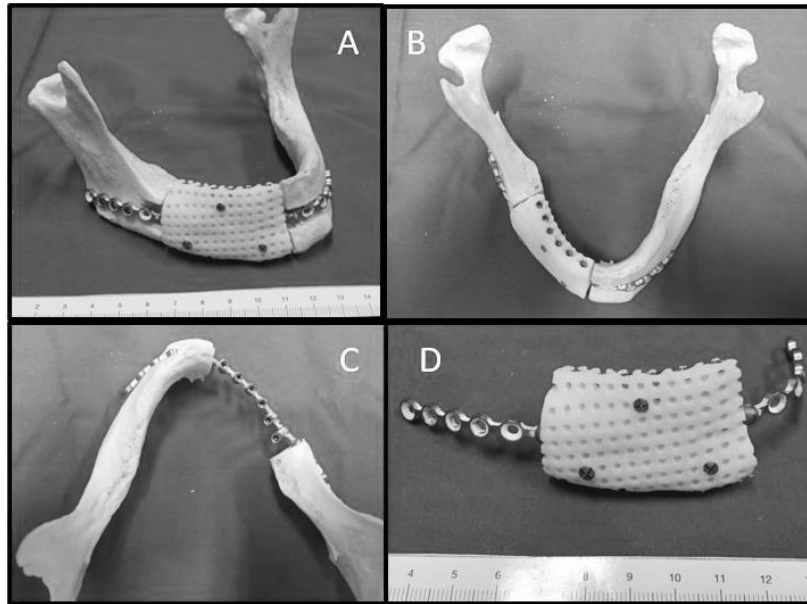


Fig. 1. Custom-made model of mandibular reconstruction segment (A,B,C), with the ϵ -PCL scaffold and relative plate (D).

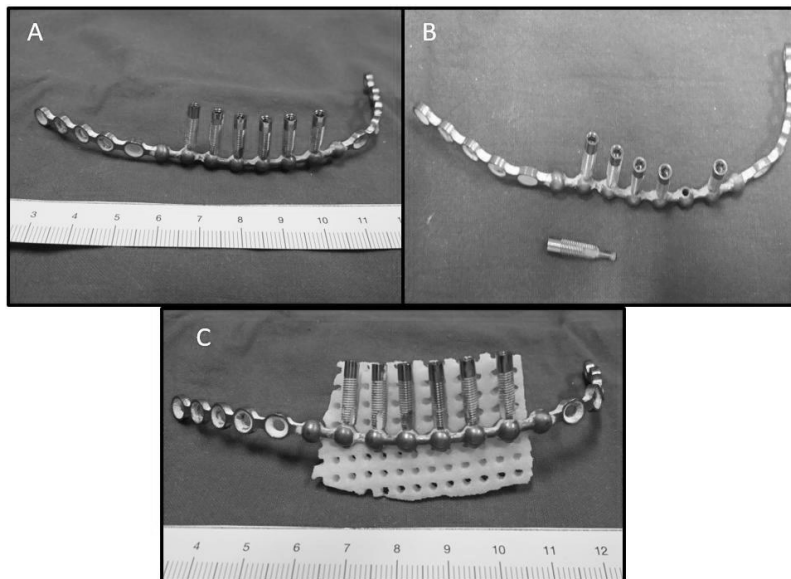


Fig. 2. Surgical plate with tridimensional components and modified dental implants (A, B) upholstered in ϵ -PCL (C).

this biomaterial through rapid prototypization and additive manufacturing, as well as its bio compatibility, could guarantee a diffuse use of this material. The osteoinductive properties and the possibility to add growth factors and BMSC improve the possibility to use this reconstructive system (11, 12).

In 2014, some Authors described the possibility to reconstruct a segmental defect of mandibular body using an ϵ -PCL scaffold combined with bone marrow homolog cells (ϵ -PCL-BMP) in a primate model similar to human. They wanted to valid the use ϵ -PCL-BMP and its efficacy compared to a control PCL group. The mechanic results show a major bearing capacity in the first group. Those researchers, after a six-month period, valued, in their sample, the appearance of complications (infections, inadequate mechanic resistance of the plate) (9).

Warncke at all. describes the use of bone without antigens; they created a titanium mesh cage filled

with bone mineral blocks and infiltrated with recombinant human bone morphogenetic protein and 20 mL of the patient's bone marrow, obtaining an etherotopic osteoinduction, but a low mechanical resistance and a rapid resorption (6).

The innovation of our reconstructive system is represented by a complex system, in which we overcame the problem of mechanical resistance using a tridimensional ergonomic plate. We tried to reduce the risk of the plate's exposure by coating the entire plate with ϵ -PCL scaffold and hydroxiapatite, which guaranteed osteoinduction. Moreover, another advantage is to have the possibility to change the length of implants and to have a prosthetic rehabilitation. The insertion of growth factors and BMSC through the implant is another good characteristic of the system as, after the operation, it activates the ϵ -PCL scaffold.

Our proposal would be to start an animal

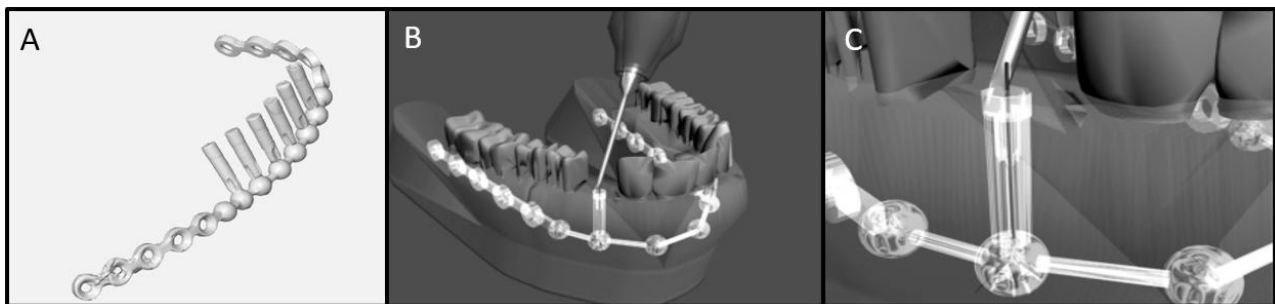


Fig. 3. 3D computer-model of surgical plate (A) and relative modified implants (B, C).

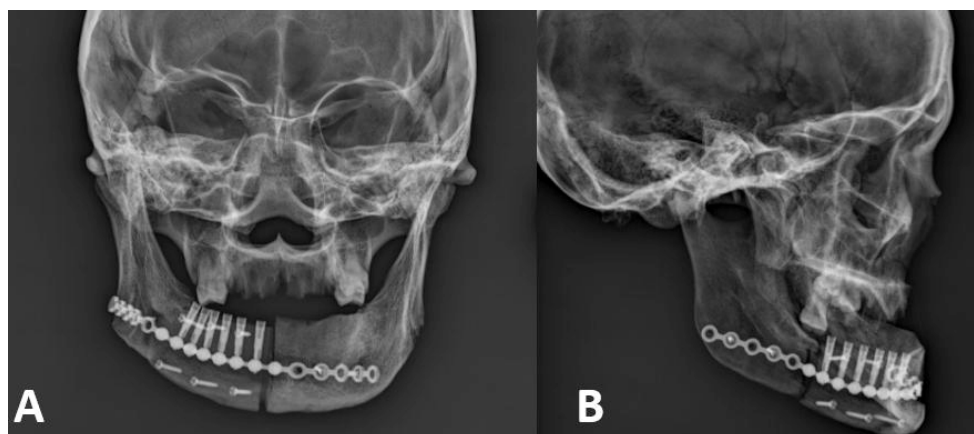


Fig. 4. Cranial X-ray in antero-posterior (A) and latero-lateral view (B) in human cadaver.

experimentation to verify the entire reconstructive system, creating a collaboration with biomedical engineering laboratories. This project could represent in the future a good alternative method for mandibular reconstruction in selected cases, even if at present this system is not valid in humans.

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LETTER TO THE EDITOR

UPDATE ON THE USE OF ANTIBIOTIC PROPHYLAXIS IN PATIENTS WITH HEART DISEASE

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To the Editor,

The oral ecosystem hosts more than 700 different bacterial species: each lesion in this environment represents for these microorganisms a way into the blood stream, generating a bacteremia. In patients with chronic diseases it can cause a worsening of the clinical picture (1, 2). The bacteremia generated by dental procedures could cause bacterial endocarditis in subjects with heart disease (3, 4). Bacterial endocarditis is an infrequent disease with an incidence between 3 and 10 cases per 100,000 population, but is characterized by a high mortality: almost 100% if not treated, and between 20-30% if treated properly (5, 6). Antibiotic prophylaxis before dental procedures would reduce the risk of bacteremia, in order to avoid bacterial endocarditis in patients with heart disease (3, 7).

Staphylococcus aureus, which is mainly present on the skin, is now the main agent responsible for the disease with between 15 and 40%, to the detriment of *Streptococcus viridans*, which comes from the mouth (6, 8-10). It is necessary to carry out a systematic review in order to provide up-to-date indications for the management of patients at risk of contracting bacterial endocarditis after dental treatment.

MATERIALS AND METHODS

For this work a research on the database of Medline, Science Direct and Cochrane Library was carried out using as key words: 'infective endocarditis' and 'antibiotic prophylaxis'. Only articles published from 2009 to the present day were considered. Duplicates were excluded from the list, and only those related to the use of antibiotic prophylaxis in dentistry were selected. Primary studies, bibliographic reviews, meta-analyses and guidelines have been included in the review. Finally, a table was created that summarizes the contribution of the various studies. The articles were then classified according to type, year of publication and main conclusions, summarizing them in a table and highlighting the most important data in bar and pie charts.

RESULTS

Twenty-nine articles were considered for analysis, including 4 guidelines, 20 bibliographic reviews, 1 meta-analysis and 4 primary studies (Fig.1). More than half were published in the last 5 years. The distribution of articles by year and type is summarized in (Fig. 2). The content of the articles

Key words: infective endocarditis; antibiotic prophylaxis; dental guideline

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according to the factors indicated in the methods are summarised in Table I. The main conclusions are reported in Figs. 3 and 4. Fig. 3 shows the different opinions of the authors regarding the existence of a relationship between dental treatment and the appearance of bacterial endocarditis. Only 5 studies confirm this relationship, while the majority have some doubts (12 articles), and 5 authors do not comment on it. The majority of studies recommend prophylaxis, others suggest to modify the guidelines, while only one author would completely stop this practice (Fig. 4).

DISCUSSION

The relationship between dental treatments and the appearance of bacterial endocarditis, on which the use of antibiotic prophylaxis is based, is a very complex issue. The studies analysed show uncertainty on the subject, so much so that only 5 articles, including 4 guidelines, confirm its existence, estimating a ratio of one case of endocarditis for every 150,000 dental procedures. Many people are doubtful about the subject (42%) or even prefer not to comment on it (17%). On the contrary, with regard to

Table I. *European guidelines for dental procedures in which antibiotic prophylaxis is recommended*

Author and year of publication	Type of patients	Type of orthodontic treatment	Type of antibiotic recommended
ESC, 2015 [4]	a) valve prostheses b) previous bacterial endocarditis c) cyanotic congenital heart disease d) congenital disease repaired with a prosthetic material.	ESC 2009 + note: no prophylaxis to treat superficial caries	ESC 2009
AHA, 2017 [28]	a) valve prostheses b) previous bacterial endocarditis c) congenital heart disease without repair or with complete repair. d) cardiac transplantation with valve regurgitation.	\	ESC 2009
Millot, 2017 [37]	ESC 2015	ESC 2015	ESC 2015
Tubiana, 2017 [40]	With valve prostheses	No difference between high or low risk	\
Chen, 2018 [41]	\	Invasive treatments	\
Hong, 2010 [17]	With venous catheter	Invasive treatments	\
Thornhill, 2011 [15]	With valve prostheses	Invasive treatments	3g Amoxicillin 1h before treatment
Falces, 2012 [13]	ESC 2009	\	\
Leong, 2012 [43]	Orthodontic patients	Put on and take off bands	2g Amoxicillin 1h before treatment
Gregor, 2013 [8]	Esc 2009 + men between 75-79 years old + patients with comorbidity	ESC 2009	ESC 2009
Alvarez, 2013 [26]	\	\	Depends on antibiotic resistance: ex Clindamycin not recommended for extractions
Brondani, 2013 [18]	AHA 2007 + consultation with the attending doctor	Treatments that produce significant oral bleeds and/or exposure to potentially contaminated tissues	\
Falces, 2013 [24]	ESC 2009	Invasive treatments	ESC 2009 Because they are effective in preventing <i>S. viridans</i> endocarditis

the use of antibiotic prophylaxis in patients at risk of endocarditis, the scientific community seems much more in agreement: the majority would recommend its use according to the existing guidelines. Instead, 38% of the authors claim that a change in clinical protocols is necessary, and probably owe their opinion to the data collected in the United Kingdom after the 2008 revelation that the National Institute for Health and Clinical Excellence (NICE) advised against antibiotic prophylaxis for all patients at risk of endocarditis in any dental procedures. The prescription rate decreased by 78.6%, but the increase in bacterial endocarditis cases remained constant and in line with pre-2008 values (11). On the contrary, the subsequent analysis of the UK events led to the conclusion that there are some types of patients at very high risk, such as those with valve prostheses, who therefore need prophylaxis. It should also be noted that NICE's current position has shifted to a softer approach, which supports the customization of treatment and interdisciplinary communication between dentist and cardiologist to assess the patient's level of risk (11). The only article that advises against antibiotic prophylaxis should be seen only in the context of patients with venous catheters and not in relation to all the others, on which the authors do not express themselves. A certain homogeneity of thought can also be found from the point of view of the patients who receive prophylaxis, i.e. high-risk patients, indicated in

the ESC 2015 guide (Table I). Patients of medium and low risk are excluded from the indications. This group includes immunosuppressed patients, those with hip prosthesis and with mitral valve prolapse, rheumatic heart disease, bicuspid valve disease, calcified aortic stenosis, or congenital heart conditions (i.e. ventricular septal defect, atrial septal defect, or hypertrophic cardiomyopathy).

The dental procedures for which antibiotic prophylaxis is recommended are those considered to be at high risk of bacteraemia, i.e. those indicated in the European and American guidelines (Table I). On the other hand, we must consider that the need for antibiotic prophylaxis is linked to the incidence of bacteraemia in each case. The probability of bacteraemia during a dental procedure involving the gum is considered to be between 10 and 100%. The data indicate highly variable percentages, depending on the treatment in question (Table II).

The best way to avoid bacteraemia is good plaque control with thorough oral hygiene and periodic dental check-ups, not only to reduce the possibility of daily bacteraemia, but to maintain a healthy mouth that does not require invasive treatments (6). For the posology and administration of prophylaxis, the dose recommended by the European guidelines of 2009 (Table I), which gives the use of b-lactamics as first choice antibiotics, is widely accepted. However, it is important to note that studies show that amoxicillin is effective in reducing the frequency of bacteraemia

Table II. *Probability of bacteraemia during dental procedures*

<i>Dental treatment</i>	<i>Risk of bacteraemia</i>	
Extraction	10-100%	
Periodontal surgery	36-88%	
Curettage	in general	8-80%
	<i>Healthy patient</i>	10-21.6%
	<i>With gingivitis</i>	20-29%
	<i>with periodontitis</i>	51.2-75%
Professional hygiene	0-40%	
Periodontal survey	20-43%	
Intraligamental anesthesia	96.6%	
Endodontics	0-31%	
Dam placement	9-32%	
Orthodontic bands placement	7.5-10%	
Points removal	5-16%	

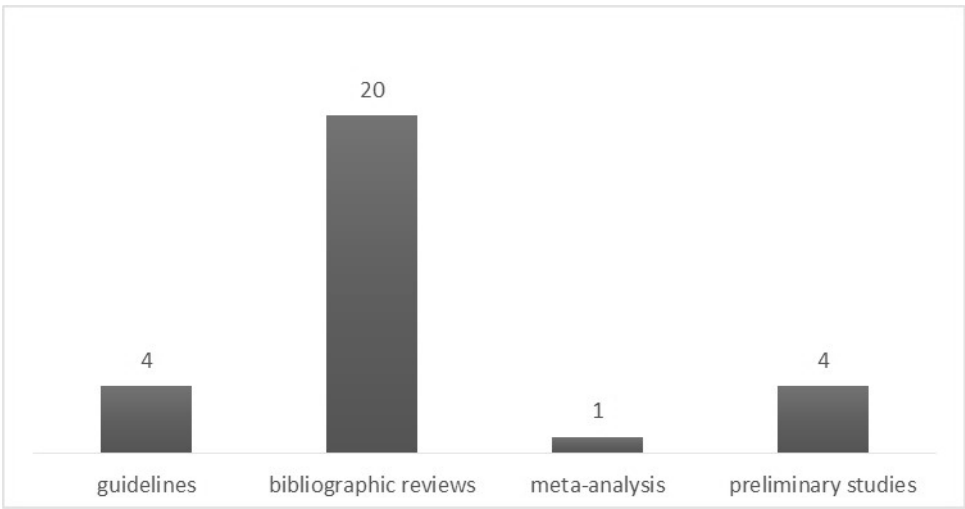


Fig. 1. Articles considered for analysis

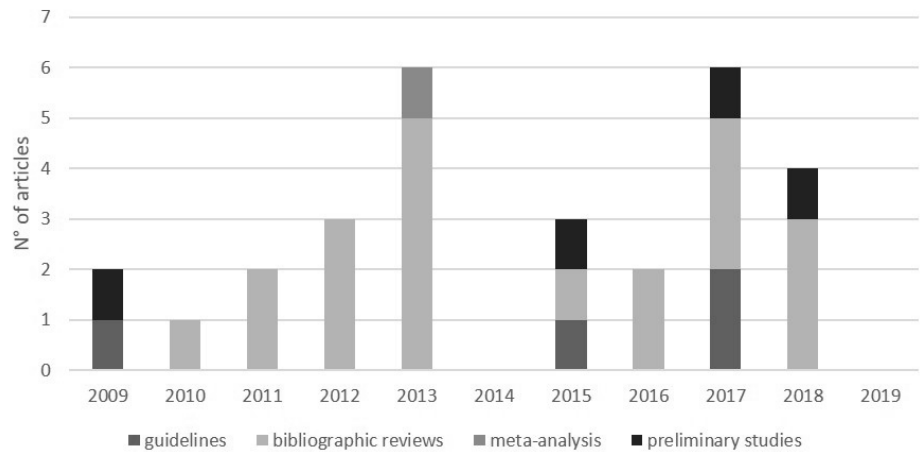


Fig. 2. Distribution of articles by year and type

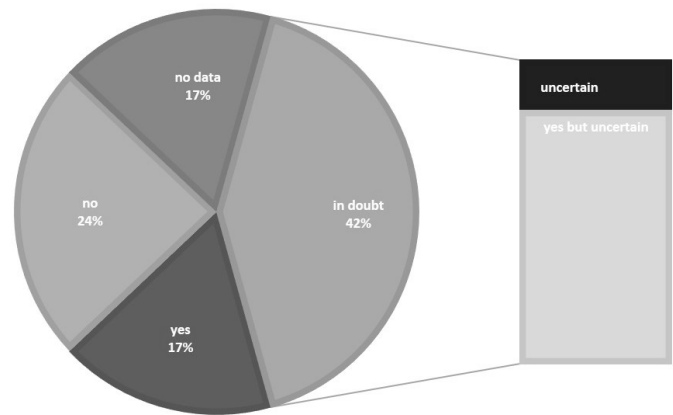


Fig. 3. Authors' opinions regarding the existence of a relationship between dental treatment and the appearance of bacterial endocarditis

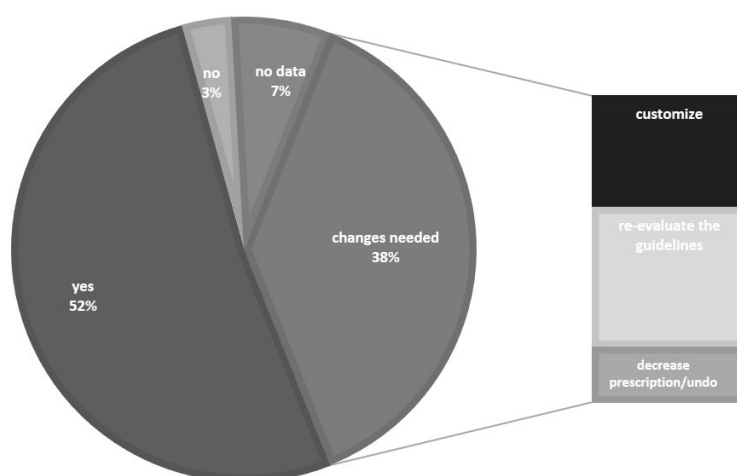


Fig. 4. Authors' opinions regarding antibiotic prophylaxis

but, on the other hand, is not able to eliminate it in 100% of cases. On the contrary, clindamycin does not seem to be effective in preventing either bacteraemia or infection of the surgical site. However, one author points out that, although clindamycin is less effective in preventing bacteraemia and probably useless in case of extractions, it is effective against the bacterial species responsible for endocarditis. Moreover, while amoxicillin is considered a very safe drug, clindamycin appears to be associated with adverse fatal reactions, mainly for secondary infections with *Clostridium difficile*. On this subject, some authors propose the use of chlorhexidine rinses to reduce the bacterial population. There is evidence that this is effective in reducing the incidence of bacteraemia, however, not everyone believes that the benefit is sufficient to justify its use as a substitute for antibiotic prophylaxis.

Data collected in the UK estimate that reducing the use of antibiotic prophylaxis for patients at risk of endocarditis would save between 6.6 and 9.8 million euros. If it were reduced to high risk patients alone, the savings would exceed 4.8 million of euros. Moreover, the advantage would be not only economic, but also in terms of quality of life. However, it should be added that the cost of antibiotic prophylaxis is much lower than the cost of treating the disease (5).

Moreover, in the case of antibiotics, the creation of possible antibiotic resistance must always be

closely monitored. In Europe, data from 2015 cite 671,689 deaths due to bacteria resistant to antibiotics during that year, with an incidence of 131/1 million and a mortality of 6.44/1 million. These figures become more surprising when comparing the consequences on the quality of life of these patients, expressed in DALY (Disability-adjusted life year) with those of other diseases during the same year and same geographical area. Infections caused by bacteria resistant to antibiotics have a DALY of 170/1 million, corresponding to the those of HIV, influenza and tuberculosis together (12, 13). In this, the greatest responsibility lies with western countries and their great abuse of drugs. The dentist alone plays a key role in this fight as he is responsible for a large number of antibiotic treatments. As far as the use of prophylaxis is concerned, many authors raise doubts about its possible role in the development of resistance (5, 6). On the other hand, others comment that there are no studies showing that a single dose causes resistance (3); indeed, antibiotic prophylaxis, due to its function of preventing bacterial diseases that require long antibiotic therapy, prevents the emergence of new resistant strains. Although the relationship between dental treatments and the appearance of bacterial endocarditis is not evident, antibiotic prophylaxis remains a recommended therapy for high-risk patients undergoing treatments involving mucosal perforation and manipulation of the periapical and gingival region, such as implant

placement. Many question marks remain in clinical practice. The dentist must therefore rely on the guidelines promulgated by the ESC while waiting for new indications.

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LETTER TO THE EDITOR

EXTRACORPOREAL SHOCKWAVE THERAPY FOR PLANTAR FASCIITIS AND GASTROCNEMIUS MUSCLE: EFFECTIVENESS OF A COMBINED TREATMENT

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To the Editor,

Plantar fasciitis (PF) is a very common disease that occurs at least once in a lifetime in 10% of the population (1). The plantar fascia is a thick, fibrous connective tissue sheet that runs from the medial tubercle of the calcaneal process to the metatarsophalangeal joints. The plantar fascia supports the plantar arch in absorbing dynamic shocks. In 83% of patients with plantar fasciitis, there is a contracture of the calf muscles, which is responsible for reducing ankle dorsiflexion during the last phase of the gait cycle (2). The patient assumes an antalgic gait in order to balance the lack of the ankle dorsiflexion with an external rotation of the foot or leg ('bouncy gait') (3). During the day, an average of 5000-7000 steps traumatizes the plantar fascia, because of moments of harmful longitudinal tension.

Extracorporeal Shockwave Therapy (ESWT)

induces bio-stimulation effects on tissues. For the treatment of plantar fasciitis with ESWT, the level of evidence of the studies is B with a success rate of 65% (4). These clinical results are due to the known biological effects of inflammation modulation, neo-angiogenesis and pain control (5, 6). According to the recent literature, the application of ESWT combined with specific stretching program and orthoses is responsible for better results and more stable over time (7). Furthermore, the authors reported that application of ESWT on muscle tissue for the treatment of spasticity, fibromyalgia, ossifying myositis is linked to finding decontracting, trophic and regenerative actions (8, 9). Analyzing the data of previous scientific literature, researchers have not sufficiently verified the efficacy of a combined ESWT treatment for the plantar fascia and for gastrocnemius musculature in patients with plantar fasciitis. We hypothesized that the patients affected

Key words: ESWT; fasciitis plantar; gastrocnemius muscle; stretching; range of motion; ankle

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by plantar fasciitis who receive shock waves (SW) for fascia could benefit from an additional treatment for the gastrocnemius musculature.

MATERIALS AND METHODS

The local Ethics Committee approved this prospective randomized study and written consent was obtained from the patients in accordance with the World Medical Association Declaration of Helsinki. For twelve consecutive months, 109 patients affected by PF who had undergone ESWT for PF were enrolled in the study.

The inclusion criteria were: diagnosis of PF and physical examination findings (plantar fascia tenderness and heel presson test); plantar heel pain more than 3 months; first-step pain in the morning during the previous week rated at least 2 on the pain scale (VAS 0–10); aged 18 years or older. The exclusion criteria were as follows: previous surgery of ankle, foot or lower leg; potential contraindications to ESWT (cancer or current or previous infections of the affected area, a cardiac pacemaker, or pregnancy or planning to become pregnant, epilepsy, coagulation disorders due to pathologies or drugs); conservative treatment (i.e. physical therapy, acupuncture, massage therapy, chiropractic treatment or local steroid injections) for PF administered in the previous 4 weeks; the presence of neurologic signs due to nerve root compression; other causes of heel pain (including tarsal tunnel syndrome, calcaneal fracture, ankle or foot instability, arthritis of the foot or ankle, rheumatoid arthritis, spondyloarthropathy, gout, neurogenic claudication, peripheral neuropathy); neurological pathologies responsible for spasticity of the posterior muscular chain.

Patients were randomized 1:1 into two groups: group A was treated with shock waves both for plantar fascia and for the muscle mass of gastrocnemius, whilst group B only for plantar fascia. Treatments were randomized according to a sequential randomization list using Stata MP12 statistical software. At the first session, all patients received the indication of insoles and stretching exercises. The clinical outcomes were recorded at baseline and 3 months after the last treatment session. Pain intensity was evaluated by Visual Analog Scale (VAS). The ankle range of motion (plantar flexion and dorsiflexion) was measured using an international manual goniometer device. Disability functional outcomes included the

Lower Extremity Functional Scale (LEFS), the Foot Functional Index (FFI) and American Orthopedic Foot and Ankle-Hindfoot Scale (AOFAS). Roles and Maudsley (RM) Score was recorded at the 3rd month. On enrolment, the lateral X-ray calcaneal and/or an echography of the plantar fascia were evaluated. For each patient, the epidemiological and anthropometric variables (age, gender, weight, height, BMI, smoking habit) and the clinical characteristics (site of the pathology, heel spur, duration, previous therapies, clinical outcomes and SW treatment protocol) were recorded.

A focalized electromagnetic shockwave device (Minilith SL1, Storz Medical, Tägerwil, Switzerland), equipped with in-line ultrasound-guidance Aloka SSD 900 (Aloka Co., Ltd. Tokyo, Japan), was used. The shockwave therapy was performed on the patient in the prone position and was administered once a week, for 3 sessions. Group A was treated with ESWT on the heel region and the gastrocnemius, whilst Group B was treated only on the plantar fascia. At each treatment session, 2000 impulses at an energy flux density (EDF) between 0.05 and 0.12 mJ/mm² and a rate of 4 pulses per second (4 Hz) were applied in correspondence of the insertion of plantar fascia for both groups. In group A, for the treatment of the gastrocnemius muscle, 800 impulses were delivered at an EDF of 0.05 mJ/mm² in the third medium of the muscle mass.

An expert physiotherapist conducted the first stretching treatment session in order to demonstrate the exercises program. The patient was instructed to hold each stretch for a count of 30 seconds, to repeat the stretch 10 times, and to perform these stretching exercises 3 times daily up to three months follow-up. The specific stretching of the plantar fascia was obtained by toe dorsiflexion with one hand and palpation of the plantar fascia with the other hand in order to ensure that it was taut. For gastrocnemius stretching the patient was seated on a firm surface while holding one leg straight in front of him/her and a towel was looped around the ball of his/her foot. The patient then pulled the towel towards himself/herself.

Sample size

A ROM value of 30° at enrollment (T0) was assumed for both groups, with an average value at the end of therapy (T1) of 36% in group B and 40% in group A, with a standard deviation in both groups of 5%. For the analysis of

the results, Student's *t*-test for independent data was used. Moreover, a significance level (α) of 0.01 and the power of the test at 90% were set; therefore, a sample size of 98 subjects was found which, assuming 20% of subjects lost to follow-up, brought the final sample to 120 patients, 60 in group B and 60 in Group A. The sample size was made using STATA MP15 software. The completed forms were inserted into a database built with Office Excel software and analyzed using Stata MP15 software. Continuous variables were expressed as mean $\pm$ standard deviation and range or as median, interquartile range (IQR) and range, categorical variables as proportions. The Skewness and Kurtosis test was used to evaluate the distribution of continuous variables and, for those not normally distributed, a normalization model was constructed. To compare normal or normalized continuous variables between groups, Student's *t*-test for paired data (parametric) was used, to compare the non-normalizable Wilcoxon rank sum test (non-parametric) was used and to compare these variables between groups and times, ANOVA analysis for repeated measurements was used; to compare the categorical variables between groups *chi*-squared test and the Fisher's exact test were used.

To evaluate the association between differences at T1 and T0 of VAS, of Ankle-Hindfoot Scale, of Lower Extremity Functional scale, of Foot function Index, of ROM, of plantar and dorsal flexion, and the group variables (group A/group B), multivariate linear regression was used; the correlation coefficients were calculated, with the indication of 95% confidence interval (95% CI) and Student's *t*-test. A *p*-value of 0.05 was considered significant for all tests.

RESULTS

At the end of treatment, the study sample was made up of 109 subjects, of whom 53/109 (48.6%) in group A and 56/109 (51.4%) in group B; 11 subjects had dropped out of the study at follow-up (Tables I and II).

ANOVA analysis for repeated measures revealed that there was a statistically significant association in the comparison between the two time-points (T1 and T0) for VAS ($F=175.6$; $p=0.000$), Ankle-Hindfoot Scale ($F=128.7$; $p=0.000$), Lower Extremity Functional scale ($F=168.9$; $p=0.000$), Foot Function Index ($F=140.1$; $p=0.000$), ROM ($F=130.0$; $p=0.000$), plantar flexion ($F=111.3$; $p=0.000$), dorsal flexion

($F=105.8$; $p=0.000$); A statistically significant association resulted in the comparison between the two groups in reference to dorsal flexion ($F=8.9$; $p=0.004$); there was a statistically significant difference between time-points and groups with regard to ROM ($F=17.2$; $p=0.000$) and to dorsal flexion ($F=54.4$; $p=0.000$). No statistically significant associations emerged in the comparison of variables between groups and in the interaction between times and groups ($p>0.05$). The average value of Roles and Maudley Score is 2.2 ± 1.1 (range: 1-4), without statistically significant differences between groups (control: 2.1 ± 1.1 ; range = 1-4 vs treatment: 2.3 ± 1.1 ; range = 1-4; $z=1.2$; $p=0.227$). With the ANOVA analysis for repeated measures, no statistically significant associations resulted in the comparison between groups and times for the EDF ($p>0.05$).

Applying multivariate analysis, no statistically significant association was observed for VAS, Ankle-Hindfoot Scale, Lower Extremity Functional Scale and Foot function Index, plantar flexion at the two follow-ups (T1 and T0) ($p>0.05$); there was a statistically significant association between ROM and the group variable (group A/group B) at T1 and T0 (coef.=6.4; 95% CI=3.0-9.8; $p=0.000$), whilst no statistically significant difference was found for the other determinants ($p>0.05$); for the comparison of dorsal flexion and the group variable (group A/group B) there was a statistically significant association at T1 and T0 (coef. = 7.2; 95% CI = 4.3-10.0; $p=0.000$), whilst no statistically significant difference was found with the other determinants ($p>0.05$).

DISCUSSION

This study compared two ESWT protocols for plantar fasciitis, in association with stretching exercises. Applying ESWT for fasciitis and muscle mass of gastrocnemius in the same session, better results were found in terms of recovery of dorsal and plantar flexion.

Plantar fasciitis is due to local biomechanical overload, which alters the gait cycle and traumatizes the plantar fascia. According to the literature, in patients with plantar fasciitis, authors reported a limitation of the tibio-tarsal dorsiflexion linked to

Table I. Sample characteristics at baseline, by group (group A/group B). Data are expressed as an average +/- standard deviation or percentage and with statistical analysis.

Variable	Group A (ESWT for plantar fascia and gastrocnemius muscle + stretching exercises) (n=53)	Group B (ESWT for plantar fascia + stretching exercises) (n=56)	Total (n= 109)	Test	p
Age; mean±SD (range)	58.4±11.3 (33-81)	59.6±10.9 (32-81)	59.0±11.0 (32-81)	t=0.6	0.569
Females; n (%)	35 (66.0%)	33 (58.9%)	68 (62.4%)	$\chi^2=0.6$	0.444
Height (m); mean±SD (range)	1.65±0.09 (1.50-1.85)	1.66±0.09 (1.48-1.85)	1.66±0.09 (1.48-1.85)	t=0.2	0.808
Weight (kg); mean ±SD (range)	78.2±12.5 (57-110)	80.0±13.5 (50-108)	79.1±13.0 (50-110)	t=0.7	0.464
BMI (kg/m ²); mean ±SD (range)	28.6±4.0 (20.2-40.1)	29.1±4.4 (17.9-39.7)	28.8±4.2 (17.9-40.1)	t=0.6	0.520
Treated foot; n (%)					
• Right	26 (50.9%)	27 (48.2%)	53 (48.6%)	$\chi^2=0.0$	0.930
• Left	26 (49.1%)	29 (51.8%)	56 (51.4%)		
Heel spur	35 (66.0%)	30 (53.6%)	65 (59.6%)	$\chi^2=1.8$	0.185
Smoke; n (%)	8 (15.1%)	9 (16.1%)	17 (15.6%)	$\chi^2=0.0$	0.888
Previous therapy; n (%)					
- physiokinesi- therapy	44 (83.0%)	38 (67.9%)	82 (75.2%)	$\chi^2=3.4$	0.067
- NSAIDs/ cortisone	9 (20.5%)	14 (36.8%)	23 (28.1%)	$\chi^2=2.7$	0.100
- orthopedic insole	24 (54.6%)	6 (15.8%)	30 (36.6%)	$\chi^2=13.2$	0.000
- infiltration	26 (59.1%)	5 (13.2%)	31 (37.8%)	$\chi^2=18.3$	0.000
- ESWT	5 (11.4%)	5 (13.2%)	10 (12.2%)	$\chi^2=0.1$	1.000
	7 (15.9%)	16 (42.1%)	23 (28.1%)	$\chi^2=6.9$	0.008

a possible contracture of the gastrocnemius (10, 11). A greater tension of the gastrocnemius muscle could increase the stress on the Achilles tendon, resulting in an antalgic gait to limit the absence of ankle dorsiflexion that overloads the plantar fascia. Previous studies confirmed the central role of the lengthening of the posterior muscular chain in the treatment of plantar fasciitis (10, 11). Stretching exercises may provide short-term pain relief and improvements in calf-muscle flexibility. In a recent study, the effect of ESWT on the gastrocnemius muscle in patients affected by spasticity was monitored by ultrasound, revealing an improvement of measurements of Achilles tendon length, muscle fascicle length, muscle thickness, pennation angle

and the passive ROM of the tibio-tarsal joint (12). In the treatment of plantar fasciitis, the administration of SW, both at the insertion of the plantar fascia and at the gastrocnemius muscle, could be effective as it produces a bio-stimulating effect on the fasciopathy; furthermore, it could improve the effects of stretching exercise by reducing the muscle tension of the posterior muscle chain.

In conclusion, we report better results in terms of ROM values for the patients who had undergone SW therapy for plantar fascia and gastrocnemius muscle. On the basis of our findings, we suggest the possibility of administering this protocol in association with stretching exercises, in cases of patients affected by plantar fasciitis and with a reduction of passive ankle range of motion.

Table II. Mean $\pm$ SD and VAS variable VAS, Ankle-Hindfoot Scale, Lower Extremity Functional scale, Foot function Index, ROM and Roles and Maudsley Score by group and detection time. Data are expressed as an average $\pm$ standard deviation and range.

Variable	Group	T0	T1
VAS	Group A	7.5 $\pm$ 1.5 (5-10)	4.5 $\pm$ 2.9 (0-10)
	Group B	7.5 $\pm$ 1.8 (3-10)	4.8 $\pm$ 2.8 (0-10)
	Total	7.5 $\pm$ 1.7 (3-10)	4.6 $\pm$ 2.9 (0-10)
AHS	Group A	43.8 $\pm$ 27.5 (16-84)	73.7 $\pm$ 27.1 (16-100)
	Group B	44.9 $\pm$ 27.6 (16-85)	71.1 $\pm$ 26.6 (16-100)
	Total	44.3 $\pm$ 27.4 (16-85)	72.4 $\pm$ 26.7 (16-100)
LEFS	Group A	31.7 $\pm$ 15.5 (0-53)	51.1 $\pm$ 21.4 (0-80)
	Group B	29.6 $\pm$ 19.0 (0-60)	48.9 $\pm$ 20.7 (0-80)
	Total	30.6 $\pm$ 17.4 (0-60)	49.0 $\pm$ 21.0 (0-80)
FFIP	Group A	66.1 $\pm$ 11.8 (44-88)	43.4 $\pm$ 25.9 (0-88)
	Group B	68.1 $\pm$ 14.5 (42-88)	46.7 $\pm$ 23.5 (0-88)
	Total	67.1 $\pm$ 13.3 (42-88)	45.1 $\pm$ 24.7 (0-88)
ROM (°)	Group A	29.0 $\pm$ 7.9 (15-46)	41.6 $\pm$ 12.5 (15-64)
	Group B	29.8 $\pm$ 9.3 (16-52)	35.7 $\pm$ 12.4 (17-65)
	Total	29.4 $\pm$ 8.6 (15-52)	35.6 $\pm$ 12.8 (15-65)
Plantar flexion (°)	Group A	15.6 $\pm$ 4.8 (7-28)	22.1 $\pm$ 7.0 (7-36)
	Group B	16.4 $\pm$ 5.7 (8-30)	21.3 $\pm$ 8.4 (9-38)
	Total	16.0 $\pm$ 5.3 (7-30)	21.6 $\pm$ 7.7 (7-38)
Dorsal flexion (°)	Group A	13.4 $\pm$ 3.8 (8-21)	19.6 $\pm$ 6.0 (8-28)
	Group B	13.4 $\pm$ 4.3 (6-25)	14.4 $\pm$ 4.9 (6-27)
	Total	13.4 $\pm$ 4.1 (6-25)	16.9 $\pm$ 6.0 (6-28)
Roles and Maudsley	Group A	-	2.3 $\pm$ 1.1 (1-4)
	Group B	-	2.1 $\pm$ 1.1 (1-4)
	Total	-	2.2 $\pm$ 1.1 (1-4)

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LETTER TO THE EDITOR

POTENTIAL ROLE OF CLODRONATE IN PAINFUL HIP PROSTHESIS

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To the Editor,

Scales assessing the outcome of joint replacement surgery consider two parameters: pain and functional recovery. A higher score is attributed to pain during the overall assessment. It is the absence of pain rather than full motor recovery which indicates that surgery has been completely successful; its persistence, instead, even partial, is a sign to the patient that the surgical intervention has not been fully successful or even that it has failed. The functional outcome of the operated joint can be influenced by an inadequate rehabilitation. According to the Danish registry, the incidence of painful hip prosthesis is estimated to be 12.1%, 1.5 years after hip replacement and it has significant repercussions on the patient's social life (1). Furthermore, there is a correlation between the type of implant used for the hip replacement and the incidence of pain: 7% for prostheses with conventional coupling (metal-on-polyethylene, ceramic-on-polyethylene), 15% for prostheses with metal-on-metal coupling and 18% in cases of hip resurfacing (2).

Bisphosphonates (BPs) have been shown to significantly reduce implant failure. Moreover, they could play a very important role in the treatment of

painful hip prosthesis, starting from the preoperative period, when inflammation of the joint induces a metabolic impairment of the bone with specific bone marrow lesions. In addition, during the postoperative period, BPs can help in the rehabilitation process by reducing pain. Clodronate (CLO), in particular, plays an important role by virtue of its analgesic properties and of its anti-inflammatory action towards macrophages, which are protagonists of wear debris disease. In this Letter, we summarise the etiology of hip prosthetic pain, problems encountered post-surgery, the importance of bone mineral density (BMD), and use of BPs.

Epidemiology and etiology

During the post-operative period, the hip joint is painful for a period of time which varies from patient to patient, as well as the time it takes for the pain to improve. Characteristics of the semiological aspects of pain include: time of appearance, immediate, early-onset (in the first 6 months) or late-onset (after at least 1 year and after an interval of wellbeing); type of onset [acute type of pain (mainly mechanical correlated with joint activity, weight bearing, specific stresses or mobilization, or also inflammatory, i.e.

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more continuous, constant and nocturnal compared to the former type)]; intensity (assessed with a visual analog scale (VAS) related to responsiveness to analgesics); associated with functional limitation, i.e. lameness or incapacity to walk.

The pain of the prosthetic joint is usually multifactorial. Pain in a joint replacement arises from diverse, often multiple, stimuli of a mechanical and/or inflammatory origin. The most frequent causes are: local complications in the immediate post-operative period; early infection; mechanical problem of articulation between the prosthetic components; prosthesis-bone impingement; periprosthetic osteometabolic alterations; and septic or aseptic mobilization (loosening) of the implant.

Hip pain post-surgery

Excluding infection, pain in the hip joint area is predominantly mechanical in origin: pain with weight-bearing, walking, when starting movements or with a specific movement, with prolonged standing, with the use of devices. A pain in the groin (whether or not it radiates to the gluteal, posterior or obturator area) mainly indicates a problem of articulation between the prosthesis components. A middle-thigh pain or crural pain, with secondary gonalgia or radiating to the leg, usually suggests a problem of osseointegration and stress shielding of the stem. A trochanteric or posterior pain is related to the surgical gesture (access path, detached bone fragments, inadequate repositioning of detached tendons).

Pain in the immediate post-operative period is specifically correlated to the surgical intervention. Dislocation of the prosthetic components (frequency 1-10%) can occur at any time from day 1 up to 5 weeks after surgery due to various causes: impingement, loosening of the joint, malposition of the components, too-early mobilization of the same, insufficient muscle tension of the pelvis-trochanter muscles; the dislocation causes a very acute pain of evident clinical importance. Intraoperative bone lesions (frequency 0.1-8%), particularly in the area of the trochanter, generate pain specifically in the area of the lesion.

Correction of significant heterometries can result in neuropathic pain due to stretched nerves. Early-onset pain, strictly speaking, appears after (within a few

weeks) the intervention and becomes chronic, with a constant intensity, sometimes worsening gradually, and has a multifactorial etiology. Sometimes the joint to be replaced has already been under metabolic stress due to persistent inflammation of the arthritic hip (3, 4). Added to this stress, there is also the surgical trauma and the conflicting interaction between two significantly different modules of stiffness.

Bone stress can be attributed in most cases to oversizing of the femoral component, especially with a distal "tip" that generates distal hypertrophy, limited to the mantle in Gruen zone 3-5 of the femur and a concomitant proximal osteo-rarefaction due to bypass of the load with spongiosization of the cortex (5). Subacute, superficial or deep infection represents the most serious complication.

Instability of the prosthetic joint can cause pain of a multifactorial pathogenesis; a subtle subluxation due to slight malpositioning or impingement. The clinical picture is more striking when there is a dislocation of the components with significant problems of malpositioning in relation to the angle of anteversion or inclination of the stem, the orientation of the prosthetic cup, or the lever-arm and, therefore, problems with the correct tension of the pelvitrochanteric muscles. The clinical diagnosis is based on radiographic or CT examinations.

Periarticular heterotopic ossifications (PHO), present in 15-25% of the cases, are a complication of hip surgery with a multifactorial etiology stemming from an underlying genetic predisposition. Postoperative treatment with indomethacin or with CLO can resolve the periarticular inflammation and has a specific capacity to prevent PHO. BPs have been primarily used as inhibitors of calcifications and resorption: they are able to bind hydroxyapatite crystals, thus inhibiting crystal growth.

Chronic pain already begins in the post-operative period, varying in terms of its continuity and intensity; initially defined as immediate or subacute; it takes on a chronic character because of its duration following the intervention, and the constant nature and intensity of its manifestation.

Late-onset pain appears after 1 year post-surgery, after a variable period of both general well-being and local well-being in the hip region. It generally has a

slow onset and progressively worsens, tending to be, or become over time, continuous and highly intense. It can express numerous problems of the prosthetic joint which are defined as secondary if these are the consequence of trauma. They are defined as primitive if they primarily affect the prosthesis as a pathological evolution of the new joint normality. In septic loosening, pain has the same semiological characteristics as aseptic pain, but it differs in terms of its onset which is more acute, more intense, more difficult to localize, not simply mechanical, but also nocturnal, or inflammatory. In aseptic loosening, pain is often significantly correlated with the site of the problem, later confirmed by radiographic evaluations.

A predominantly inguinal pain generally expresses pathology of the prosthetic cup. It is a pain that manifests when initiating a movement, or passing from one position to another, or from torsion on the inferior limb in both supine and upright position. Groin pain is a characteristic in patients with a femoral prosthesis.

Femoral pain (middle thigh-pain) is an expression of a problem prevalently of the femoral component of the prosthesis (radiolucency, osteolysis, component subsidence, varization, loosening, etc.). It is reported by the patient as a pain of the thigh, mainly antero-lateral, defined as worsening or constricting, continuous, progressively worsening during prolonged upright stance and walking, typically mechanical in the case of aseptic loosening, or it can also be of an inflammatory nature in its osteometabolic genesis (stress shielding, osteoporosis).

Greater trochanter pain syndrome is a relatively common pathology (occurring in 1.8% of the general population), more frequent in females and obese people, and often associated with low back pain. Pain due to mobilization of both the components is caused by combined femoral and acetabular loosening; wear debris disease starting from the acetabular polyethylene causes a foreign body granuloma that induces femoral and, subsequently, acetabular periprosthetic osteolysis with radiographic pictures of loosening of the components.

Hip prosthesis and BMD

The decrease of periprosthetic bone mineral density (BMD), ranging from 3 to 15%, probably

due to periprosthetic bone remodeling, is a common finding in the first months after prosthesis surgery. There are data showing that the insertion of prosthesis into the bone is able to cause bone cell and matrix damage, with consequent inflammation leading to osteoclast activation (6).

It is recognised that the most important mechanism of action of BPs is the inhibition of osteoclasts. The risk of aseptic hip prosthesis loosening was associated to bone quality by Nixon and colleagues (7). These findings could be the rationale for the use of BPs in preventing periprosthetic bone loss. Indeed, an association between BP use and implant survival after total primary arthroplasty has been shown. The rate of arthroplasty revision was significantly lower and the implant survival was significantly longer in patients who were identified as BP users (8).

Collectively, these data form the basis for use of BPs in the prevention and in the treatment of periprosthetic bone loss. Moreover, the efficacy of CLO on pain, in association with its efficacy in preventing prosthetic mobilization, could be the rationale for treating some particular cases of painful prosthesis. In particular, those prosthesis where the surgical treatment was correct and there are no pathologic bone remodeling, dislocations, heterotrophic ossifications, impingements, infections or other complications (9, 10).

CLO, a first generation, non-nitrogen containing BP, has a particular mechanism of action which leads to anti-inflammatory activity, linked to the apoptosis of macrophages as well as to central and peripheral prolonged anti-nociceptive effects (11). The analgesic effects are evident in osteoporotic vertebral fractures, in avascular osteonecrosis, in osteoarthritis and in CRPS-1 syndrome, where CLO is successfully used. CLO is generally well tolerated: in particular, in osteonecrosis of the jaw, which is considered the most severe possible side-effect of BPs, CLO has been rarely applied, mainly in patients who had undergone previous treatment with other BPs (12). A small trial conducted on 91 patients with total uncemented hip arthroplasty showed that intramuscular CLO administration after surgery, for 12 months, at a weekly dose of 100 mg, was able to reduce bone periprosthetic loss ($p < 0.05$).

The reduction was evident in all periprosthetic Gruen zones except zones 4 and 5 (10). This finding could be a good reason for using CLO (and generally all BPs), in osteoporotic patients, to avoid aseptic loosening of the prosthesis. In our personal experience, CLO can be successfully administered with a brief course of 2.0-2.1 grams every 3 months because, after this period, the effectiveness of the drug tends to decrease. CLO can be used with daily intravenous vials (300 mg in saline solution) for 7 days or daily intramuscular vials (200 mg) for ten days (11).

Moreover, CLO could be effective in wear debris disease, where the role of osteoclasts is very important. Shima and colleagues recently demonstrated that CLO has analgesic effects exerting a direct action on neurons, where the drug enters and acts on glutamate or Adenosine Triphosphate (ATP) pain transmission pathways. The Authors concluded that CLO may be representative of a new class of analgesic drugs (12). Moreover CLO, as an inhibitor of purinergic chemical transmission, can also inhibit vesicular ATP release with consequent analgesic effects. *In vivo*, CLO is more effective than other therapeutic agents in attenuating neuropathic and inflammatory pain in mice, without affecting basal nociception. Finally, CLO, as shown in algodystrophic syndromes, is a potent inhibitor of bone marrow oedema, which is probably another important factor in periprosthetic pain (11).

CONCLUSIONS

Patients undergoing prosthetic hip replacements experience a high degree of functional recovery; the number of operations is increasingly annually and are also performed on younger patients. The consequence is a new standard of "normality" for joint prostheses, which guarantees an optimal functioning of the prosthesis for two decades or more. There are multiform pathological situations correlated to the prosthetic components and their tribology, to the modality of implantation, and to the processes of wear and tear over time. Today, pharmacological protection of the periprosthetic bone has assumed a central role in maintaining normal bone-prosthesis interaction. BPs have been shown to

play an important role in this sense, starting from the preoperative period when inflammation of the joint induces a metabolic impairment of the bone with specific bone marrow lesions. CLO, in particular, plays a key role by virtue of its anti-inflammatory and analgesic activity: both these characteristics lead to the hypothesis that this drug could be useful in the treatment of PHO, particularly in implants without orthopedic or infectious complications, as well as during the rehabilitative process.

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LETTER TO THE EDITOR

**PRIMARY SCHOOL SCREENING OF FLATFOOT IN 6-YEAR-OLD CHILDREN:
A DESCRIPTIVE STUDY TO PLAN A REHABILITATIVE APPROACH**R.G. BELLOMO¹, L. PEZZI², T. PAOLUCCI³, A. PORRECA⁴ and R. SAGGINI³¹*Department of Biomolecular Sciences, University of Study of Urbino Carlo Bo, Urbino, Italy;*²*Department of Medical and Oral Sciences and Biotechnologies, School of specialty in physical and rehabilitative medicine, University G. d'Annunzio Chieti, Italy;* ³*Department of Medical and Oral Sciences and Biotechnologies, Physical and Rehabilitative Medicine, University G. d'Annunzio Chieti-Pescara, Chieti, Italy;* ⁴*Department of Economics, University G. d'Annunzio Chieti-Pescara, Chieti, Italy**Received September 17, 2019 – Accepted January 20, 2020*

To the Editor,

The term 'flatfoot' is generally defined as a medical condition in which the foot does not have a normal medial longitudinal arch in weight-bearing position (1). Typically, children are born with flexible flat feet which in the first decade of their lives develop into neutral feet with the structuring of the medial plantar arch. Usually the flat foot is clinically asymptomatic, but it can alter the biomechanics of the lower limbs and lumbar spine, increasing the risk of pain and injury. The arches enable the foot to be cushioned and levered during locomotion, first acting as a shock absorber (being adaptive and flexible) and then as a lever (rigid and resistant) (2). The configuration of the plantar arch is determined by age and genetic factors and its rapid development is observed between 2 and 5 years of age. In fact, at 6 years of age a neutral morphological structure of the plantar arch can be seen (3, 4). Bordin et al. reported an incidence of flatfoot of 16.4% in 243 primary school pupils (3rd and 4th class - age range 8-10 years) (5).

Current evidence suggests that it is appropriate to observe (follow-up study and scholar screening) an asymptomatic child with flat feet within the first

6 years of life (4). Moreover, the use of the term "normal" or "neutral" or "healthy" in relation to foot posture is misleading in categorizing the pediatric foot, because a flatfoot posture is a normal finding at specific ages and because the foot posture of a developing child is age-dependent, changes over time (6). Therefore, the aim of this observational study was to evaluate the prevalence of flatfoot in 6-year-olds, an age that is critical for medial plantar arch development and related factors, such as BMI, ligamentous laxity, and sex.

MATERIALS AND METHODS

An observational cross-sectional study was conducted from January 2017 to June 2017, enrolling 183 Caucasian children from the first-grade school classes. Two physiatrists collected anamnestic data (age, sex, weight, and height for anthropometric values); BMI was calculated by dividing body weight by the square of the height (kg/m²). The subject was considered underweight if his BMI was below 18.5, normal weight between 18.5 and 24.9, overweight between 25 and 29.9, and obese at 30 or higher. BMI was used to classify children in 3 weight

*Key words: pes planus; posture; pediatric; baropodometry**Corresponding Author:*

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levels underweight, normal weight, overweight/obese according to the definition of obesity for children and adolescents of the Italian Institute of Public Health and the Italian Ministry of Health. Plantar support and foot posture were evaluated by clinical non-quantitative inspection and based on footprint parameters by podoscopic assessment. The podoscope allows one to evaluate the footprint, supplying secondary, indirect information on heel alignment and the toes, and was used to diagnose flatfoot by an expert physiatrist respecting the classification of Denis (7). Static heel pronation for the left and right foot, referring to the calcaneal motion in respect to the orientation of the talus at the subtalar joint, was evaluated and given 1 of 5 grades of malalignment: neutral, minor-medium, medium, medium-severe and severe (8).

Baropodometry test was performed to evaluate the foot load symmetry. In particular, the static baropodometry test was used to calculate the average result of the distribution of foot pressure load, expressed as percentage of the maximum load value. The sensorized mat measured the pressures under the feet and allowed us to identify the center of pressure (CoP) i.e., the linear projection of the body's center of mass (CoM) along the antero-posterior and latero-lateral directions. We considered the static load of the left and right feet (Foot Static Load - FSL Left and Right, CoP X= the latero-lateral direction of the body's center of pressure, CoP Y= the antero-posterior direction of the body's center of pressure). Dynamic baropodometry was performed by monitoring 6 walks on a 2.5-m (on average) sensorized footboard: the deambulation was assessed by capturing the progression of foot pressure during gait cycle. The following parameters were considered for the dynamic baropodometry: left and right foot progression dynamic load (Foot Dynamic Load - FDL Left and Right); left and right stride frequency or cadence (SF, strides/min), measured as the inverse of time between 2 consecutive strides, and walking speed (WS, m/s), measured as the stride length multiplied by the stride frequency reported in strides/sec; finally, left and right foot progression angle (FPA), defined as the angle between the line from the calcaneus to the second metatarsal and the

line of progression, averaged from heel strike to toe off during the stance phase of walking for each step (toe-in angle is positive, and toe-out angle is negative). Ligamentous laxity was assessed using the Beighton Score (BS); this scale ascertains the presence of joint hypermobility in the wrist, fifth metacarpal phalanx articulation, elbow, knee and lumbo-sacral spine (bending forward, in position). The BS yields a score of 9 points; thus, the usual arbitrary limit of 5 of 9 or higher indicates joint hypermobility (9). After receiving information on the study, the parents of each child provided consent to participate; recruitment and evaluation procedures met the Helsinki Declaration on human experimentation and the local ethical approval was obtained.

Statistical analysis

Statistical analyses were performed for continuous quantitative variables FSL (left and right), CoP X, CoP Y, FDL (left and right), WS (left and right), SF (left and right) and FPA (left and right) measuring the mean and standard deviation, minimum and maximum values for all parameters. Then, the variables were transformed into dummy variables and analyzed with the remaining dichotomous variables in a summary table of absolute and percentage frequencies. Finally, a contingency table was constructed to describe the joint distribution of each variable regarding the type of plantar pathology; in particular, the absolute and percentage frequencies are reported in respect to the sample size for a better understanding of the distribution. The data were entered using SPSS, version 12.0.1 (SPSS, Chicago, IL, USA).

RESULTS

The sample comprised 183 6-year-olds, composed of 81 females and 102 males with no vestibular or auditory deficit, dyslexia, attention deficit and language; the possible presence of visual deficits were corrected by lenses.

As shown in Table I, 107 (58.5%) children (47 female and 60 male) had bilateral normal plantar support. Thirty-five children (16 female and 19 male)

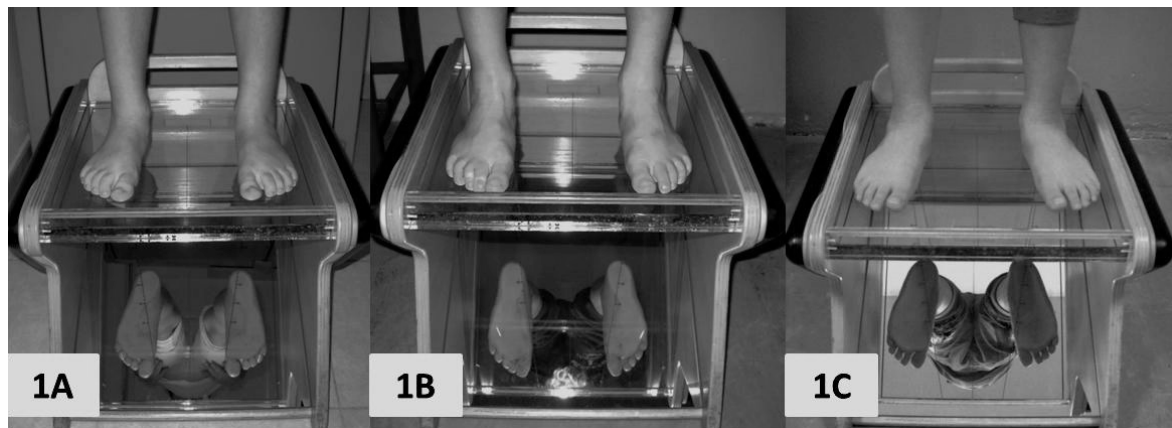


Fig. 1. Clinical non-quantitative inspection of foot shape by a Podoscopic assessment, using classification based on the Denis method. **A)** Grade I flat foot; **B)** Grade II flat foot; **C)** Grade III flat foot.

Table I. Continuous quantitative variables of static and dynamic baropodometry; measuring the mean and standard deviation, minimum and maximum values for all parameters are reported.

Baropodometric parameters	Mean	SD	Min	Max
FSL Left	52.54	±7.88	29.56	78.39
FSL Right	47.46	±7.88	21.61	70.44
COP X	-5.46	±11.16	-40.74	26.44
COP Y	-23.90	±12.93	-56.69	21.99
FDL Left	52.09	±6.71	20.53	82.49
FDL Right	47.91	±6.71	17.51	79.47
WSLeft	0.72	±0.15	0.18	1.13
WS Right	0.73	±0.13	0.28	1.05
SF Left	0.22	±0.05	0.10	0.55
SF Right	0.22	±0.06	0.12	0.70
FPA Left	15.85	±4.36	2.34	35.54
FPA Right	10.69	±4.17	0.72	25.73

FSL: foot static load, the average result of the distribution of foot pressure load(left and right); CoP X: centre of pressure, the latero-lateral direction of the body's center of pressure; CoP Y: the antero-posterior direction of body's center of pressure; FDL: foot dynamic load, the average result of the distribution of foot pressure load (left and right); WS: walking speed, m/s, measured as the stride length multiplied by the stride frequency reported in strides/sec; SF= stride frequency or cadence (strides/min) measured as the inverse of time between 2 consecutive strides; FPA= foot progression angle, the angle between the line from the calcaneus to the second metatarsal and the line of progression, averaged from heel strike to toe off during the stance phase of walking for each step.

Table II. Contingency table describing the joint distribution of each variable with regard to the grade of flatfoot; the absolute and percentage frequencies are reported in respect to the sample size.

Flatfoot Clinical parameters		Grade I N(%)			Grade II N(%)			Grade III N(%)		
		bil	Left	Right	bil	Left	Right	bil	Left	Right
sex	F	16(8.7)	2(1.1)	(1.6)	5(2.7)	4(2.2)	0(0.0)	0(0.0)	1(0.5)	1(0.5)
	M	19(10.4)	5(2.7)	4(2.2)	14(7.7)	4(2.2)	3(1.6)	3(1.6)	0(0.0)	0(0.0)
BS	<1	12 (6.6)	4(2.2)	2(1.1)	6 (3.3)	2(1.1)	2(1.1)	2(1.1)	0(0.0)	0(0.0)
	1-3	21(11.5)	2(1.1)	4(2.2)	12(6.6)	5(2.7)	1(0.5)	0(0.0)	1(0.5)	1(0.5)
	4-6	1 (0.5)	1(0.5)	1(0.5)	1 (0.5)	1(0.5)	0(0.0)	1(0.5)	0(0.0)	0(0.0)
	>7	1 (0.5)	0(0.0)	0(0.0)	0 (0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
HP Right	1	18 (9.9)	4(2.2)	2(1.1)	6 (3.3)	3(1.6)	1(0.5)	0(0.0)	0(0.0)	0(0.0)
	2	1 (0.5)	0(0.0)	1(0.5)	1 (0.5)	0(0.0)	0(0.0)	0(0.0)	1(0.5)	0(0.0)
	3	13 (7.1)	2(1.1)	4(2.2)	6 (3.3)	5(2.7)	1(0.5)	1(0.5)	0(0.0)	1(0.5)
	4	3 (1.6)	1(0.5)	0(0.0)	4 (2.2)	0(0.0)	1(0.5)	1(0.5)	0(0.0)	0(0.0)
	5	0 (0.0)	0(0.0)	0(0.0)	2 (1.1)	0(0.0)	0(0.0)	1(0.5)	0(0.0)	0(0.0)
HP Left	1	11 (6.1)	3(1.7)	0(0.0)	4 (2.2)	1(0.6)	2(1.1)	0(0.0)	0(0.0)	0(0.0)
	2	4 (2.2)	0(0.0)	1(0.6)	0 (0.0)	0(0.0)	0(0.0)	1(0.6)	1(0.6)	0(0.0)
	3	18 (9.9)	4(2.2)	3(1.7)	10(5.5)	3(1.7)	1(0.6)	0(0.0)	0(0.0)	1(0.6)
	4	1 (0.6)	0(0.0)	1(0.6)	3 (1.7)	2(1.1)	0(0.0)	2(1.1)	0(0.0)	0(0.0)
	5	1 (0.6)	0(0.0)	2(1.1)	2 (1.1)	2(1.1)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
NW	F	10 (5.5)	0(0.0)	2(1.1)	0 (0.0)	2(1.1)	0(0.0)	0(0.0)	1(0.5)	0(0.0)
	M	11 (6.0)	1(0.5)	3(1.6)	7 (3.8)	3(1.6)	0(0.0)	1(0.5)	0(0.0)	0(0.0)
OW	F	5 (2.7)	1(0.5)	1(0.5)	4 (2.2)	2(1.1)	0(0.0)	0(0.0)	0(0.0)	1(0.5)
	M	6 (3.3)	4(2.2)	1(0.5)	5 (2.7)	1(0.5)	3(1.6)	1(0.5)	0(0.0)	0(0.0)
UW	F	1 (0.5)	1(0.5)	0(0.0)	1 (0.5)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
	M	2 (1.1)	0(0.0)	0(0.0)	2 (1.1)	0(0.0)	0(0.0)	1(0.5)	0(0.0)	0(0.0)
FSL	Right	10 (5.5)	4(2.2)	2(1.1)	6 (3.3)	4(2.2)	1(0.5)	1(0.5)	0(0.0)	1(0.5)
	Left	25(13.7)	3(1.6)	5(2.7)	13(7.1)	4(2.2)	2(1.1)	2(1.1)	1(0.5)	0(0.0)
COP_X	Right	8 (4.4)	3(1.6)	1(0.5)	3 (1.6)	3(1.6)	1(0.5)	1(0.5)	0(0.0)	1(0.5)
	Left	27(14.8)	4(2.2)	6(3.3)	16(8.7)	5(2.7)	2(1.1)	2(1.1)	1(0.5)	0(0.0)
COP_Y	front	1 (0.5)	0(0.0)	0(0.0)	2 (1.1)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
	back	34(18.6)	7(3.8)	7(3.8)	17(9.3)	8(4.4)	3(1.6)	3(1.6)	1(0.5)	1(0.5)
FDL	Right	16 (8.7)	2(1.1)	3(1.6)	7 (3.8)	2(1.1)	1(0.5)	0(0.0)	0(0.0)	0(0.0)
	Left	19(10.4)	5(2.7)	4(2.2)	12(6.6)	6(3.3)	2(1.1)	3(1.6)	1(0.5)	1(0.5)
FPA	Right>Left	2 (1.1)	0(0.0)	4(2.2)	2 (1.1)	4(2.2)	0(0.0)	0(0.0)	1(0.5)	0(0.0)
	Left>Right	33(18.0)	7(3.8)	3(1.6)	17(9.3)	4(2.2)	3(1.6)	3(1.6)	0(0.0)	1(0.5)
SV	Right>Left	15 (8.2)	5(2.7)	2(1.1)	8 (4.4)	2(1.1)	2(1.1)	2(1.1)	0(0.0)	0(0.0)
	Left>Right	16 (8.7)	2(1.1)	5(2.7)	9 (4.9)	6(3.3)	1(0.5)	1(0.5)	1(0.5)	1(0.5)
	Right=Left	4 (2.2)	0(0.0)	0(0.0)	2 (1.1)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
SF	Right>Left	16 (8.7)	0(0.0)	2(1.1)	11(6.0)	1(0.5)	0(0.0)	1(0.5)	0(0.0)	0(0.0)
	Left>Right	14 (7.7)	4(2.2)	1(0.5)	2 (1.1)	3(1.6)	1(0.5)	2(1.1)	0(0.0)	0(0.0)
	Right=Left	5 (2.7)	3(1.6)	4(2.2)	6 (3.3)	4(2.2)	2(1.1)	0(0.0)	1(0.5)	1(0.5)

F: female; M: male; bil: bilateral; BMI: body mass index; NW: normal weight; OW: overweight; UW: underweight; BS: Beighton Score; HP: Heel Pronation; FSL: foot static load, the average result of the distribution of foot pressure load(left and right); CoP X: centre of pressure, the latero-lateral direction of the body's center of pressure; CoP Y: the antero-posterior direction of body's center of pressure; FDL: foot dynamic load, the average result of the distribution of foot pressure load (left and right); WS: walking speed, m/s, measured as the stride length multiplied by the stride frequency reported in strides/sec; SF: stride frequency or cadence (strides/min) measured as the inverse of time between 2 consecutive strides; FPA: foot progression angle, the angle between the line from the calcaneus to the second metatarsal and the line of progression, averaged from heel strike to toe off during the stance phase of walking for each step.

Table III. Clinical data of children, sample number and percentage.

Clinical parameters		N (%)
sex	F	75 (41%)
	M	108 (59%)
Neutral foot	Bilateral	107 (58.5%)
	left	1 (0.5%)
	right	6 (3.3%)
Grade I flatfoot	Bilateral	35 (19.1%)
	left	7 (3.8%)
	right	7 (3.8%)
Grade II flatfoot	Bilateral	19 (10.4%)
	left	8 (4.4%)
	right	3 (1.6%)
Grade III flatfoot	Bilateral	3 (1.6%)
	left	1 (0.5%)
	right	1 (0.5%)
BMI	normal weight	107 (58.5%)
	Overweight	52 (28.4%)
	Underweight	24 (13.1%)
BS	<1	69 (37.7%)
	1-3	105 (57.4%)
	4-6	8 (4.4%)
	>7	1 (0.5%)
HP right	Neutral	103 (56.6%)
	Minor-Medium	8 (4.4%)
	Medium	51 (28%)
	Medium-Severe	15 (8.2%)
	Severe	5 (2.7%)
HP left	Neutral	71 (39.2%)
	Minor-Medium	16 (8.8%)
	Medium	65 (35.9%)
	Medium-Severe	19 (10.5%)
	Severe	10 (5.5%)

F: female; M: male; BMI: body mass index; BS: Beighton Score; HP: Heel Pronation

had grade I bilateral flatfoot (Fig. 1A) and 7 children had grade I unilateral flatfoot of the left foot, versus 7 of the right foot. Furthermore, 19 children had grade II bilateral flatfoot (Fig. 1B); 8 children had grade II flatfoot of the left foot compared with 3 of the right foot. Three children had grade III flatfoot support bilaterally (Fig. 1C), versus 1 each of the left and right foot. None of the 183 subjects reported foot pain under static and dynamic conditions. In total, 31.1%

of the sample had bilateral flat plantar support to varying degrees (19.1% grade I, 10.4% grade II, and 1.6% grade III). The data show that male and female sex are not considered a predisposing factor for flatfoot. With respect to the visual, non-quantitative inspection and the footprint parameters of calcaneal pronation of the flatfoot, 15 showed medium-severe pronation of the right foot, versus 5 children with severe pronation. For the left foot, 19 children showed medium-severe pronation compared with 10 with severe pronation. As seen in Table II, based on the static baropodometric examination, 183 children distributed the body weight on the left: on average, the percentage of the load on the left was 52.54% versus 47.46% for the right foot. Moreover, the shift of the center of gravity on the Y axis (CoP Y: antero-posterior axis) was negative (away from the ideal reference position of center of gravity an average of 23.9 mm toward the hind foot). Therefore, 44.5% of flat foot children experienced a retreat in center of gravity: 48 (26.6%) grade I, 28 (15.3%) grade II, and 5 (2.6%) grade III. The displacement in the center of gravity on the X axis (CoP X: middle-lateral axis) was -5.46 mm (a negative value indicates that the subject moved from the ideal reference position of center of gravity toward the left), in 34.4% of flat foot children. The pre-positioned barycenter is present in 2.8% of flat foot children. The right and left foot load in the dynamic baropodometric analysis was, respectively, 47.91% and 52.09%, consistent with the static baropodometric foot load value. The foot progression angle (FPA) for the right was $10.69^{\circ} \pm 4.17$ and $15.75^{\circ} \pm 4.36$ for the left; the right foot stride frequency (SF) was 0.22 ± 0.06 s compared with 0.22 ± 0.05 s for the left. The speed of advancement of the center of gravity (expressed in meters per second as walking speed) for the right foot was 0.73 ± 0.13 m/sec 0.72 ± 0.15 m/sec for the left.

Regarding the assessment of ligamentous laxity by BS, 69 of 183 children (37.7%) had a BS <1, indicative of the absence of ligamentous hyperlaxity; 4.9% (9 of 183) had a BS that suggested ligamentous hyperlaxity (> 4).

As shown in Table I, 52 children (28.4%) were overweight, based on BMI, of whom 35 presented with bilateral or unilateral flat plantar support. Table

III shows that 11 overweight children (6%) had grade I bilateral flatfoot versus 5 (2.7%) with grade I left single-sided flatfoot and 2 (1%) with grade I unilateral right flatfoot. Specifically, 18 children had grade I bilateral or unilateral flatfoot and were overweight, per the BMI stratification.

With regard to grade II flatfoot, 9 overweight children (4.9%) had bilateral flatfoot, 3 overweight children (1.6%) had left flatfoot, and 3 overweight children (1.6%) presented with right unilateral flatfoot: in total, 15 overweight children had grade II bilateral or unilateral flatfoot. With regard to grade III flatfoot, 1 overweight child (0.5%) had bilateral flatfoot, and 1 overweight child (0.5%) developed right flatfoot: thus, 2 overweight children had grade III bilateral or unilateral flatfoot.

Furthermore, 24 children (13.1%) had a BMI that was indicative of being underweight (Table I). Among underweight children, as shown in Table III, 3 (1.6%) had grade I bilateral flatfoot, 1 (0.5%) had grade I left flatfoot, 3 (1.6%) had grade II bilateral flatfoot, and 1 (0.5%) had grade III bilateral flatfoot.

In total, 107 participants (58.5%) had a normal weight, based on BMI (Table I): 41 (22.4%) had bilateral flatfoot. Of those with grade I flatfoot, 22 children (11.6%) had bilateral flatfoot, 1 (0.5%) had left flatfoot, and 5 (2.6%) had right flatfoot (Table III). Regarding grade II flatfoot, 7 children (3.8%) had it bilaterally, and 5 (2.6%) had left flatfoot. One child each (0.5%) had grade III bilateral flat plantar support and grade III left flat plantar support.

DISCUSSION

Regarding the aim of this observational study, the data show that 31% of children have bilateral flatfoot at 6 years of age. In the literature, flexible flatfoot is common in most children aged under 10 years, and its incidence is approximately 45% in preschoolers (10). The findings of this present study are at least 25% higher than in the literature: despite its widespread prevalence, flatfoot is often a difficult topic to define as the lack of high-level evidence to guide treatment indications perpetuates some confusion. Transversal epidemiological studies have shown that flatfoot is a normal form of the

foot in the first several years of life. Morley et al. reported a prevalence of flatfoot of 97% in children aged 2 years or younger, as defined by the width ratio between the heel and medial arch (3, 11). This rate declined with age, and only 4% of 10-year-old patients had flat feet. A similar trend was reported by Staheli et al., with 54% of 3-year-olds with flatfoot; this value fell to 26% in 6-year-olds, demonstrating that 28% of such cases disappear at age 6 years (11). These data underline that the age between 4 and 5 years is an important period for the development of the median longitudinal arch. Several factors can predispose children to the development and persistence of flatfoot and, in our study, the majority (58.4%) of participants had normal plantar support, with a prevalence of bilateral flatfoot of 31.1%.

Moreover, the increase in childhood obesity is one of the most serious health challenges worldwide, and this trend can be a direct consequence of a sedentary lifestyle and altered eating habits. The results of this study show high BMI in 28.4% of schoolchildren, 10% of whom were females and 18% were males. Other studies have confirmed that obesity correlates with the persistence of flatfoot in children (12).

Although flatfoot is considered to be the most common condition in pediatric age, it is unknown at what age children develop neutral flatfoot and, in the absence of obvious pathology, when and if flatfoot could be defined as “pathological”. The results from a screening of plantar foot support in a primary school allowed the clinicians to identify abnormalities in plantar support at an early stage. It is important to promptly identify pathological flatfoot so as to act with rehabilitative exercises and insoles.

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LETTER TO THE EDITOR

TREATMENT OF CHRONIC PERSISTENT SPINAL PAIN WITH DICLOFENAC MESOTHERAPY IN PATIENTS WITH SPONDYLARTHROSIS

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To the Editor,

Chronic spinal pain persists 6 months after an injury and beyond the usual course of an acute disease or a reasonable time for a comparable injury to heal. It is seen in 25% (for neck pain) to 60% (for back pain) of patients, one-year or longer after the first episode (1). The intradermal administration (mesotherapy) of analgesic drugs has been used to relieve local musculoskeletal pain (2-4). The aim of this retrospective study is to investigate the intradermal efficacy of diclofenac complexed with β -cyclodextrin (Akis[®], IBSA Farmaceutici Italia) versus lysine acetylsalicylate mesotherapy, in patients with chronic persistent spinal pain for spondylarthrosis. Data were extracted from the consecutive records of patients treated with mesotherapy between January 2014 and July 2016 at a center of rehabilitation medicine in Italy (Unit of Physical Medicine and Rehabilitation, Teaching Hospital Foundation "Agostino Gemelli", Catholic University of Sacred Heart, Rome, Italy) following the approval of the study by the ethics committee of the institution (protocol number, 33220/16; ID :11206) and after informed, signed consent to

participate in the trial. Inclusion criteria were: age ≥ 18 years, presence of spinal pain lasting for 12 weeks or more with no cause identified, VAS >5 cm, no previous treatment with mesotherapy, no other pharmacological, physical, or exercise-based treatment during mesotherapy and until 3 months of follow-up. Exclusion criteria were: presence of neurologic diseases, psychiatric disorders, vertebral fractures, severe rheumatic disease, oncologic diseases. Patients received two mesotherapy sessions per week for a total of five sessions. In each treatment, patients were injected with either 2 mL of a drug mixture containing 0.5 mL of sodium diclofenac (Akis[®], IBSA Farmaceutici Italia, 50 mg/mL) plus 0.5 mL of lidocaine hydrochloride 2% and 1 mL of physiological saline solution (Group A), or 2 mL of a drug mixture containing 0.5 mL of lysine acetylsalicylate (Flectadol[®], Sanofi S.p.A. 500 mg/2.5 mL) plus 0.5 mL of lidocaine hydrochloride 2% and 1 mL of physiological saline solution (Group B). The choice of the use of different drugs depended on the availability in the clinic. Demographic and clinical data, values of VAS (0-10 cm) and Short-Form McGill Pain Questionnaire (SF-MPQ) (5)

Key words: chronic spinal pain; intradermal therapy; rehabilitation; mesotherapy

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Table I. Demographic, clinical characteristics and baseline values of outcome measures at baseline of patients in diclofenac (Group A) or lysine acetylsalicylate (Group B) mesotherapy group analysis

	Group A N = 49 Mean and SD	Group B N = 51 Mean and SD
Age, years, mean ($\pm$SD)	61.2 (13.1)	64.1 (15.8)
Gender, n (%)		
Female	39 (64.7)	39 (76.5)
Male	10 (35.3)	12 (23.5)
Occupational status, n (%)		
Retired	27 (55.1)	16 (50.0)
Employed	17 (34.7)	5 (15.6)
Self-employed	0	1 (3.1)
Student	2 (4.1)	1 (3.1)
Housewife	3 (6.1)	7 (21.9)
Secondary school	33 (67.3)	7 (21.2)
High school	6 (12.2)	13 (39.4)
University degree	5 (10.2)	1 (3.0)
missing	4 (8.1)	18 (9.18)
Comorbidities, n (%)		
Cardiovascular	12 (24.5)	4 (7.8)
Respiratory	1 (2.0)	2 (3.9)
Patients with >1 comorbidities, n (%)	31 (63.3)	17 (33.3)
None	2 (4.1)	10 (19.6)
Missing	4 (10.2)	18(35.3)
VAS T0	7.47 $\pm$ 1.20	7.20 $\pm$ 1.64
SF-MPQ T0	16.7 $\pm$ 6.51	19.9 $\pm$ 10.5

SD: standard deviation; VAS: visual analog scale; SF-MPQ: Short-Form-McGill Pain Questionnaire

Table II. Mean values and standard deviations of outcome measures at T0, T1, T2, T3 of patients in diclofenac (Group A) or lysine acetylsalicylate (Group B) mesotherapy group analysis.

	Group A N = 49 Mean and SD	Group B N = 51 Mean and SD	P level
VAS T0	7.47 $\pm$ 1.20	7.20 $\pm$ 1.64	0.349
VAS T1	2.96 $\pm$ 1.42	5.16 $\pm$ 2.53	<0.001*
VAS T2	3.35 $\pm$ 1.62	5.22 $\pm$ 2.53	<0.001*
VAS T3	3.71 $\pm$ 1.48	5.08 $\pm$ 2.88	<0.049*
SF-MPQ T0	16.7 $\pm$ 6.51	19.9 $\pm$ 10.5	0.053
SF-MPQ T1	4.02 $\pm$ 5.05	15.02 $\pm$ 12.66	<0.001*
SF-MPQ T2	4.52 $\pm$ 5.05	13.10 $\pm$ 10.08	<0.000*
SF-MPQ T3	3.29 $\pm$ 4.07	12.00 $\pm$ 4.55	<0.001*

ANOVA *p*-value two independent samples comparison, **P* level<0.05. Paired samples test significant results between groups; SD: standard deviation; VAS: visual analog scale; SF-MPQ: Short-Form-McGill Pain Questionnaire

were extracted from the patient records before the first treatment (T0), at the end of the five-week treatment (T1), and at 4 (T2) and 12 weeks (T3) of follow-up. Data of 100 patients were analyzed, 49 treated with diclofenac mesotherapy (Group A) and 51 with lysine acetylsalicylate mesotherapy (Group B). Patients in both group A and group B reported severe pain at T0 (Table I). Diclofenac mesotherapy (group A) was associated with significant reduction of VAS and SF-MPQ at each evaluation, compared with lysine acetylsalicylate mesotherapy (Table II).

DISCUSSION

This retrospective study showed that diclofenac mesotherapy is an effective treatment to reduce chronic persistent spinal pain in patients with spondylarthrosis for 3 months. Diclofenac-based mesotherapy was associated with significantly greater improvements in pain severity than lysine acetylsalicylate-based mesotherapy. Both treatments were well tolerated, and the benefits were sustained over 3 months of follow-up.

The experience of pain is recognized as being subjective and multidimensional. Therefore, self-report of pain intensity is considered of prime importance in the assessment. The improvements in pain intensity achieved with diclofenac-based mesotherapy in this study represent a clinically relevant difference, that is perceived by patients as adequate analgesic control, according to accepted outcome measures reported in the literature (6). This study had a number of limitations. Firstly, the retrospective study design did not allow us to make extrapolation regarding results. A possible placebo effect in both groups could also be present in this study. Despite these limitations, mesotherapy appears to be a

valid strategy for improving persistent chronic spinal pain, limiting the systemic exposure to NSAIDs. These promising findings need to be confirmed in prospective randomized controlled trials.

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LETTER TO THE EDITOR

ROLE OF GENDER DIFFERENCES ON DENTAL AESTHETICS IN ADOLESCENT AND YOUNG POPULATION

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To the Editor,

Functions of body aesthetics are integral parts of personality and complexity which must be taken into account especially in adolescence and early adulthood. However, it is not always possible to highlight the relationships with psychopathologies intuitively connected because the adolescent is not aware of the relationships between discomfort and aesthetic alteration. The relational value of the face is intuited by the subjects after a path of significant internalization (psychotherapy, introspection, other reflexive practices). Dental aesthetics can therefore significantly affect the well-being of adolescents and young adults, influencing important factors such as body representation and self-esteem. The impact of oral features on the bio-psychosocial functioning of these subjects was analyzed with particular reference to gender and age. Some authors have suggested that younger subjects are more affected by the effect of oral blemishes on their self-esteem in the same way as women, who appear more severe in reference to their appearance (1-9). Dento-facial features have a strong impact, especially on the female gender, with

significant consequences on the representation of the self (10), on oral health (11, 12) and indirectly on the social sphere (13-16). From an aesthetic-relational point of view, subjects with greater security are perceived as more attractive and have a better social impact, unlike those with oral disorders, who present a negative body image and a higher level of isolation. From previously research it is clear that problems related to malocclusions, cavities, untreated decays or injuries have significant consequences on oral aesthetics, affecting self-esteem and, indirectly, individual well-being (14). Above all, the presence of anomalies of the anterior incisors, more visible than the posterior teeth, can predict a negative self-perception (15, 16). These results support the hypothesis of a direct relationship between oral health and self-esteem, despite some long-term follow-ups not having detected a significant relationship between the aforementioned elements.

Given the above, we hypothesize: significant gender differences related to the psychological impact of oral health; significant correlations among age, self-confidence, social impact, psychological

Key words: dental aesthetics; oral health; well-being; self-confidence

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impact, aesthetic concern, convictions and oral health; relationships among such predictor covariates such as age, gender, crooked and spaced teeth and outcomes such as self-confidence, psychological impact, conviction and self esteem, as indicated through regression analysis.

MATERIALS AND METHODS

One hundred and ninety subjects were selected from private dental practices, with the help of students from the degree course in Dentistry and Dental Prosthetics of the University of Messina. The observation group consists of 71 males (M) and 119 females (F), between 14 and 29 years (Mean: 23.8; SD: 3.27) (Table I).

The following were used for the evaluation of oral health and self-esteem: i) Psychosocial Impact of Dental

Aesthetics Questionnaire (PIDAQ) (20), for the analysis of the quality of oral life through the following subscales (21): reasons to search orthodontic treatment; self-confidence from the dental point of view; social and psychological impact; aesthetic concern; patient's beliefs. A five-point Likert scale was used, ranging from 0 (no impact of dental aesthetics to QoL) to 5 (maximal impact of dental aesthetics) for each item.

ii) Oral Health Impact Profile (OHIP-14) (22), based on patient experience, focusing on the negative impact of oral conditions. Seven different dimensions are considered (23): functional limitation; physical pain; psychological distress; physical deficiency; psychological disability; social incapacity; handicap.

iii) Rosenberg Self Esteem Scale (24), which allows a global assessment of self-esteem (25), through a measurement of both negative and positive feelings associated with the self.

Table I. Absolute frequency for percentage and categories. Distributions are reported according to gender.

Sex	Frequency	Percentage
Male	71	37
Female	119	62.6
Total	190	100

Table II. Descriptive analysis for numerical data (PIDAQ, OHIP-14, Rosenberg Self Esteem Scale)

	Mean	Standard Deviation
Age	23.8	3.27
Crooked Teeth	3.79	1.20
Spaced Teeth	3.35	1.25
Skeletal Protrusion	3.85	1.22
Indicated by the Dentist	3.58	1.34
Self-Confidence	20.28	6.06
Social Impact	12.88	5.02
Psychological Impact	11.38	4.68
Aesthetic concern	4.77	2.88
Convictions	13.84	4.34
Oral Health	11.15	9.65
Self\Esteem	17.62	2.60

Statistical analysis

The numerical data were expressed as mean and SD and the categorical variables as number and percentage. The non-parametric approach was used because examined variables were not normally distributed, as verified by Kolmogorov Smirnov test.

Spearman's test was applied in order to evaluate the correlation between age, self-confidence, social impact, psychological impact, aesthetic concern, convictions, oral health and self-esteem. The Mann Whitney test was used in order to compare all numerical variables between male and female subjects. Linear regression model was estimated in order to assess the dependence of some outcome variables (self-confidence, social impact,

psychological impact, aesthetic concern, convictions, self-esteem, oral health) from age, gender, crooked teeth, spaced teeth, skeletal protrusion, indicated by the dentist. Statistical analyses were performed using SPSS 22.0 for Window package. A P-value of < 0.050 was considered to be statistically significant (Table II).

RESULTS

To highlight directions among age, self-confidence, social impact, psychological impact, aesthetic concern, convictions, oral health and self-esteem, Spearman correlations are reported in Table III. Significant inverse correlations were found

Table III. Nonparametric correlations.

	Age	Self-confidence	Social Impact	Psychological Impact	Aesthetic concern	Convictions	Oral Health	Self-Esteem
Age	1.00	-	-	-	-	-	-	-
Self-confidence	0.067	1.00	-	-	-	-	-	-
Social Impact	0.097	0.502**	1.00	-	-	-	-	-
Psychological Impact	0.135	0.560**	0.591**	1.00	-	-	-	-
Aesthetic concern	0.048	0.610**	0.528**	0.620**	1.00	-	-	-
Convictions	0.116	0.260**	0.019	0.012	0.096	1.00	-	-
Oral Health	0.034	0.200**	0.356**	0.272**	0.262**	0.064	1.00	-
Self-Esteem	0.027	0.082	0.115	0.026	0.091	0.023	0.169*	1.00

* $p < 0.05$ level (2-tailed); ** $p < 0.01$ level (2-tailed)

Table IV. Linear regression analysis for numerical data

	Age			Sex			Crooked Teeth		
	β	χ^2/t	p	β	χ^2/t	p	β	χ^2/t	p
Self-confidence	0.091	1.25	0.0213	0.226	3.08	0.02*	0.079	0.711	0.478
Psychological Impact	0.154	-2.08	0.039*	0.021	0.279	0.781	0.178	1.58	0.115
Conviction	0.087	1.20	0.231	0.116	1.59	0.12	0.217	1.97	0.049*

* $p < 0.05$ level (2-tailed); ** $p < 0.01$ level (2-tailed)

among social impact and self-confidence, suggesting that a decreasing level of self-confidence corresponds to increasing social impact level. Significant inverse correlations were also shown between psychological impact and self-confidence; thus if psychological impact increases, self-confidence decreases and viceversa. The correlation between psychological impact and social impact was positive and significant, demonstrating that the improvement of psychological impact increases with social impact. The correlation between aesthetic concern and self-confidence was instead inverse. The correlation between aesthetic concern with social impact was positive and significant, demonstrating that the improvement of aesthetic concern increases with social impact. The correlation between aesthetic concern and self-confidence was instead inverse. Significant and positive correlations were found among convictions and self-confidence; thus the improvement of convictions increases with self-confidence. Significant inverse correlations were evident between oral health and self-confidence, suggesting that the decreasing level of oral health corresponds to increasing self-confidence level. Significant positive correlations were found among oral health and social impact, psychological impact and aesthetic concern; thus, when oral health increases, each of the three factors also increases.

Finally the correlation between self esteem and oral health was positive and significant, demonstrating that improvement of self esteem increases with oral health.

To highlight significant differences between groups (gender and age), the Mann Whitney Test was used. Significant gender differences were found in terms of gender, in reference to variables such as self-confidence and convictions; the *p*- values of the Mann Whitney Test were respectively 0.003 for self-confidence and 0.041 for convictions. No significant differences were found in relation to age.

Independent variables were considered in order to highlight dependences. Specifically age, sex, and crooked teeth were considered. Their psychological role was considered as relevant for self-confidence, psychological impact and conviction. The reported data show significant dependences among age and

psychological impact, sex and self-confidence, crooked teeth and conviction. All the above-mentioned independent variables were in significant and positive relation with the reported psychological phenomena. In line with reported differences, the role of sex was relevant in reference to self-confidence (Table IV).

DISCUSSION

The results of this study highlight how dental aesthetics and oral health can affect the psychological well-being of adolescents and young adults. The data underline that the characteristics and appearance of the face play a crucial role for these subjects, with significant consequences on self-perception, self-esteem and quality of life. In line with numerous studies, the data obtained from our research shows that dental aesthetics has a great influence on the psychological well-being of the F gender, with significant repercussions on the body image and adaptation.

Despite Flores et al. having obtained opposite results, in our study significant differences emerged regarding gender; the dento-facial aspect affects the variables "self-confidence" and "convictions" especially in the female population, with significant consequences on individual well-being. The first factor refers to body representation and self-perception, the second one to beliefs about oral aesthetics and how it can promote health, work and social success.

From these results, it is also possible to explain the inverse correlation between aesthetic concern and social impact; as explained by Claudino and Traebert, the positive perception of the body image is connected to a lowering of the social inhibitions mentioned above. In this sense, it is evident that self-confidence is significantly associated with the psychological impact and the variable related to aesthetic concern; a high level of self-confidence can be linked to lower levels of stress and to a better self-perception, guarantor of the individual well-being. These data explain the existence of a significant connection between dental morphology and psychological characteristics and the positive correlation that emerged the social

and the psychological impact with oral health. As highlighted in the studies by Kavaliauskiene et al. high levels of satisfaction related to oral health are associated with psychosocial well-being. On the other hand, the authors themselves point out that dental well-being can prevent the appearance of anxiety-depressive symptoms, emphasizing the link between the aforementioned elements. Finally, our studies have shown that even patients' beliefs play an important role as they were positively correlated with self-confidence. In fact, it seems that those who believe that good dental aesthetics can promote individual well-being and social success are more satisfied with their appearance. With regard to the analysis of the regressions, the study of the dependencies between the examined variables showed that age can affect the psychological well-being of the adolescent. It would appear that younger subjects are more affected by the effect of oral problems on their appearance, with a significant impact on psycho-social functioning. Especially for younger people, social relations and well-being itself depend directly on physical attractiveness, therefore aesthetic alteration can have a direct impact on psychological components, such as body representation and self-esteem, ultimately influencing the subject's quality of life. Even the presence of crooked teeth can have significant repercussions on patients' beliefs, influencing the value attributed to dental aesthetics. According to these results, in fact, misalignment of teeth can affect the individual's appearance and socio-work success, with consequences on quality of life.

Contrary to the data found in literature few trends have emerged regarding the impact of dental aesthetics on self-esteem. This could be due to the tool used, therefore we propose applying a different one, or it is due to the relationship between observer and the observed, not particularly profound to allow the user the real expression of his problems? We must also consider that several and different factors can affect facial and dental aesthetics; nowadays 3D Low-Dose X-ray protocols are available that lead an accurate orthognatodontic evaluation with a low radiological risk for the patients. The results of our research once again highlight the importance of dental aesthetics

in the lives of adolescents and young adults, with particular reference to the female gender, emphasizing the relevance of psychological parameters in orthodontic research and clinical practice.

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LETTER TO THE EDITOR

SEASONAL MONITORING OF SERUM IgE AND BLOOD EOSINOPHIL VARIABILITY MAY LEAD TO A BETTER SEVERE ASTHMA PHENOTYPING AND TO A CORRECT BIOLOGIC PRESCRIPTIONB. SPOSATO^{1,2}, M. SCALESE³ and P. ROGLIANI^{2,4}¹*Azienda USL Toscana Sud-Est Pneumology Department, "Misericordia" Hospital, Grosseto,*²*Experimental Medicine and Systems, "PhD program" Department of Systems Medicine University of Rome "Tor Vergata";* ³*Clinic Physiology Institute, National Research Centre, Pisa, Italy* ⁴*Respiratory Unit, department of Experimental Medicine, University of Rome "Tor Vergata" Rome, Italy**Received October 11, 2019 – Accepted February 5, 2020*

To the Editor,

According to Global Initiative for Asthma Guidelines (GINA) (1), we should consider adding on type 2-targeted biologic drug [anti-immunoglobulin E (IgE), anti-IL5/anti-IL5R, anti-IL-4R] for patients who have eosinophilic/allergic biomarkers with frequent exacerbations or poor symptom control, although treated with high doses of inhaled corticosteroid/long-acting β -agonist (ICS/LABA) and/or who need oral corticosteroid maintenance. Guidelines (1) suggest choosing one of the above-mentioned biologic medications on the basis of allergic sensitization and/or blood eosinophil counts, considering also some factors that may predict a good response [higher number of blood eosinophils, fractional exhaled nitric oxide (FENO), exacerbations, childhood/adult asthma onset and nasal polyps].

However, the different pathways of allergic or eosinophilic asthma indicating anti-IgE or anti-IL5/anti-IL5R may overlap in some patients, and it may therefore be difficult to choose the right treatment with a possible consequent lack of improvements or reduced outcomes in asthma. In this case, it is not always easy to understand whether eosinophilic

asthma is a pathway independent of IgE-induced allergic activation, consequent to allergen exposure. In fact, the latter leads to the activation of Th2 cells and the production of cytokines, interleukin IL-4, IL-5, IL-9, and IL-13, resulting in IgE production (2), eosinophilia and mast cell activation, whereas ILC2 cells (type 2 innate lymphoid cells) are involved in the innate immune response, that is independent of allergen sensitization, producing IL-5 and IL-13 (2, 3) with consequent eosinophilia.

Allergic asthmatics may have a serum IgE level increase during pollen seasons and a reduction in all other periods (4). These seasonal IgE variations may also influence the number of blood eosinophils in the severe allergic asthma phenotype. In fact, other authors have demonstrated a seasonal variability of eosinophil counts in asthmatics (5). One out of 6 patients, whose average count was above the 150 cells/mm³ cut-off value, had initial counts below such value (5). Therefore, a monitoring of IgE and eosinophil levels in the different seasons may lead to divergent results and therefore to a different indication on the choice of biologic treatment in severe asthmatics showing an overlapping phenotype.

Key words: biologic treatment; severe asthma; blood eosinophils; IgE, season; prescription

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We report a case of a severe asthmatic studied in two different seasons (winter and summer) for a biologic treatment prescription. The subject was a 66-year-old man with uncontrolled severe allergic asthma (sensitized to *Dermatophagoides*, *Parietaria*, *Cypress*). His clinical history was remarkable for the following: he was a non-smoker with allergic asthma from the age of 12; his severe asthma was diagnosed in 2014 associated to nasal polyps that had been surgically treated but with no other significant comorbidities; he had no suggestive history of occupational respiratory disease. Even though he was being treated with high doses of ICS/LABA, Tiotropium and leukotriene inhibitors, he had experienced 7/8 asthma exacerbations per year during the previous 4 years with several admissions to the emergency room and some hospitalizations. He had frequently used oral corticosteroids (almost every month), even for long periods of time. In early March 2019, he showed a serum IgE level of 1,300 U.I./ml and blood eosinophil counts of 950 cells/mm³ that indicated the prescription of a biologic therapy. Since he had a high number of eosinophils and a high IgE level associated with an elevated body weight, an omalizumab treatment was not suggested, while an indication for anti-IL-5/IL5R was given. For some reasons, the beginning of treatment was delayed and when the patient was retested at the end of June his total serum IgE and blood eosinophil counts were 904 U.I./ml and 100 cells/mm³, respectively. His last treatment with oral corticosteroid had been in early April for ten days. The pollen exposure reduction had probably led to a decrease in IgE and a consequent normalization of the blood eosinophil number. This result led to reconsider the type of biologic drug to be prescribed, thus correctly orientating towards the use of omalizumab (Fig. 1). After 4 months of Omalizumab treatment (early September 2019 to the end of January 2020), the patient was revalued. He did not show any asthma exacerbations, and oral corticosteroids had been used only once for a few days during the that period. The asthma control test (ACT) increased from 12 to 20. Pre-treatment FEV₁ was 1.81 L - 55% (July 2019), whereas post-treatment FEV₁ was 2.2 L - 66.2% (end of January 2020).

DISCUSSION

This case shows a serum IgE reduction during summer, when allergen exposure is low, with a consensual normalization of blood eosinophils, which allows us to consider the patient was affected by an allergic asthma rather than by an eosinophilic asthma phenotype. This normalization should not occur in innate-immune-induced eosinophilic asthma. In fact, eosinophilic asthmatics, who are poorly responsive to a trial of systemic corticosteroids, showed baseline high blood eosinophil counts associated to IgE low levels (6), suggesting that eosinophils and IgE may be unrelated as the former may be innate-immune induced. Consequently, Omalizumab becomes the best therapeutic choice for such subjects affected by a severe allergic asthma phenotype.

We know that serum IgE concentrations show variability over time in subjects with moderate to severe allergic asthma (4). IgE levels in allergic patients are influenced by different seasonal allergenic exposure. In fact, some authors have found that Japanese cedar pollen-IgE production is mainly associated with the degree of allergen exposure (7). Furthermore, a recent study on pediatric patients observed that serum allergen specific IgE levels measured during asthma exacerbations were elevated compared with the values observed when asthma was under control (8), confirming that allergen exposure, increasing IgE production, can be a trigger of exacerbations. In our case, in early March, when IgE was measured for the first time, allergen exposures were higher but decreased in summer, as demonstrated by lower IgE values measured at the end of June. In the patient's area, the cypress pollen concentration peak (which disappears in May) is usually observed in February-March (9). Dust mite exposure, instead, can be constant during autumn/winter/spring but is lower in summer. Indoor *Dermatophagoides pteronyssinus* 1 (Der p 1) antigen concentrations in homes can be higher in cold months in comparison to hot months (10). Furthermore, in summer, there is a significant reduction of allergic symptoms in dust-mite-induced respiratory allergy (11). Even though the *Parietaria* pollen is a perennial allergen during the whole year, it shows lower concentrations in summer in Italy and

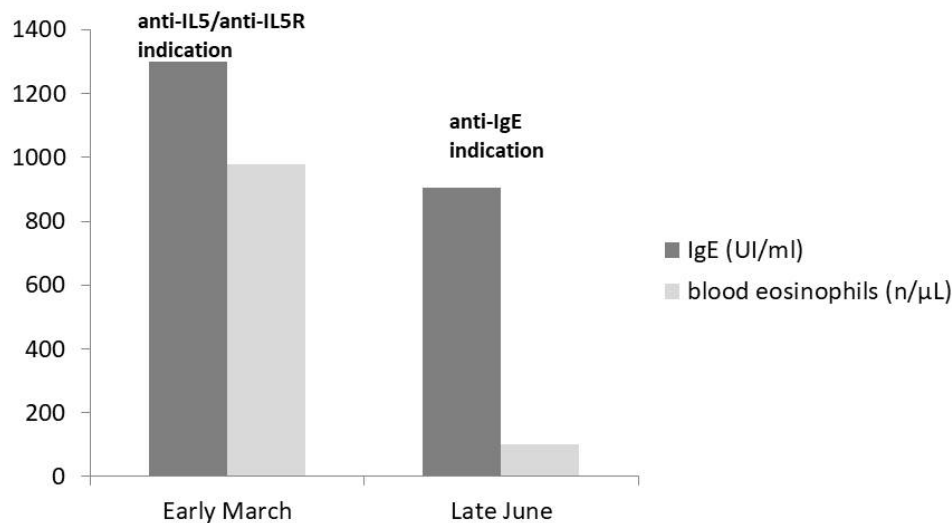


Fig. 1. Severe asthmatic patient with a seasonal variability in serum IgE and blood eosinophils that led to a different indication for biologic prescriptions

especially in the patient's area (9, 12).

Variability in IgE levels seems to have influenced clinical decisions for anti-IgE therapy (dosing and/or treatment choice) in approximately 40% of patients (4). As already stated, blood eosinophils can also show a significant variability in different measurements (5). In 17% and 22% of asthmatics, whose average counts were above the 150 or 300 cells/mm³ cut-off values respectively, had initial counts below these cut-off values (5). These data suggesting the use of a single eosinophil or IgE count as a criterion for eligibility for specific therapeutics may be inadequate to identify all patients with potential benefits.

After 4 months of omalizumab treatment, the patient did not show any significant asthma exacerbations, he used oral steroids only once for few days and showed a significant improvement in symptoms and lung function, demonstrating that the choice of the anti-IgE therapy was the right one.

Our case not only confirms that there is a seasonal variability of eosinophil counts and IgE levels, but also highlights that the variability of measurements may allow to properly differentiate allergic from eosinophilic asthma phenotypes in overlapping subjects, enabling to choose the most suitable biologic drug (anti-IgE vs anti-IL5/anti-IL5R).

In conclusion, on the basis of our case, we observed that blood eosinophil measurements in overlapping subjects in the different seasons (with high/low allergen exposure), may lead us to change the diagnosis of presumed eosinophilic asthma to allergic asthma and therefore indicate the right biologic treatment in cases of overlapping severe asthmatics. Therefore, in patients with overlapping eosinophilic and allergic phenotypes, according to the biomarkers measured in winter, it is advisable to monitor such biomarkers in the other seasons before deciding which biologic drug to prescribe.

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LETTER TO THE EDITOR

SCIENTIFIC METHODS IN DENTAL RESEARCH: CRITICAL ANALYSIS OF THE LITERATURE AND EXPERT OPINION ON THE MATTER

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To the Editor,

Recently, dentistry topics have been well reported in the scientific literature (1), however, the general impact and presence of dentistry within top-class scientific journals is still weak. Typically, the scientific dental community is quite heterogeneous: in fact, it involves dentists, academics, scientists and students. On the contrary, the number of dental journals is scarce, as is their impact factor.

Researchers certainly know that the Impact Factor (IF) is one of the most influential indicators: recently, a worldwide debated topic was related to the aim of describing the overall quality of a scientific journal and whether the IF unquestionably defines the scientific reputation of a journal within the scientific community.

Currently, the highest impact factor reported in the ISI-category of ‘Dentistry’ has been attributed to the journal “Periodontology 2000” with IF 6.22. On the other hand, in the ISI-category of ‘Oncology’, the IF reaches higher values: for example, the journal “CA Cancer J Clin” reaches an IF 244.59. In this context, do we really assume that oncology-related topics are

somewhat 30 times more interesting than dentistry?

Of course, different disciplines may have different ways to communicate, publish and share their topics; moreover, high-impact journals are more likely to be cited by several scientists in different fields. The interest in dentistry-related publications strongly depends on the quality of the data obtained, and on the potential clinical applications of such data; in this light, dentistry urges to link its topics to biosciences, to general medicine, and even to basic research (2).

In the present study, we searched the key word “dentistry” on PubMed, to assess whether the selected articles were related to dental sciences or to different matters.

MATERIALS AND METHODS

Searching strategy

We analyzed the impact of the key word “dentistry” in the title, in the abstract, and in the main text of articles published in top-journals previously selected: the main aim was to evaluate whether the topic discussed in these articles was relevant or not to dental fields. Searching for

Key words: dentistry; evidence-based medicine; dental journals; scientific research; dental education

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the key word “dentistry” and “journal name” [journal] in PubMed, we analyzed all the items reported. Eight highly-impacted journals were considered in our research, as they represent a balanced selection of high-impact journals and those most recognized worldwide. We first debated to include top dental journals such as “Journal of Dental Research”, “Dental Materials” and so on; however, we felt that those journals are certainly committed and well focused on dentistry. Thus, we decided only to investigate the following journals:

- JADA (Journal of the American Dental Association) ISSN: 0002-8177
- PLOS One (Public Library of Science) ISSN: 1932-6203
- Nature ISSN: 1476-4687
- Science ISSN: 1095-9203
- JAMA (Journal Of The American Medical Association) ISSN: 0002-9955
- PLOS Medicine ISSN: 1549-1277
- JCI (Journal Clinical Investigation) ISSN: 0021-9738
- Scientific Reports ISSN:2045-2322

The time frame of the selected articles ranged from 1973 to 2018.

The main criterium for selecting a scientific journal was its impact factor, and its overall impact to the scientific community: the 2017 Journal Citation Report (JCR) total Impact Factor of the journals selected in this investigation was 164.9.

RESULTS

The analysis of the 8 selected journals showed that the key word “Dentistry” was reported in 13,473

articles, however, only 767 articles reported such key word in the title and only 232 articles reported it in the abstract (Table I).

With the aim of understanding the overall impact of the dental sciences in the journal “Nature”, we searched for the key word “dentistry” AND “Nature” [journal] on PubMed: 101 items were reported. Surprisingly, after a brief reading of the titles and the abstracts related to these 101 selected articles, we found that none of them really focused on dentistry until 1986.

The same research was carried out for the journal “JAMA” which reported the word “dentistry” in 225 published articles, only 5 of which reported such word in the title.

Our further analyses showed that the highest percentage of articles reporting the key word “Dentistry” belonged to the journal “JADA”, undoubtedly one of the most important journals typically focused on dentistry; JADA reported 9,562 articles and 743 titles containing the key word “Dentistry”.

The data obtained from the journal “PLOS One” were particularly suggestive about the substantiation of our starting hypothesis; in fact, we found 2,365 articles, but only six titles contained the key word “Dentistry”.

We found extremely interesting the analysis made on the key word “dentistry” AND “Science” [journal] in PubMed: in fact, only 118 items were reported, and after a brief reading of the titles and the abstracts of these 118 selected articles, we deduced that none of them were really related to dentistry

Table I. The key word “Dentistry” has been reported in the main text, in the title or in the abstract of scientific publications of the journals selected for this research.

Journals	Dentistry - main text	Dentistry - title	Dentistry - abstract
JADA	9562	743	218
PLOS ONE	2365	6	12
NATURE	101	1	1
SCIENCE	118	12	0
JAMA	225	5	1
PLOS MEDICINE	35	0	0
JCI	145	0	0
SCIENTIFIC REPORTS	922	0	0

until the year 2012. In this case, the articles were mainly focused on molecular biology or forensic medicine, even to paleontology, but relatively few were closely related to dental fields.

Finally, no article and no title containing the key word “Dentistry” was found in the journals “JCI”, “PLOS Medicine” and “Scientific Reports”.

After this preliminary screening, we investigated the presence of the key word “Dentistry” in the abstract of those articles reporting the key word “Dentistry” in their title. Following a careful analysis, we found 232 abstracts containing the key

word “Dentistry”, although we must report that some articles reported no available abstract on PubMed and PMC.

In a further step, we critically read the whole text of articles containing the key word “Dentistry” both in the title and in the abstract. The results clearly show that the highest percentage of these articles was focused on “new concepts in dentistry” and “innovative techniques and materials”, highlighting how the research and development (R&D) themes are undoubtedly the most appreciated by editors and readers.

Table II. Topics summarized in macro-categories.

	JADA	PLOS	NATURE	SCIENCE	JAMA	PLOS MED	JCI	SCI REP
	ONE							
Evolution of dentistry	25	/	/	/	/	/	/	/
Women in dentistry	6	/	/	/	/	/	/	/
New concepts in dentistry	123	/	1	9	2	/	/	/
Guidelines in dentistry	29	2	/	/	/	/	/	/
Preventive and community dentistry	47	/	/	/	/	/	/	/
Oral diseases	12	/	/	/	2	/	/	/
Dentistry and systemic diseases	39	/	/	/	/	/	/	/
Innovative techniques and materials	91	/	/	1	1	/	/	/
Use of drugs in dentistry	20	/	/	/	/	/	/	/
Non-invasive dentistry	5	/	/	/	/	/	/	/
Aesthetic dentistry	21	/	/	/	/	/	/	/
International protocols	52	1	/	/	/	/	/	/
Adverse reactions and technical errors in dentistry	7	2	/	/	/	/	/	/
Dietology and dentistry	4	/	/	/	/	/	/	/
Anesthesiology	12	/	/	/	/	/	/	/
Clinical dentistry	30	/	/	/	/	/	/	/
Pediatric dentistry	5	1	/	/	/	/	/	/
Dentistry and laws	14	/	/	/	/	/	/	/
Ethics in dental research	7	/	/	/	/	/	/	/
Evolution of dental specialties	37	/	/	1	/	/	/	/
Public and private dentistry	8	/	/	/	/	/	/	/
Dentistry and social media	29	/	/	/	/	/	/	/

Moderate interest has also been reported for articles dealing with the “evolution of dentistry”: these articles typically emphasize the history of dentistry and on the state of the art of the traditional techniques and technologies in the dental field.

Although we found that the topics related to “general dentistry” were mainly reported in JADA articles, we have tried to summarize the other topics most represented published in the selected journals. Table II shows the different topics distributed within the selected articles (Table II).

DISCUSSION

In our article, we have tried to understand whether dentistry-related topics are substantially represented in the top journals. For this purpose, we selected only articles reporting the key word “dentistry”. After a careful analysis, we assessed that these articles mainly focused on matters belonging to the categories of “Evolution of dentistry and general concepts” and “Innovative techniques and materials”. Such articles reported some interesting concepts related to innovation in dentistry, and the way in which dentistry has improved over past years thanks to the discovery of new biomaterials and novel techniques.

Few articles were focused on clinical research in dentistry, while several articles were focused on severe infections, bacteria superinfections, novel allergens, and acute respiratory diseases in dentistry, reporting authoritative considerations on air pollution and substances that daily pollute water and air.

The less-impacting articles were based on the use of 3D CAD/CAM-based digital technologies in implant dentistry (3).

We noticed that decayed teeth still represent a social and healthcare issue in many countries (4). We believe, on the other hand, that several impacting topics could be of interest for the general population, such as several diseases still frequently affecting oral health (5), or the use of stem cells for the growing branch of regenerative dentistry (6, 7). Of course, we do understand that dentistry is of interest mainly to dentists or to emerging dental journals (8), but the real challenge is to make dentistry attractive also with such closely related topics.

In our work, we have analyzed the prevalence and the overall coherence of the key word “dentistry” in some top-class journals. Nowadays, clinical dentistry is mainly focused on topics of general interest, such as bone scaffolds or osteonecrosis after oral surgery or the impact of syndromes on clinical management (9, 10). The interest of patients, on the other hand, is currently on aesthetic dentistry (11) and stem cells for biomedical applications (12).

A novel and safe evidence-based practice in dentistry would be the main and most reliable way for ensuring the scientific growth of this medical science in the near future. After our study, we provocatively launched an overall alert on the scientific quality of dental research to the dental scientific community. In fact, despite dentistry being the subject for several research topics, it is possible that “dentistry-related” topics are still not interesting enough to reach a wider number of readers and to attract more attention from top-journals. Our opinion is that the truth is between the two: although the most debated topics in dentistry are still heavily related to clinical aspects, sometimes “exceptionally” overestimated and overestimated, it is equally true that some novel concepts, such as “regenerative dentistry” or “nanodentistry” have merits. Of course, we hope to see a general improvement of this trend in the near future.

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