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EDITORIAL

HAEMATOPOIESIS: “LIVING IN THE SHADOW” OF STEM CELL DIFFERENTIATION

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Haematopoiesis is one of the most well understood stem-cell associated processes. It is a process in which pluripotent hematopoietic stem cells (HSCs) self-proliferate and differentiate into all types of blood cells. The process takes place in marrow of the flat bones in adults, however its location changes several times through embryonic and foetal development. Given the broad range of blood cells and the major differences in their build and function, together with the fact that their numbers need to be maintained within relatively narrow margins in order to maintain homeostasis despite changing environmental conditions, makes the whole process of haematopoiesis highly regulated and depending on a variety of growth factors. When influenced by those, HSCs undergo several irreversible steps, with every next one committing them to an even more specialised fate, ending with all the specific types of mostly short-lived blood cells, that are unable to proliferate on their own and need constant replenishment from the HSC pool. Because the process of haematopoiesis is the only source of all the members of the group of cells performing a range of highly important roles in functioning of the organism, significant damage to the underlying stem cells can cause a range of severe diseases. Many treatments are suggested for managing their symptoms or slowing progress, with bone marrow transplant being one of the only ones that offer possible permanent solution and, despite being a relatively risky procedure, is being widely performed, with the methods constantly improving in order to achieve progressively better results in both treatability and survivability of the patients

Stem cells are one of the greatest discoveries of recent medical science history. Being pluripotent cells of almost unlimited potential for differentiation

into all of the tissues found in the adult organism makes them a highly interesting topic which, when properly understood, would spark giant

Key words: haematopoiesis, stem cells

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breakthrough in modern medicine. However, the way in which they are regulated to create an extremely complicated organism, with all the tissues present in the right place and amount is still far from being fully comprehended (1). One of the most well analysed processes involving stem cells, knowledge of which is already widely used in therapies that often lead to treatment of associated diseases, is haematopoiesis. This complex chain of interactions, causes a singular type of progenitor cells to develop into a broad range of lineage committed intermediates, that give rise to a variety of cell types that significantly differ in almost every biochemical and morphological aspect (2). Almost every cell that is created as a result of this process is essential for proper functioning of both the developing and adult organism. Because of that, even small abnormalities in the process of haematopoiesis can cause significant consequences to the person affected. A broad range of diseases, associated with both inherited and acquired defects in that process, and their usual severity, causes a great need for research in this field. For this reason, many scientific groups try to broaden the understanding of HSCs. This process, apart from the development of clinical applications for better treatment of haematopoiesis-associated diseases, such as leukaemias and aplastic anaemia, broadens the understanding of blood progenitor stem cells, hence stem cells overall (3). This will hopefully not only allow better treatment of patients and increase their survivability, but also improve the methods of detection and early prevention of many of those diseases, possibly with the application of molecular and genetic basis of HSC functioning. In this review, we present an overview of the current knowledge regarding haematopoiesis as a process, as well as analysing the examples of diseases caused by its defects, and the possible methods of their treatment.

OVERVIEW OF HAEMATOPOIESIS

Haematopoiesis is a process in which pluripotent hematopoietic stem cells (HSCs) proliferate and specialise to become all the types of blood cells. Cells of blood require constant replacement, with an average lifespan of 100-120 days for red blood

cells and only hours/days for some types of white blood cells and platelets (4). For this reason, there is a constant number of HSCs present in all the development stages and in adult humans. They constantly proliferate, and differentiate in a highly-regulated way, in order to maintain optimal numbers of blood cells.

In adults, HSCs can be found in bone marrow. However, they change their location several times before ending in their final destination. At the early stages of embryonic development haematopoiesis starts after gastrulation. Precursors of HSCs arise from mesoderm and migrate towards yolk sac where they aggregate in groups called “blood islands” and initiate red blood cell production. As the embryo grows rapidly, the early embryonic hematopoietic stem cell pool becomes insufficient which leads to excessive HSC formation, and proliferation takes place during that period. Definitive HSCs are documented to arise from AGM-aorta-gonadomesonephros (location near the ventral wall of the dorsal aorta), with other sources including umbilical and vitelline arteries (5). Developing liver is probably colonised by these cells (some studies suggest that yolk sac HSCs also take part in that process) and serves as a main hematopoietic organ until the late foetal period. There is some debate whether placenta functions as a hematopoietic organ, as it also harbours a large pool of HSCs during foetal development. However, there is no direct evidence for this and some research suggests that the size of that pool has more to do with the fact that placenta lies on the way from the dorsal aorta to foetal liver, and HSCs are probably just nurtured in the placental niches temporarily on their way from AGM (6). This hypothesis is more likely as, at later stages of development, the number of HSCs in the placental pool decreases, as the liver pool continues to expand.

Foetal liver is the main site of HSC proliferation and differentiation. However, the blood cells are not produced there *de novo*, but are rather seeded travelling via the bloodstream from the yolk sac and AGM via umbilical vessels. The cells then proliferate, until they reach a maximum of ~1000, after which their number stays relatively constant and after time starts to decline. With gestation ongoing, the way in

which differentiation proceeds also changes. At the early stages of development, proerythroblasts and colony-forming erythroids are present, which is an indicator of erythropoiesis being the main focus. At later stages, other progenitor cells arise, starting to differentiate in other types of blood cells (5).

After the bones form, HSC start to migrate into bone marrow niches in which they will be found in the adult organism. The exact way in which the switch from liver to bone marrow haematopoiesis occurs remains poorly understood. However, it is known that HSCs start circulating in the blood stream before the ossification of bones occurs. When blood vessels invade developing bones, establishing circulation, HSCs seed into blood marrow niches and establish a bone marrow hematopoietic pool. In young humans, these pools are located in marrow of long bones (femur, tibia). This changes in adults when haematopoiesis ceases in long bone marrow, and continues only in flat bones (pelvis, sternum), vertebrae and cranium. However, not all the parts of that process are conducted in blood marrow, as maturation of blood cells in some cases takes place in blood or tissue. A good example of this are T lymphocytes, that need to mature in an antigen-free environment prior to assuming their function, which is why some lymphocyte precursors migrate to thymus in order to complete differentiation. There, a selective process occurs in order to eliminate all the cells that are unable to recognise other cells and antigens properly (only 2-4% of cells succeed, the rest undergo apoptosis) (7). There is also evidence that B lymphocytes use spleen as a secondary maturation site (8).

HSC differentiation is tightly regulated in order to maintain the optimal numbers of all the blood cell types. These numbers are not always constant and vary highly depending on the environmental condition. Causes such as bleeding or infection may cause the need for quick drastic change in production patterns in order to maintain homeostasis, or nullify external conditions (9). There are multiple growth factors which, when acting upon HSCs in different combinations and periods, induce their differentiation into the whole range of products. Stages of that differentiation are usually irreversible, with cells

losing their potency to differentiate into some type of cells along the way, to the moment when they cannot differentiate any further. The key growth factors include Stem Cell Factor (SCF), involved in self-renewal and development of blood cells, Interleukins (IL) that regulate their proliferation and maturation, and Colony Stimulating Factors (CSFs) that stimulate formation of specific cells from progenitor or intermediate product cells. These growth factors, when acting upon HSCs, initiate signal transduction pathways which leads to activation of transcription factors, which, depending on their combination and time of expression, influence HSCs to differentiate into a specific type of product cells.

HSCs differentiate into one of two types of progenitor cells. The first type, common lymphoid progenitor cells, arise when exposed to IL-2, IL-7, IL-12 and additional factors such as TGF- β . These will later differentiate into B and T lymphocytes (white blood cells) influenced mostly by a range of interleukins. The second type are common myeloid progenitor cells, that arise in response to IL-1, IL-3, IL-6 and SCF. These cells will later differentiate into two further subtypes. Granulocyte Macrophage-Colony stimulating factor (GM-CSF) will cause differentiation into Granulocyte Macrophage progenitors. These progenitors will differentiate into granulocyte progenitors that give rise to all types of granulocytes, and monocytes that will later become macrophages. Other common myeloid progenitors will become erythrocyte-megakaryocyte precursors. That transition is triggered by exposure to SCF, IL-3 and GM-CSF. Cell fates of erythrocyte-megakaryocyte precursors are determined by two factors: when exposed to cytokine-thrombopoietin (TPO), they differentiate into megakaryocytes, big bone marrow cells responsible for production of thrombocytes (platelets) (10); and when exposed to erythropoietin (EPO), a glycoprotein hormone, erythrocyte-megakaryocyte precursors differentiate into erythrocytes (red blood cells).

The cells that are the result of HSC differentiation play a crucial role in the organism. Erythrocytes transport substrates and products of respiration, granulocytes and lymphocytes take part in defence against antigens, and platelets are necessary for

clotting which makes them necessary in healing many injuries. Therefore, as HSCs are the starting point of all differentiation, diseases of HSCs tend to cause severe conditions which often affect many types of blood cells.

A good example of an HSC-associated disease is leukaemia. It is a term describing a group of cancers that result in accumulation of immature or abnormal blood cells, however the exact causes are still to be discovered and different types of leukaemia are believed to have different causes. Nevertheless, there are several risk factors associated with leukaemia such as non-ionizing radiation, smoking, chemicals such as benzene, and having undergone chemotherapy (11). There is also strong proof of genetic predisposition to leukaemia, as multiple cases have been observed when many members of the same family are affected. Most types of leukaemia involve production of large amounts of faulty white blood cells by damaged HSCs. Accumulation of those cells, that cannot perform their function, leads to suppression of other blood cell production, which results in multiple associated health problems. Lack of a sufficient amount of blood platelets causes easy bruising and problems in bleeding suppression in even the smallest of wounds, as they are the cells responsible for blood clotting, hence initial wound closure. Lack of functional white blood cells, that play a major role in fighting pathogens, results in the general inability to fight infections, which leads to a significant increase in their frequency. Finally, lack of red blood cells may lead to anaemia, which will negatively impact the ability of the organism to transport respiratory gasses, which is the main function of those cells. Other major symptoms dependent on the spread of disease include swelling of spleen and liver (caused by tissue penetration by cancer cells), nausea (caused by abnormal white cell accumulation in lymph nodes) and finally, if cancerous cells penetrate the nervous system, headaches and, less often, seizures or coma (11-12).

Anaemia can also arise directly as a result of HSC damage by both genetic and environmental causes, an example of which can be aplastic anaemia (AA), which is characterised by the stem cells being unable to produce functional blood cells. Specifically, as

the term 'aplastic' suggests, its cause is the inability to generate mature blood cells which can result in several types of deficiencies such as pancytopenia (lack of all types of blood cells), anaemia (lack of red blood cells), leukopenia (lack of white blood cells) and thrombocytopenia (lack of platelets). All these deficiencies carry subsequent symptoms, similar to those described in the previous paragraph where leukaemia was characterised. As with leukaemia, the exact cause of AA is unknown. However, there are also several suggested risk factors that influence developing AA, such as chemicals like benzene, or use of certain drugs. These are mostly case reports, with insufficient research conducted to consider them definite. There is strong evidence that ionizing radiation exposure is associated with development of AA, with famous cases such as Marie Curie who died of AA after working unprotected with radioactive materials, as there was little knowledge on the effects of ionizing radiation at that time.

Both leukemia and AA, as associated with stem cells, are relatively hard to treat. A major obstacle is the fact that they are usually diagnosed by symptoms, and bone marrow biopsy is the only way to confirm the exact cause. There are several suggested treatments, however the only fully effective one, that is focused on causes rather than suppressing symptoms is bone marrow transplant. However, it is a very risky procedure, which means that it is usually only conducted when the symptoms are unmanageable and the person affected is otherwise healthy, and under 30 years of age. There are several steps that need to be undertaken before conducting the transplant. Firstly, a donor needs to be found. There are several databases that currently serve to recognise potential donors. The donor, usually siblings or close relatives, must share tissue type (human leukocyte antigen) with the recipient in order to minimise the possibility of graft versus host disease. However, if there is no related donor to be found, bases such as the National Marrow Donor Program, exist in order to identify potential unrelated donors. Alternatively, autologous (host-to-host) graft can be performed which means the chance of graft rejection is almost zero. However, autologous graft is only possible if healthy stem

cells can be obtained from the host, or if frozen cells which had been conserved as a preventive measure are already available (4).

In the first case, bone marrow is stimulated to proliferate by introduction of HSC proliferation factors, in order to cause “leakage” of HSCs into the bloodstream. Then blood is extracted from the host, and stem cells separated. Subsequently, “purging” of cells is conducted in order to remove cancerous stem cells from the pool. The remaining healthy cells are then placed in a cryo-container, awaiting transplant. If there is no possibility of extracting enough healthy HSCs for transplant, it is also possible to “repair” damaged stem cells *ex vivo* using gene therapy, in order to return them to the host. This method is not yet widely used, however there is a large amount of research undergoing to make it widely available, with some successful clinical trials already conducted (13). None of these processes are required if healthy HSCs are already available, being extracted previously as a pre-emptive measure. There is much debate currently about the sources of such stem cells, with one of the leading sources of these being umbilical cord blood. The process involves harvesting blood from the umbilical cord shortly after birth, and separating stem cells from the rest of blood contents *in vitro*. It is a relatively easy way to obtain a source of HSCs, which are then frozen for potential future use, either in the donor or a close relative (14).

Regardless of the origin of the transplanted stem cells, the recipient needs additional procedures before the transplant can proceed. Firstly, a prolonged treatment with high dose chemotherapy is conducted, sometimes combined with additional radiotherapy treatment. These steps are necessary to maximally lower the number of cancerous white blood cells in the organism, as well as cause bone marrow ablation (damage to the point where it is not able to produce any new blood cells. Then, previously stored cells are transported to the patient’s bloodstream, where they take over the function of damaged HSCs and resume proliferation and differentiation. These steps are usually sufficient for autologous transplant (with HSCs coming from the host), as there is almost no chance of graft versus host disease (15). However

if the transplant is allogenic (coming from a donor different than host) additional measures must be taken to mitigate the effects of possible rejection. This is achieved by administration of immunosuppressive medicine to the host prior to and through the transplantation process. After the transplant, a period of convalescence is required for the bone marrow cells to achieve necessary numbers and optimise levels of recipient blood cells that were lowered by pre-transplantation ablation of original HSCs.

Bone marrow transplant is a potential cure for a range of diseases, including a range of leukaemias, lymphomas, myelomas and anaemias, that are often otherwise untreatable, with other therapies mostly focused on suppressing symptoms. Any transplant is a risky procedure associated with a relatively high mortality rate. One study published in 2003 estimated that the mortality rate for a group of patients, who had undergone HSC transplant between 1974 and 1998 and survived at least 2 years after the procedure, 22% have died (most 2-5 years after transplant). The main causes of death included relapse of primary disease and graft versus host disease, thus bone marrow transplant is only performed for life-threatening conditions. However, the increasing knowledge of stem cells, development of better medicines, improvements in transplantation, post-transplantation care techniques and growth of databases of potential donors will lead to an improvement in the numbers of successfully performed HSC transplants and a greater patient survival.

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EDITORIAL

CROHN'S DISEASE AND EXTRA INTESTINAL GRANULOMATOUS LESIONS

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Crohn's disease (CD) is an inflammatory bowel disease with a multifactorial etiology. Clinical features include mucosal erosion, diarrhea, weight loss and other complications such as formation of granuloma. In CD, granuloma is a non-neoplastic epithelioid lesion, formed by a compact aggregate of histiocytes with the absence of a central necrosis, however, the correlation among CD and the formation of granulomas is unknown. Many cases of granulomas in the extracellular site, related to CD, have been reported in the literature. These granulomas, at times, represented the only visible manifestation of the pathology. Extra intestinal granulomas have been found on ovaries, lungs, male genitalia, female genitalia, orofacial regions and skin. From the data in the literature it could be hypothesized that there is a cross-reaction of the immune system with similar antigenic epitopes belonging to different sites. This hypothesis, if checked, can place CD not only among inflammatory bowel disease but also among inflammatory diseases with systemic involvement.

Crohn's disease (CD) is an inflammatory bowel disease (IBD) characterized by a chronic inflammatory process. The etiology of CD is multifactorial and involves a combination of genetic and environmental factors. The set of these factors generates a condition known as dysbiosis that, by altering the eubiotic equilibrium of the intestinal flora, leads to a continuous and massive activation of the lymphoid tissue associated with the intestine (GALT) (1, 2), and the establishment of a chronic inflammatory state, characterized by the release of various chemical mediators of inflammation, such as

heat shock proteins (HSP) (2, 3).

CD involves the entire gastrointestinal tract from the mouth to the anus and manifests itself with clinical features including mucosal erosion, mucus-bloody ulcers, diarrhea, weight loss, and abdominal pain (1-3). Among the complications of the disease, the most important are predisposition to dysplasia and colorectal cancer (due to the generation by TNF-alpha of a metaplastic alteration of the genetic material of the enterocyte and/or colonocyte), visceral stenosis and onset of granulomas (4).

This article focuses on the general

Key words: Chron's disease, granuloma, epithelioid lesion, extra intestinal manifestations

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characteristics of the granulomatous lesion in Crohn's disease, analyzing and discussing possible immunopathological phenomena leading to granuloma formation.

Granulomatous lesions in CD

Granuloma is a non-neoplastic lesion the potential causes of which can be broadly classified as infections or non-infectious immune reactions (4-6). It is caused by a cell-mediated immune reaction to harmful substances and infectious agents that resist rapid immune destruction and are "seized" to prevent damage to the surrounding tissue. Granuloma formation is a chronic inflammatory reaction, and is triggered by a flogistic process leading to activation of T helper 1 lymphocytes and recruitment of macrophages and other inflammatory cells in the site of the lesion. Inflammation gradually destroys the harmful agents, creating debris and forming scar tissue (5). Lymphocytes are, in turn, encircled by fibroblasts that, always under the stimulus of $\text{TNF-}\alpha$, produce extra-cellular matrix, surrounding and isolating the antigens (6).

After some antigen exposure and processing, the release of $\text{IFN-}\gamma$, $\text{TNF-}\alpha$, and IL-2 (5, 6) allows for activation of macrophages, immune cell recruitment, epithelioid cells and giant cells as well as greater T cell proliferation (5, 6) arranged in this order from the center to the periphery (Fig. 1).

Regarding their histological features, granulomas may be of varying morphological appearance, most commonly epithelioid (4). An epithelioid granuloma is a compact aggregate of histiocytes (epithelioid cells) with or without giant cells, with no central necrosis and with the formation of histiocyte aggregates located in the most superficial part of the mucosa or associated with damaged crypts (7). Although not found in all patients with CD, granuloma, given its incidence, could be considered a pathognomonic element of the disease (6, 7).

The formation of granulomas in CD is currently a topic of discussion in the literature, as such, its symptomatology often overlaps with that of other pathologies. Generally, a CD granuloma has the following characteristics: (i) the presence of approximately 0.75 granulomas per section with a maximum width of 95 μm ; (ii) microgranulomas at the inflammation sites; (iii) location of granulomas at the sigma rectum junction; and (iv) no necrosis in the center of the granuloma (7).

Extra intestinal granulomatous lesions related to CD

Although the CD-associated granuloma is found in the intestinal environment, there are numerous cases of extra intestinal granulomas associated with CD. Table I summarizes some examples of cases found in literature.

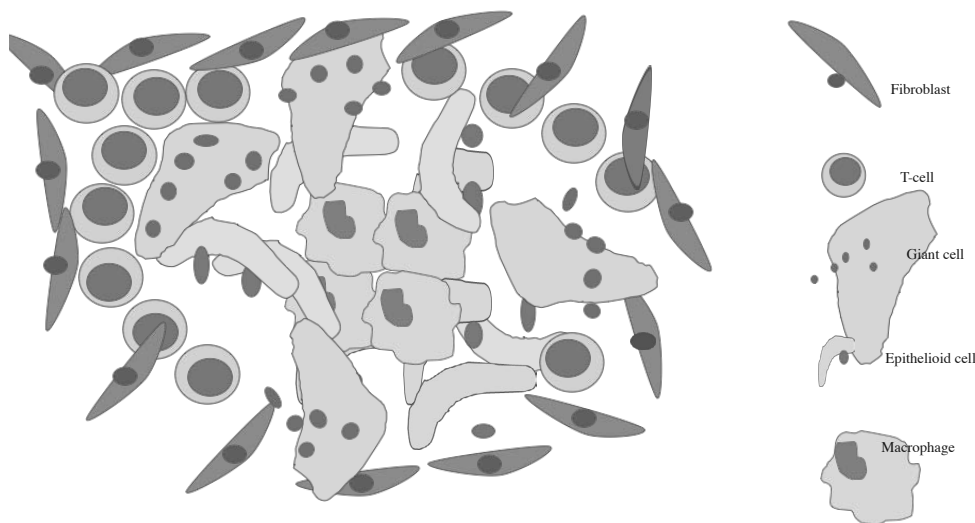


Fig. 1. Cell infiltrates within a granuloma. From the center to the periphery, there are macrophages, epithelial cells, giant cells and T cells, surrounded by a layer of fibroblasts.

Ovarian

The literature (between 1975 and 2015) reported 15 cases of ovarian cancer associated with CD. In most cases, an ovarian granulomatous lesion was derived from intestinal-ovarian fistulation, whereas, in one case, there was absence of fistulation and development of ovarian granuloma with an intact capsule (8).

Cutaneous

CD granulomatous lesions were found in patients with psoriasis and suppurative Hidradenitis (HS).

In relation to psoriasis, a single case was reported in which a patient with CD had skin lesions with histopathological features of psoriasis and granulomatous inflammation (9).

In relation to HS, there are three HS cases of patients who then developed CD. It is a pathology that produces perianal skin lesions clinically indistinguishable from CD. It is also important to note that, especially in a 38-year-old patient, axillary and perianal lesions were observed in areas not affected by HS-related flogos and presumably of epithelioid granulomatous attributable to CD (10).

Pulmonary

CD-related granulomatous lung involvement has been highlighted by two cases reported in the literature. The first case concerned a pediatric patient (11 years old) with a persistent symptomatology of the respiratory tract (cough and dyspnea) that did not respond to any type of drug. Pulmonary nodules were detected by CT scan and subsequently subjected to biopsy. The histopathology highlighted the granulomatous nature of these nodules, with features similar to those of a CD granuloma. The presence of CD was subsequently confirmed (11). The second case concerned a 44-year-old patient with recurrent pneumonia. Diagnostic investigations highlighted the presence of pulmonary nodules due to granulomatous lesions to be ascribed, in the first instance, to sarcoidosis. However, subsequent diagnostic investigations demonstrated a chronic inflammation of the intestine compatible with CD diagnosis (12).

Orofacial

Oral CD manifestations are well covered in the literature (1) and some studies have highlighted

Table I. *Summary of extra intestinal granulomatous lesions related to CD.*

Localization	Age	Intestinal involment (Yes/No)
Ovarian	Variable	Y
Skin	44	Y
Skin	38	Y
Pulmonary	11	Y
Pulmonary	44	Y
Lips	Variable	Only two cases (out of 3)
Penis and scrotum	18	Y
Ano – genital	21	Y
Vulvar	47	N
Vulvar	Variable	Only one case (out of 3)
Orofacial	Variable	Y
Orofacial	14	Y

the appearance of granulomatous CD lesions in the orofacial region. A first study describes three cases of patients with granulomatous cheilitis as a CD presentation. Two of the three patients also had intestinal involvement with typical CD lesions, but one patient had lip lesions as the only manifestation of the disease. Another study describes five pediatric cases (7 to 15 years of age) with non-caseous granulomatous in the oro-facial region. In two patients, the diagnosis of CD was prior to the discovery of granulomas, while in the other three the diagnosis of CD was made within 3 to 24 months after the discovery of orofacial involvement (13). A third study reports the case of a 14-year-old patient undergoing histological examination of orofacial tissues, which showed the presence of granulomas and giant cells. In the same patient, a condition of sideropenic anemia was also reported, suggesting the endoscopic examination of the colon. Colonoscopy was positive for CD granulomatous colon lesions (13).

Male genitalia

The first case of male genitalia involvement in CD describes an African-American patient suffering from CD (diagnosed at age 8 years) with swelling of the penis. He underwent surgery for circumcision and lymphangectomy of the penis. Histological examination showed interstitial edema and granulomas, leading to a metastatic CD diagnosis. A second study describes 25 patients with history of penile and scrotum swelling caused by ano-genital granulomatosis (AGG). The presence of abdominal pain in the same patients led to a diagnosis of CD, thus correlating AGG to CD (14).

Female genitalia

Reported studies show a case of a 47-year-old patient with vulvo-perineal granulomas being the only manifestation of the CD (15), and three cases of vulvar giant cell epithelial granulomas. In two of the three cases, there was no intestinal involvement and vulvar granulomas were the only manifestation of the CD (16).

In conclusion, based on the reported studies in the literature, one could say that patients who develop

granulomas are those with a more severe form of the disease which is confirmed by the fact that one patient may be negative at the time of diagnosis and positive at a later stage. Such a fact suggests that the presence of granuloma is related to a greater severity of the pathophysiological course of the CD. The presence of inflammatory-granulomatous lesions could represent the first signs of the disease. In fact, in many of the cases described, the extra-intestinal manifestations of CD appeared before those of the intestines. On this basis, one could also formulate a hypothesis regarding the pathogenesis of extraintestinal CD manifestations. In particular, one could hypothesize that there are cross-reactions of the immune system with similar antigenic epitopes belonging to different sites. This hypothesis, if checked, can place the CD not only among IBD's but also among inflammatory diseases with systemic involvement, however, further studies are needed in this regard.

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EDITORIAL

**DIFFERENT SIGNALS INDUCE MAST CELL INFLAMMATORY ACTIVITY:
INHIBITORY EFFECT OF VITAMIN E**

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Vitamin supplementation in disease reduces morbidity and mortality in humans by promoting the activation of different genes which influence several pathways. The purpose of this article is to clarify the role of vitamin E in mast cell inflammation. Vitamin E is a fat soluble antioxidant which protects from low-density lipoprotein (LDL) oxidation. Vitamin E promotes a barrier function and anti-inflammatory responses by binding the regulatory domain of protein kinase C α (PKC α) (a regulator and antagonist of heart failure) and decreases the activation of NF- κ B, a proinflammatory transcription factor, causing the generation of cytokines/chemokines and mast cell activation. Mast cells participate in innate and acquired immunity and inflammation. Several factors, including cytokines and chemokines, regulate the development and migration of activated mast cells. Mast cells generate and release inflammatory compounds in asthma and allergic diseases and have a detrimental effect on the vessel wall, which can be inhibited by vitamin E. Vitamin E inhibits histamine release generated in activated mast cells, increases calcium Ca²⁺ uptake and prevents the oxidation of unsaturated fatty acids. Vitamin E is relatively non-toxic, however, administered at very high doses may suppress normal hematological response as well as causing other adverse effects. Therefore, vitamin E may be beneficial in the prevention of diseases mediated by mast cells and can have special value in the treatment of asthma and allergic diseases; however, the exact mechanism by which vitamin E acts is still unclear, thus warranting future research.

Here, in this paper, we report the synthesis of the data of obtained in our laboratory experience over the past 30 years on mast cells (MCs), inflammatory cytokines/chemokines and allergy, and a summary of updated findings reported in the scientific literature found in a PubMed search (1).

Research efforts in the past decade have

highlighted that vitamin supplementation in diseases reduces morbidity and mortality in humans. Vitamins represent a promising candidate for inflammatory and autoimmune diseases as they exert an inhibitory effect on inflammation and promote the activation of different genes, influencing several pathways, including cellular

Key words: vitamin E, mast cells, cytokines, inflammation, allergy

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metabolism and biosynthesis, and modulation of cell proliferation and differentiation (2).

Vitamin E

Vitamin E was discovered in 1936 from a dietary fertility factor in rodents, and was given the name "tocopherol" (CH₃, H, CH₃, H) which in Greek language means: *tókos* = birth, and *phérein* = carry (to carry a pregnancy).

Vitamin E, a fat-soluble antioxidant consisting of multiple natural and synthetic forms, is a scavenger for free radicals and comprises two analogue groups: four tocopherols (T) (alpha, beta, gamma, delta) and four tocotrienols (T3) (alpha, beta, gamma, delta). γ -tocopherol is present at high concentrations in plant seeds and vegetable oils, and α -tocopherol is the most biologically active form of vitamin E. α -tocopherol is in 10-fold higher concentrations in tissue than γ -tocopherol and there is also 10-fold more scavenging of ROS by α -tocopherol than by γ -tocopherol *in vivo*. Alpha and gamma tocopherol are lipid soluble and differ by a methyl group that is present in the alpha but not in the gamma.

Vitamin E and other antioxidants reduce the production of damaging oxidants, and promote a barrier function and anti-inflammatory responses (3). During inflammation, vitamin E binds the regulatory domain of PKC α , but γ -tocopherol acts as an agonist and increases PKC α activity, while α -tocopherol is an antagonist and decreases PKC α . It has been reported that α -tocopherol inhibits VCAM-1 and ICAM-1 activation of PKC α in endothelial cells, an effect opposed by γ -tocopherol. Alpha-tocopherol decreases lipid peroxidation and enhances LDL resistance to oxidation; while γ -tocopherol is more potent than α -tocopherol and inhibits LDL oxidation, malondialdehyde (MDA) formation, and superoxide anion generation, and therefore oxidative stress (4).

Vitamin E inhibits the generation of reactive oxygen species (ROS) formed when fat undergoes oxidation. ROS diffuse through the cell membrane (5), and stimulate p38MAPK, oxidizing and activating intra-cellular PKC α . Vitamin E acts mainly in the lipid phase and inhibits the chain reaction of lipid peroxidation, therefore, prevents the spread of free radicals.

However, the antioxidant effect of vitamin E is not strictly related to the protection from oxidation.

Vitamin E decreases the activation of NF- κ B, which is a proinflammatory transcription factor, causing the release of inflammatory mediators, such as cytokines/chemokines in some tumor cells (6).

LPS may activate TLR4 mast cell receptor, leading to the activation protein-1 (AP1) and nuclear factor κ B (NF- κ B) involved in transcription regulation of cytokine/chemokine gene expression and, therefore, leading to inflammation which may be inhibited by vitamin E. Vitamin E is a modulator of signal transduction and gene expression in several cell types and inhibits protein phosphatase 2A, protein kinase B and smooth muscle cell proliferation by inhibiting protein kinase C (7).

In platelets, alpha-tocopherol, especially at high doses, decreases the release of proinflammatory cytokines and chemokine IL-8, and lipid peroxidation, superoxide (O₂⁻) production, plasminogen activator inhibitor-1 (PAI-1), protein kinase C, 5-lipoxygenase, tyrosine-kinase, cyclooxygenase-2, C reactive protein (CRP) levels and ROS, limiting toxic effects of reactive oxygen species released during inflammation, in *in vivo* study (8).

There are low plasma vitamin E levels in asthma subjects where MCs play a leading role.

Reduced maternal intake of vitamin E is associated with an increase of incidence of asthma and wheezing in children. The demonstration that vitamin E is protective in allergic diseases, including asthma, is demonstrated by the high concentration of this vitamin in the plasma of individuals living in countries with the lowest prevalence rate for asthma. Therefore, vitamin E helps to prevent and/or improves inflammation, and allergic diseases, including asthma (9).

The same authors reported that γ -tocopherol blocks airway hyperreactivity and inhibits certain immune cells, such as granulocytes activated by endotoxin, a classical immunostimulant *in vitro* and *in vivo*, with beneficial effects in induced experimental allergic inflammation in the lung of experimental animal model. In addition, vitamin E regulates the signals for leukocyte and significantly reduces lung eosinophil recruitment when challenged with

foreign antigen (10). However, in some particular cases, the effect of α -tocopherol may counteract the effect of the γ -tocopherol giving contrary results, with opposite regulatory functions during leukocyte activation and allergic inflammation. Since vitamin E has antioxidant activities, it may have a potential beneficial effect on inflammatory disorders, autoimmune diseases and allergy.

Moreover, vitamin E is involved in modulating apoptosis, migration, secretion and cell proliferation (11). Several human clinical trials have shown an improvement in the surrogate markers of inflammatory diseases and vascular function by vitamin E treatment. It also prevents several inflammatory diseases mediated by MCs by modulating signal transduction and gene expression. Therefore, an increasing body of evidence suggests the involvement of vitamin E in inflammation and allergy where MCs play a key role.

Mast cells

Mast cells are involved in innate, acquired immunity, autoimmunity, immunological diseases and inflammation (12). MCs maintain homeostasis under the control of peptides, hormones and environmental factors, and they are activated through cross-linking of their surface high affinity receptors for IgE, leading to immediate degranulation (in seconds), and release stored mediators such as histamine, heparin, tryptase, chymase, kinogenases, carboxypeptidase A3 and TNF (13). MC *de novo* synthesize inflammatory compounds such as growth factors, leukotrienes, prostaglandins, NO, and some cytokines/chemokines, released later selectively without degranulation.

Mast cell activation and inflammation

Cytokines are inflammatory and anti-inflammatory proteins that mediate different inflammatory states (14-15); while chemokines are cytokines which have the capacity to recruit and activate leukocytes, mediating inflammatory reactions. Several factors, including inflammatory and anti-inflammatory cytokines and chemokines, regulate the development, migration, growth, survival and local activation of MCs (Fig. 1).

The allergic immune reaction can be mediated by IgE (hypersensitivity type I) or other non-IgE mechanisms. MCs can be activated not only by IgE and specific antigen, but also, some MC populations can be activated via innate mechanisms, for example TNF, IL-1, IL-33, stem cell growth factor/cKit, stem cell factors (SCF), complement C3a, C5a, LPS, pathogen-associated molecular patterns (PAMPs), endothelin-1 (ET-1), and venom components.

MCs are mediators of allergy and also atherosclerosis where they accumulate in perivascular tissue and atherosclerotic plaque. According to the published international scientific literature, current consensus holds that vitamin E has positive effects on mast cell-mediated disorders. However, vitamin E very rarely causes allergy and certainly does not cause MC activation. IgE generation is mediated by cytokines such as IL-4, -5, and -13 that are produced by T helper (T_H) 2 effector cells, leading to differentiation of B cells and plasma cells (16). Li-Weber *et al.* reported that vitamin E, by blocking transcription factor, inhibits IL-4 mRNA level which is important for the IgE isotype switching in B cells and the differentiation of naïve T cells to T_H2 (16).

MC Fc ϵ RI activation involves several cytoplasmatic proteins, such as tyrosine kinases, and recruitment of adaptor molecules, including PI3K/Akt, PLCgamma/Ca²⁺/PKC and Grb2/SOS/Raf-1/MAPK pathways. In humans, MCs can be classified into MC_T, which express high levels of the MC-specific protease tryptase but little or no chymase and MC_{TC}, which express both tryptase and chymase.

MCs express their *c-kit* receptor or Fc ϵ RI during the inflammatory responses. Activation of tyrosine-kinase receptor *c-kit* leads to MC proliferation and development of inflammatory reactions.

Inflammatory MCs possess high affinity receptor for immunoglobulin E (Fc ϵ RI), and SCF receptor *c-kit*, and they are capable of increasing inflammation by inhibiting some immunosuppressant cytokines, such as IL-10, IL-4 and IL-13, generated by regulatory T (Treg) cells which have a protective effect on the development of inflammation. Treg subsets CD4⁺CD25⁺, Foxp3⁺, T cells, called natural Tregs, are important mediators of immune homeostasis and promote the establishment and maintenance of

peripheral tolerance.

Vascular Cell Adhesion Molecule-1 (VCAM-1) is an important member of the immunoglobulin (Ig) superfamily of proteins which comprises several extracellular Ig-like domains and is involved in tissue inflammation. ROS and VCAM-1 are involved in lymphocyte migration and induction of several matrix metalloproteinases where vitamin E may have an inhibitory role (17).

Toll like receptor (TLR) microbial ligands and inflammatory cytokines stimulate and induce VCAM-1 expression by stimulation of TLRs (17). MCs express TLRs, including TLR-9 and TLR-3, which can be activated by several compounds, including bacteria. This activation leads to the release of several cytokines, such as IL-8, TNF, IFN- γ , IL-6, IL-33, IL-18 and IL-1, which induce

the secretion of inflammatory Cysteiny l aspartate-specific proteases (caspases), and can be activated by pathogens through the nuclear oligomerization domain signaling pathway. Caspases, expressed as a zymogen, are composed by three groups: i) the inflammatory caspases; ii) the initiator caspases; and iii) the effector caspases, which all have a conserved cysteine in the active site and possess proteolysis activity and cleave cytokine precursors.

Chemokines and vitamin E

Chemokines are specific for leukocyte cell types that express the chemokine receptors. Chemokines IL-8, MCP-1, MCP-3, MCP-4 and RANTES exert chemo-attraction, recruitment and infiltration of leukocytes during inflammation.

Our group and other authors reported that

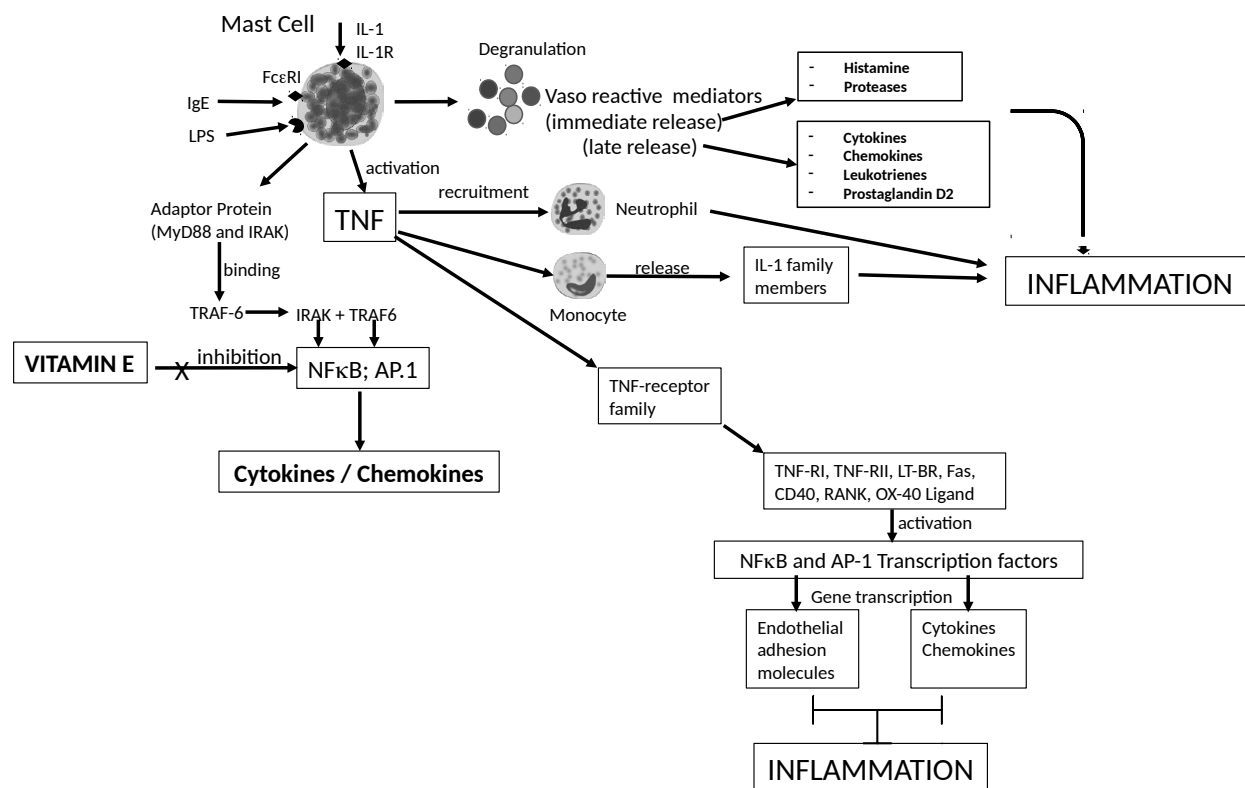


Fig. 1. Biochemical events of mast cell activation by IL-1. Mast cell network stimulated by the main activators, such as IgE and IL-1, causes the release of TNF which provokes the recruitment of inflammatory cells, such as neutrophils and monocytes, which release inflammatory cytokines and cause inflammation. In addition, the activation of mast cells leads to the production of NFκB and AP-1, which are involved in innate immunity, releasing cytokines and provoking inflammation. Abbreviations: LPS (lipopolisaccaride); TLR4 (Toll-like Receptor 4); TRAM (Transcription Mapper); TRIF (TIR domain-containing adaptor inducing IFN-beta; API (Activator protein 1); NFκB Nuclear Factor κB)

asthma, a chronic inflammatory disease of the lungs, is mediated by MCs which generate and release many inflammatory compounds: SCF, chymase and tryptase, TNF, and IL-33, which is an *alarmin* cytokine repeatedly identified as predisposing to asthma risk, allergy and inflammation.

Effects of vitamin E in some inflammatory diseases

Vitamin E has a free radical-neutralizing activity *in vitro* and *in vivo* and possesses anti-oxidative effects in the pathogenesis of allergic diseases, including asthma, by inhibiting nuclear NF- κ B level. In addition, vitamin E regulates ICAM-1 activation of PKC α but not the signal ERK1/2. However, α -tocopherol is an antagonist, and γ -tocopherol is an agonist of PKC α .

Research efforts in the past decade have highlighted that vitamin E reduces the clinical manifestations of asthma, atherosclerosis and coronary artery diseases, which are inflammatory diseases where MCs play an essential role (18). After activation, MCs have detrimental effects on the vessel wall, causing recruitment of inflammatory cells, matrix degradation, and apoptosis, all implicated in allergy and atherosclerosis. All these effects may be inhibited by vitamin E, contributing a benefit to the whole body.

Coronary artery disease patients have lower plasma concentrations of vitamin E than normal subjects. Therefore, vitamin E in human therapy has positive clinical effects with a significant reduction in cardiovascular diseases. Deficiency of vitamin E is found in several diseases and after medical treatments.

Vitamin E protects from LDL oxidation, reducing oxidized LDL. Moreover, vitamin E may have an inhibitory effect on vascular endothelial cells by reducing the expression of some pro-inflammatory cytokines/chemokines such as IL-8, adhesion molecules, and the adhesion of monocytes to the endothelial cells, improving vascular functions (19).

In addition, vitamin E decreases CRP, arachidonic acid cascade (5-LOX and COX-2, with consequent inhibition of leukotrienes and prostaglandins), tyrosin kinase and protein kinase. Therefore, vitamin E inhibits the aggregation reaction and arachidonic

acid metabolism. However, this essential vitamin has no effect on thromboxane A2 (TxA2) (detected as TxB2) generation, even in its aggregation power.

It has been also reported that in rheumatoid arthritis mediated by neutrophils, macrophages, lymphocytes and MCs, and inflammatory cytokines, such as IL-1, IL-6, TNF, IL-33, vitamin E and vitamin D exert an inhibitory effect and prevents joint damage.

Vitamin E low plasma levels are associated with elevated blood histamine levels and progress of cardiovascular diseases. Vitamin E, by inhibiting protein kinase C has been found to have an inhibitory effect on platelet adhesion, aggregation and function, and suppresses the augmentation of smooth-muscle cell. Vitamin E has an inhibitory effect on prostacyclin synthesis in human endothelial cells which is an anti-platelet aggregator and a strong vasodilator; while TxA2 has opposite effects (20).

In addition, vitamin E inhibits the expression of the scavenger innate immune receptors SR-A and CD36, which are essential for the generation of foam cells in atherosclerosis. Vitamin E may prevent the oxygenation of unsaturated fatty acids, and increase Ca²⁺ uptake, and it is able to inhibit histamine release generated in activated MC experimental animal models.

It is well known that endothelial cells express selectively specific cellular adhesion molecules which bind activated monocytes (macrophages), expressing in their surface cellular adhesion molecule E-selectin which can be inhibited by vitamin E, improving endothelial and vascular functions.

VEGF and IL-33

Vascular endothelial growth factor (VEGF) is one of the most important inducers of angiogenesis and exerts its cellular effects mainly by interacting with two high-affinity trans-membrane tyrosine kinase receptors. VEGF is released by human MCs which may be recruited by chemoattractants and secrete molecules as well as proteases (63) and inflammatory cytokines.

IL-33, or IL-1F11, is one of the newest inflammatory members of the IL-1 family, synthesized as a 31-kDa peptide precursor cleaved

by caspase-1 to generate mature cytokine IL-33 (64). This cytokine binds its receptor T1/ST2 (Toll-IL-1 receptor), an orphan member of the IL-1 receptor family, activating NF- κ B pathway, and MAP kinases which drive the production of Th2 cytokines (65-66). IL-33 activates the generation of PGD₂, an arachidonic acid product, and TNF and MCP-1 inflammatory proteins in MCs (25). We recently reported that IL-33 stimulates the release of VEGF in human MC line, and it is known to enhance experimental allergic inflammation by directly stimulating MCs to produce inflammatory cytokines (21).

Vitamins E, A, C and D

A diet rich in antioxidants, such as omega-6 polyunsaturated fatty acids (PUFAs), folate, vitamins A, C, and E, may be beneficial in the prevention of diseases mediated by MCs and could be of special value in the treatment of asthma and autoimmune disorders for which the routine immunosuppressives are contraindicated. Vitamin E, along with vitamins A, D, zinc, fruit, vegetables and n-3 long-chain polyunsaturated fatty acid supplementation, also prevents MC function in early childhood allergic disease in children at high risk of allergy and other atopic diseases. In addition, tocopherols are critical factors in the development of vascularization. In fact, vitamin E has been used to try to regulate inflammatory diseases that involve VCAM-1-mediated mast cell recruitment during allergic inflammation (22).

In conclusion, taken together, these observations suggest that increasing vitamin E intake may prevent inflammatory diseases by interfering with cytokine/chemokine transcription and translation proteins and other inflammatory MC compounds, which are potent mediators for inflammatory diseases, and allergic disorders where mast cells play an essential role (see illustration).

With regard to vitamin E in clinical studies of MC-mediated inflammatory disorders, this article sheds new light on the relationship concerning vitamin E, mast cells and inflammation, even though more studies are needed to draw and support firm conclusions on the role of vitamin E on MCs.

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RELATIONSHIP BETWEEN METHYLATION STATUS OF RASSF2A GENE PROMOTER AND ENDOMETRIOSIS-ASSOCIATED OVARIAN CANCER

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Relationship between the methylation status of the RASSF2A gene promoter and endometriosis-associated ovarian cancer (EAOC) was explored. Between January 2013 and January 2016, tissue samples were collected from 30 patients diagnosed with ovarian endometriosis cyst (EC group), 30 patients diagnosed with ovarian endometrial adenocarcinoma (OEA group) and 30 patients diagnosed with ovarian clear cell carcinoma (OCC group). Additionally, 30 cases of normal endometrium tissues were collected for the control group. The methylation status of the RASSF2A promoter was evaluated by combined bisulfite restriction enzyme analysis (COBRA). RT-PCR was used to detect the expression level of RASSF2A mRNA in tissues. Relationship between methylation status and RASSF2A mRNA expression level and the patient age, tumor clinical stage, tumor grading and pathological type were analyzed. Results showed that in the OEA and OCC groups, the methylation degrees of the RASSF2A promoter were obviously higher than that of the other two groups. The expression level of RASSF2A mRNA in the OEA and OCC groups was lower than that of the other two groups. The methylation degree of the RASSF2A promoter was related to clinical staging and grading. No relationship between the methylation degree of the RASSF2A promoter and patient's age and the pathological type of the tissue was detected. We concluded that the methylation status of the RASSF2A gene promoter could be considered an excellent indicator for early detection of ovarian cancers.

Endometriosis (EM) is one of the most common benign gynecologic diseases, and its prevalence in childbearing women is about 7-15% (1). EM can also manifest some malignant behavior, such as adjacent tissue adhesion, invasion and destruction, local and distant metastases (2). Ovaries are the most commonly engrafted sites for EM. The malignant tumor of ovarian EM is called endometriosis-associated ovarian cancer (EAOC). EAOC meets the following requirements: (i) cancer tissue is coexisting with EM and they are histologically

relevant; (ii) other types of tumor can be ruled out; and (iii) malignant transformation of EM can be microscopically observed (3). Research has confirmed that ovarian endometrial adenocarcinoma (OEA) and ovarian clear cell carcinoma (OCC) are the main histopathological subtypes of EAOC (4). Epidemiological studies have shown that EM is significantly correlated with EAOC (5).

Several genetic events are involved in the process of EM malignant transformation, such as loss of heterozygosity (LOH) on chromosome and gene

Key words: RASSF2A gene, promoter methylation, endometriosis-associated ovarian cancer

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mutation, including LINE-1, ARID1A, BAF250a and PIK3CA mutation (6). Prior studies showed that epigenetic modifications such as DNA methylation, genomic imprinting, histone modifications and RNA editing play an important role in the tumorigenesis (7, 8). Among these epigenetic modifications, DNA methylation had the highest incidence rate. It has been established that the inactivation of tumor-suppressor gene is linked to the abnormal methylation of CpG island in the promoter region (9). RAS proteins play an important role in regulation of tumor cell proliferation, differentiation and apoptosis (10). Proteins containing RAS-related domains are called RASSF family, and are significantly important negative factors that activate the related signaling pathways (11). Currently, a total of ten RASSF subtypes are known, and it has been shown that nine of them are downregulated or silenced in a variety of malignant tumors (12). Previous studies reported that RASSF2A gene expression in gastric cancer, colon cancer, ovarian cancer, pancreatic cancer and Ewing's sarcoma tumors is lower than normal (13, 14). Few studies have been carried out regarding RASSF2A gene expression and the relationship between the methylation status of the RASSF2A promoter and EOAC. In the present study, the relationship was investigated between the methylation status of the RASSF2A gene promoter and the endometriosis associated ovarian cancer (EAOC).

MATERIALS AND METHODS

Between January 2013 and January 2016, tissue samples were collected from 30 patients diagnosed with ovarian endometriosis cyst (EC), 30 patients diagnosed with ovarian endometrial adenocarcinoma (OEA) and 30 patients diagnosed with ovarian clear cell carcinoma (OCC). Additionally, 30 cases of normal endometrium tissues were collected as control. Diagnosis was confirmed by the pathological results. Neo-adjuvant chemotherapy was performed, and GnRH-a, sex hormones or antibiotics were used within 6 months before surgery. Patients' ages ranged from 35-67 years (average=45.7±12.3 years). All the patients or their families signed the informed consent. The study was approved by the ethics committee of Ningbo Women and Children's Hospital.

Research methods

The methylation status of RASSF2A promoter in the tissue samples was detected by combined bisulfite restriction enzyme analysis (COBRA). RT-PCR was used to detect the expression level of RASSF2A mRNA in tissues. The relationship between these results and the patient age, tumor clinical stage, tumor grading and pathological type was analyzed.

COBRA method

DNA samples were processed by sodium bisulfate before the target fragment was amplified by PCR. PCR products were digested by restriction enzyme, which could specifically recognize sequences rich in CG. When C residues in the recognition sequence were methylated (5mCG5mCG), PCR amplification retained the CGCG sequence, which could be recognized and cut by the restriction enzyme. In the absence of methylation of C residues in the recognition sequence, PCR amplification would transform it into TGTG, making the sequence undetectable by the restriction enzyme. Electrophoresis was conducted followed by probe hybridization and scanning quantitation for the enzyme digestion products.

Reagents and instruments: DNA mini preparation kit, PCR products purification kit (SK1261), and TaqDNA polymerase were bought from SIGMA (St. Louis, MO, USA). EZDNA Methylation Gold™ Kit was from R&D (Minneapolis, USA). PCR amplification instrument was from Santa Cruz (CA, USA); 3730 sequences analyzer was bought from Invitrogen (Carlsbad, CA, USA). DYY-8 voltage-steady and flow-steady electrophoresis apparatus was bought from GE (Fairfield, Connecticut, USA) and Gel imaging system was bought from Media Cybernetics corporation (Washington, USA).

DNA extraction

Tissue samples were placed in EP tubes, and 1200 µl xylene was added into each tube and mixed well. Samples were incubated at 60°C for 3 h before dewaxing. Samples were centrifuged at 3000 g for 10 min, and 1200 µl anhydrous ethanol was added into each tube and mixed well. Samples were centrifuged at 3500 g for 5 min and dried at 37 for 30 min. All steps were performed according to the instructions of the manufacturer.

PCR amplification

RASSF2A sequence was obtained from the Gene Bank, and Primer 3.0 software was used to design primers. Primers were synthesized by Shanghai Sangon Company, (Table I). Amplification system: DNA template 1 μ l + 10X buffer 2.5 μ l + 2.5 mmol/L dNTP 0.5 μ l + upstream primer 0.5 μ l + downstream primer 0.5 μ l + 25 mmol/L $MgCl_2$ 2.0 μ l + 10 $\times$ Taq polymerase 0.2 μ l, and water was added to reach a total of 25 μ l. Reaction conditions: reactions were repeated conducted under 95°C for 3 min, 55°C for 25 s, 94°C for 25 s, and 72°C for 30 s for 35 cycles and the production was extended at 72°C for 5 min.

Enzyme digestion detection of PCR products

Reaction system: PCR products 10 μ l + enzyme 10X buffer 1.5 μ l + 10 U/ μ l restriction enzyme 0.1 μ l + dH_2O 3.5 μ l, a total of 15 μ l. This system was incubated at 37°C overnight, and 3% agarose electrophoresis was performed to detect enzyme products. Sequencing verification: 20% of products were randomly selected for sequence verification that was conducted by Shanghai Sangon Company.

RT-PCR

Total RNA was extracted using the Trizol method, and ultraviolet spectrophotometer was used to detect the concentration and purity of RNA. Agarose gel

electrophoresis was used to determine the integrity of RNA. The reverse transcription process was completed with the TaKaRa PrimeScript™ 1st Strand cDNA Synthesis Kit. Primer sequence (Table I). Reaction system: Taq polymerase Mix 10 μ l + upstream primer 1 μ l + downstream primer 1 μ l + cDNA 2 μ l + RNAase-free water 6 μ l, a total of 20 μ l. Reaction conditions: Pre-degeneration was performed at 95°C for 3 min, degeneration at 94°C for 30 s, annealing at 57°C for 30 s, and extending at 72°C for 30 s. All these steps were repeated for a total of 35 cycles, and the production was extended at 72°C for 5 min. The procedure was terminated at 4°C. Agarose gel electrophoresis was performed with a voltage of 100 V, and maintained for 28 min. The electrophoresis results were observed with gel imaging instrument (FOTODYNE, USA), and results were shown using $2^{-\Delta\Delta Ct}$.

Statistical analysis

We used SPSS20.0 software for statistical analysis. Measurement data were expressed as mean $\pm$ standard deviation. For comparisons among groups one-way ANOVA was used. LSD-*t* test was used for comparisons between two groups. Enumeration data were expressed as cases or percentage. For comparisons among groups, *chi*-squared test was used. $P < 0.05$ meant that the difference was statistically significant.

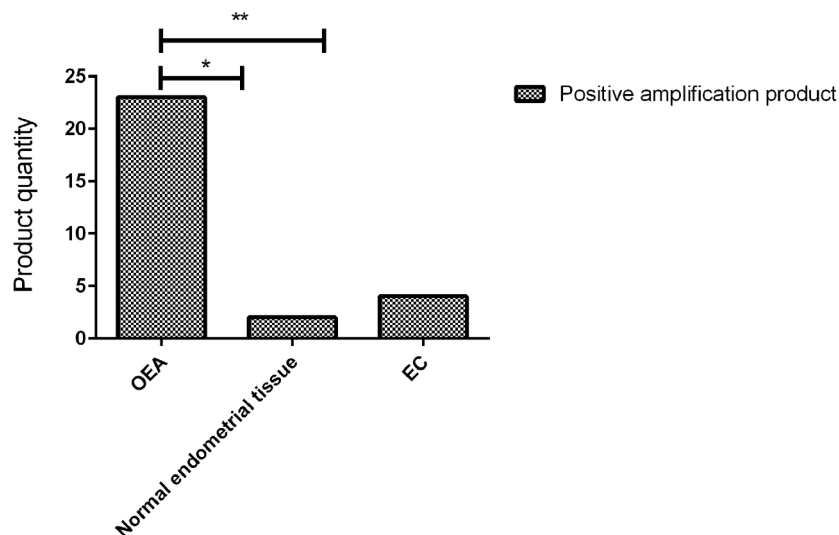


Fig. 1. Comparison of methylation degrees of the RASSF2A promoter between OEA group and normal endometrial tissue, EC group. ** $p < 0.01$, * $p < 0.05$

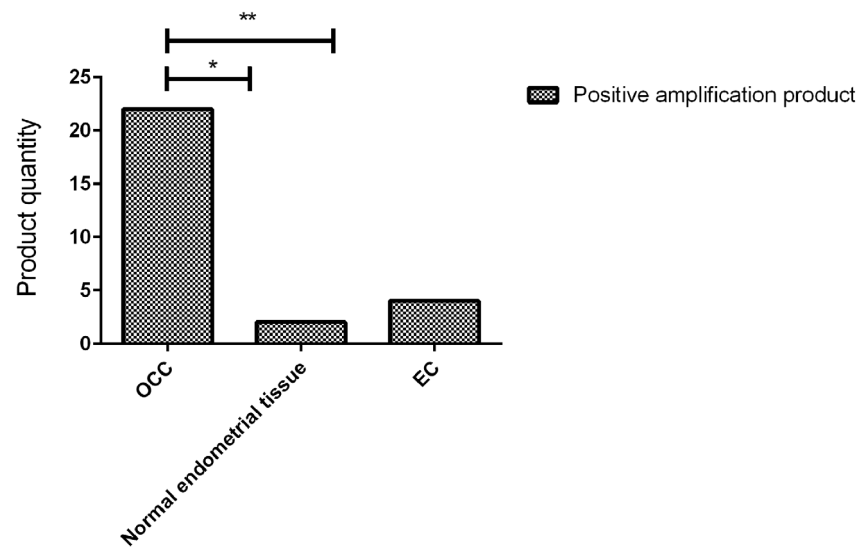


Fig. 2. Comparison of methylation degrees of the RASSF2A promoter between OCC group and normal endometrial tissue, EC group. ** $p<0.01$, * $p<0.05$

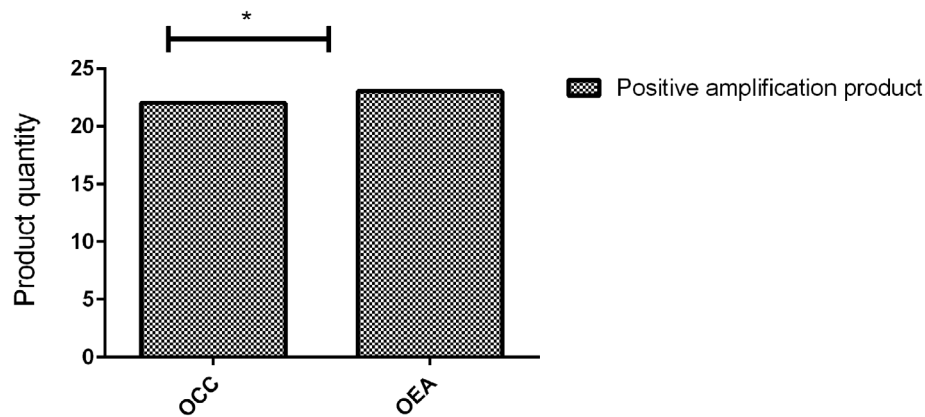


Fig. 3. Comparison of methylation degrees of the RASSF2A promoter between OEA group and OCC group. * $p>0.05$.

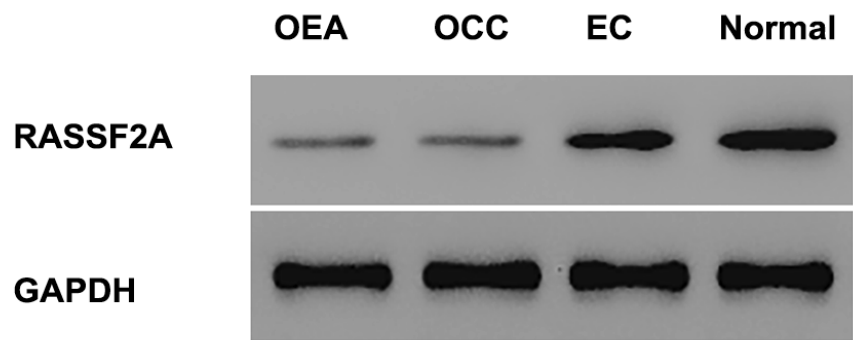


Fig. 4. Comparison of RASSF2A promoter mRNA levels between groups.

RESULTS

Comparison of methylation degrees of the RASSF2A promoter between groups

The methylation degrees of the RASSF2A promoter in OEA (76.67%, 23/30) was higher than that of normal endometrial tissue (6.67%, 2/30) and OCC (6.67%, 2/30) groups ($p < 0.05$, $\chi^2 = 30.24$; $P < 0.05$, $\chi^2 = 24.31$). Compared with the OCC group (73.33%, 22/30), the methylation degrees of the RASSF2A promoter in normal endometrial tissue (6.67%, 2/30) and EC groups (13.33%, 4/30) were significantly lower ($P < 0.05$, $\chi^2 = 27.78$; $P < 0.05$, $\chi^2 = 21.99$). The difference of methylation degrees of the RASSF2A promoter between in the OEA group and OCC group were not statistically significant ($P = 0.889$, $\chi^2 = 0.765$) (Table II, Figs. 1-3)

Comparison of RASSF2A promoter mRNA levels between groups

The expression level of RASSF2A mRNA in OEA (0.1234 ± 0.0635) and OCC (0.1425 ± 0.0724) were significantly lower than that of the EC groups (0.7249 ± 0.1424) and the normal endometrial tissue group (0.7659 ± 0.1236). The difference was statistically significant ($F = 46.535$, $P < 0.001$) (Fig. 4).

Relationship between methylation degree of the RASSF2A promoter and clinical features of patients in EAO group

The methylation degree of the RASSF2A promoter demonstrated no relation to patient's age and the pathological type of the tissue. On the other hand, relationship was established between

Table I. Primer sequence.

Gene	Forward primer	Reverse primer
Methylation primer	5'-GTTCGTCGTCGTTTTTTAGGCG-3'	5'-AAAAACCAACGACCCCCGCG-3'
Non-Methylation primer	5'-AGTTTGTGTTGTTGTTTTTAGGTGG-3'	5'-AAAAAACCAACAACCCCCACA-3'
RASSF2A mRNA	5'-GCGCCTAGAACGTGTTTTTC-3'	5'-ACTAGGCGTCCTCACATTGC-3'
GAPDH	5'-TGGGTGTGAACCACGAGAA-3'	5'-GGCATGGACTGTGGTCATGA-3'

Table II. Methylation degrees of the RASSF2A promoter.

Group	Ovarian specimen	Positive amplification product (n)	Positive ratio (%)
OEA	30	23	76.67%
OCC	30	22	73.33%
Normal endometrial tissue	30	2	6.67%
EC	30	4	13.33%
χ^2			52.208
P			0.001

Table III. Relationship between the methylation status of RASSF2A promoter and the clinical features in EAOc patients [cases (%)].

	Cases	Methylation (+)	Methylation (-)	χ^2	P
Age $\geq$ 45	28	22(78.57)	6(21.43)	0.357	0.550
Age<45	32	23(71.88)	9(28.13)		
OEA	30	23(76.67)	7(23.33)	0.089	0.766
OCC	30	22(73.33)	8(26.67)		
I~II stages	38	26(68.42)	12(31.58)	4.429	0.035
III~IV stages	22	19(86.36)	3(13.64)		
Poor differentiation	17	15(88.24)	2(11.76)	12.008	0.002
Median differentiation	28	24(85.71)	4(14.29)		
Well differentiation	15	6(40.00)	9(60.00)		

the methylation degree of the RASSF2A promoter and clinical staging and grading (Table III).

DISCUSSION

There are three subtypes of RASSF2 gene, including RASSF2A, RASSF2B and RASSF2C. CpG islands have been identified only in the promoter region of RASSF2A (15). RASSF2A can tightly bind to K-Ras in a GTP-dependent manner, but bind loosely to H-Ras (16). It has been shown that methylation can take place even before the occurrence of tumor (17). Compared with the multiple mutations in the exon regions, changes in the CpG island of gene promoter region markedly decrease the detection ranges (18). Results obtained from previous studies showed that the methylation status and methylation degree were closely associated with a variety of biological behaviors of tumors, affecting the therapeutic effects and survival outcomes (19). The methylation level of RASSF2A influences the process of gene transcription, translation and expression (20). A host of studies have confirmed that the inactivation of gene transcription mediated by the methylation of RASSF2A promoter region is a frequent event in the malignant tumors (21). The methylation of the RASSF2A promoter is involved in the occurrence and development process of a variety of solid tumors, including ovarian cancer. Since the

DNA methylation is reversible, the methyltransferase inhibitors can reverse the high methylation status of tumor suppressor genes and inhibit the tumor growth. Currently, 5-Aza-2'-deoxycytidine (5-aza-dC) is one of the most commonly used nucleoside methyltransferase inhibitors, which can effectively inhibit the growth and invasion of tumor cells (22). As RASSF2A is the most commonly methylated tumor suppressor gene in epithelial ovarian cancer, it can be used as a good molecular indicator in the diagnosis and treatment of ovarian cancer.

There are some limitations to this study. Our study did not explore the effect of RASSF2 on ovarian cancer. The deficiency of the specimen and the regional difference may be biased on the results of the experiment. Furthermore, in this study, we used neoadjuvant chemotherapy and other treatments, and do not know whether the results would have been affected. The digestibility of the digestive enzyme can only get the specific methylation status of the enzyme cut site, which has a high specificity. The negative detection can not exclude the possibility of methylation in the sample DNA. Therefore, in future research, a larger number of specimens needs to be collected and strict inclusion criteria should be customized, which can more effectively increase the accuracy and representativeness of the experimental results.

The results obtained from this study showed that

the methylation degrees in the OEA and OCC groups were obviously higher than those of the other two groups, while the mRNA expression levels were significantly lower than those of the other two groups. Tumors with higher tumor TNM staging and lower differentiation level had the higher degree of tumor malignancy, with a higher RASSF2A methylation degree. RASSF2A mRNA expression level in these tumors was lower than normal. These results suggest that the methylation status of RASSF2A gene promoter was closely related to EAOC. We conclude that the methylation status of the RASSF2A gene promoter could be considered an excellent indicator for early detection of ovarian cancers.

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IMMUNOMODULATORY EFFECTS OF T HELPER 17 CELLS AND REGULATORY T CELLS ON CEREBRAL ISCHEMIA

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The aim of the present study was to analyze the relationship between cerebral ischemia and immune effects. A total of 70 Kunming mice were randomly divided into two groups: a model group (60 mice) and a sham group (10 mice). The model group was divided into six subgroups (10 mice per group) which were categorized according to the following time periods of treatment: 6 h, 12 h, 24 h, 48 h, 72 h and 5 days. The temporary middle cerebral artery occlusion (tMCAO) mouse model was established using intracavitary suture. The degree of brain injury was evaluated by detecting the neurological deficit score (NDS). Following cerebral ischemia reperfusion, the edema of the brain tissue was aggravated, and the infarction area was increased. At 48 h, the volume of the cerebral infarction reached a peak ($44.4 \pm 3.2\%$) and then it decreased. The NDS score gradually decreased, and the nerve function was gradually restored. At 6 h, the NDS score was 4.6 ± 0.55 , whereas at the 5 d time point, it was significantly decreased ($P < 0.05$) to 2.2 ± 0.45 . Flow cytometry analysis indicated that the percentage of Th17 cells increased gradually following ischemia. At 24 h, the percentage of Th17 cells reached its maximum value ($0.70 \pm 0.10\%$) compared with the sham and the 5 d groups ($P < 0.05$). At 24 h, the percentage of Th17 cells reached the lowest value ($0.9 \pm 0.29\%$), whereas at the 5 d time point it increased significantly ($3.2 \pm 0.49\%$) compared with the normal level ($P < 0.05$). The secretion of Th17 and Treg-associated cytokines was consistent with the number of Th17 and Treg cells following ischemia. However, the levels of IL-17A in the brain tissues and the serum indicated a tendency to increase following the prolongation of ischemia. This marker reached the maximum levels on day 5. The IL-17 brain level was $77.9 \pm 5.11 \text{ pg/mL}$, whereas the serum level was $29.44 \pm 3.06 \text{ pg/mL}$. The changes in the secretion of the Th17 and Treg-related inflammatory cytokines were consistent with the changes in the cell ratio of Th17 and Treg cells. A significant correlation was noted between the two groups and the degree of ischemic brain injury. The results suggested that the functional status of Th17/Treg cells was imbalanced following cerebral ischemia.

Cerebrovascular disease is one of the diseases with high morbidity, disability and mortality; 75-80% of cerebrovascular diseases are cerebrovascular ischemic diseases (1). Immune injury following cerebral ischemia is associated with the involvement of immune cells and their cytokines, brain tissue, and the complement. All types of T lymphocytes

are usually at a state of equilibrium prior to cerebral ischemia. The onset of cerebral ischemia stimulates the transformation of the original Th0 cells into other T cells (2).

The inflammatory response occurs rapidly within a few hours following cerebral ischemia and includes local microglia, astrocyte activation and invasion

Key words: T helper cells, regulatory T cells, cerebral ischemia, immune response, cell function balance

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of macrophages and lymphocytes in the blood circulation that in turn aggravates the brain damage (3). Th17 cells are newly discovered lymphocyte subsets that exhibit distinct features from Th1 and Th2 cells. Th1 and Th2 cells specifically secrete IL-17 and play an important role in the development of autoimmune diseases and allergic reactions. Various studies have shown that Th17 cells are involved in ischemic brain injury (4).-

Treg cells are considered an important immunological class of regulatory cells in the body, which play an important role in the control of the inflammatory response and the maintenance of immune tolerance. In addition, the destruction of Th17/Treg cell balance is implicated in a variety of disorders (5). The present study aimed to investigate the association between the imbalance of the Th17/Treg cell ratio and the development of cerebral ischemic injury in a mouse model of transient cerebral ischemia.

MATERIALS AND METHODS

Reagents and instruments

The main reagents and instruments were the following: TTC (Sigma, United States); Paraformaldehyde(Tianjin Kemiou Chemical Reagent Co., Ltd.) DAPI (SANTA CRUZ, United States); Mouse lymphocyte separation solution (Tianjin Jin Mai, China); PMA(Sigma, United States); PE-IL-17, FITC-CD4 (eBioscience, United States); Treg detection kit (eBioscience, United States); Rat - anti - mouse Foxp3 and ROR γ t (eBioscience, United States); ELISA kit (Shanghai, Xitang, China); BCA Protein Quantification Kit (Jiangsu biyuntian, China); Brain stereotaxic device(Olympus, Japan); Laser Doppler cerebral blood flow monitor (Perimed, Sweden); Frozen slicer (Lycra, Germany); Fluorescence microscopy (Olympus, Japan); Electrophoresis (Bole, United States); Gel imager (Alpha, Britain); Continuous wavelength microplate reader(TECAN, Switzerland); Flow cytometry (BD company FACS Aria, United States).

Animals

A total of 70 Kunming mice were randomly divided into a model group and a sham control group. Forty-three mice (61.4%) were male, whereas the average age

of the mice was 41.3 ± 1.42 weeks; the average weight was 21.41 ± 4.28 g. All mice were acclimatized in the laboratory prior to experimental conduct, at $22 \pm 3^\circ\text{C}$ and $57 \pm 3\%$ humidity. The ratio of the number of mice in the two groups was 60:10. The model group was divided into six subgroups, 10 in each group, which were operated at 6 h, 12 h, 24 h, 48 h, 72 h and 5 d. The short right middle cerebral artery occlusion (tMCAO) model was established using a suture method. The study was approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University, China.

tMCAO model

The mice were anesthetized with an intraperitoneal injection of 8 g/L sodium pentobarbital ($M=0.08\text{g/Kg}$). The laser Doppler probe was placed in the middle of the cerebral artery supply cortex. It was located 2 mm from the front half tone point and 3 ~ 4 mm to the side of the middle seam. At this point, the measured cerebral blood flow that was proportional to the perfusion unit was set to 100% of the baseline value. The animals were sacrificed and the right common carotid artery, and the internal and external carotid arteries were separated. At the proximal end of the common carotid artery, an oblique incision was cut. The 6-0 nylon thread with the silicone end of the head was inserted via the internal carotid artery into the middle cerebral artery by oblique incision. The relative perfusion units of the cerebral blood flow were less than 20% of the baseline. The silk thread was fixed. The incision was simply sutured. Following 60 min of the suture, the thread was removed to restore blood perfusion. The cerebral blood perfusion was restored to more than 50%. The mice were maintained at 37°C during intraoperative conditions and anesthesia. The sham mice underwent the same method as the tMCAO model group with the exception that no thread was inserted.

Immunohistochemical staining

For histological analysis of mouse brains, the animals were sacrificed and the brains perfused with 0.9% saline solution followed by 4% paraformaldehyde in phosphate-buffer saline (PBS) (pH 7.4) as described in the experimental procedures. Brain tissues were paraffin embedded and cut into 10 μm sections. Following dewaxing in dimethylbenzene and graded alcohol, the

sections were placed in 3% H₂O₂ for blocking endogenous peroxidase activity and finally soaked in antigen retrieval buffer under high pressure conditions and heat. The sections were incubated for 1 h with blocking solution (5% rabbit serum), washed several times, and finally incubated overnight at 4°C with an antibody against IL-17 (1:200). The following morning the sections were rinsed, incubated for 2 h at room temperature with biotinylated rat-anti- mouse immunoglobulin G (IgG) (1:100) and stained with diaminobenzidinetetrahydrochloride (DAB). A negative control was performed by incubation in the absence of the primary antibody.

Foxp3 and ROR γ t protein Western blot detection

At each observation point, the mice were sacrificed in order to obtain brain, spleen and thymus tissues, and subsequently the tissues were rapidly placed in liquid nitrogen for long-term storage. The tissue was homogenized on ice and the protein extract was obtained. The samples were centrifuged and the supernatant was collected in order to determine the protein concentration. Each sample contained 50 μ g of protein during electrophoresis. Electrophoresis was performed with 5% and 12% SDS-PAGE. The protein was isolated and transferred to a Polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was blocked with 5% skimmed milk powder TBST solution for 1 h. Subsequently, the membrane was incubated with a rat anti-mouse Foxp3 (1:200) and/or a rat anti-mouse ROR γ t (1:200) overnight at 4°C. The following morning the membrane was washed with TBST 3 times for 10 min each time. An HRP-anti-rat secondary antibody (1:10,000) was added at room temperature for 2 h and the membrane was rinsed 3 additional times with TBST. The chemiluminescence kit (ECL) was used for protein detection and the bands were visualized in an ALPHA gel imager. Densitometry analysis software was used for the semi-quantification of the protein expression using β -actin and/or GAPDH as internal controls.

Neurological assessment

The neurological status of each animal was assessed by the Neurological Deficit Score (NDS). The assessment was conducted as follows: 0: No neurological dysfunction; 1: Inability to fully stretch the contralateral forepaws; 2: The contralateral foreleg grip was weakened during gentle pull of the tail; 3: Spontaneous movement in all directions

and opposite orientation of the animal during gentle pull of the tail; 4: Rotation and/or walk on the opposite side; 5: Walk only upon stimulation; 6: Weakened consciousness and no response to the stimulus; 7: Death

Statistical analysis

The results are expressed as the mean $\pm$ standard deviation. The neurological functional scores were compared using Kruskal-Wallis and Dunnett's tests. The remaining data were analyzed by SPSS19.0 software (IBM Company) using one-way ANOVA and LSD-*t* test. A *p*-value less than 0.05 (*p*<0.05) was considered statistically significant.

RESULTS

Gradual improvement in neurological function

With the prolongation of the reperfusion time, the neurological function indicated a trend of gradual improvement. On the 5th d (2.2 $\pm$ 0.45) the neurological function was significantly higher compared with that noted at 6 h (4.6 $\pm$ 0.55) (*p* <0.05). The neurological score was significantly lower compared with that of 6 h, whereas no significant difference was noted among the 12 h (4.2 $\pm$ 0.45), 24 h (3.6 $\pm$ 0.55), 48 h (2.8 $\pm$ 0.45), 72 h (2.4 $\pm$ 0.55) and 6 h time points (Fig. 1). No neurological deficit was reported in the sham group.

Immunology indicator changes following cerebral ischemia

The spleen mononuclear cells were analyzed by flow cytometry in order to observe the changes of Th17 and Treg cells following cerebral ischemia. The results indicated that a small amount of Th17 and Treg cells were found in the sham group following ischemia. The CD4 + IL-17 + / CD4 + ratio in the model group was increased 6 h following cerebral ischemia. At 24 h, the ratio reached the maximum value (0.70 $\pm$ 0.10%, *p*<0.05) compared with the sham and the 5 d groups, whereas it was decreased to the normal level on the 5th d (0.30 $\pm$ 0.10%). In contrast to Th17 cells, Treg cells (CD4 + Foxp3⁺) initially indicated a decreasing trend. At 24 h, they reached the lowest level (0.9 $\pm$ 0.29%) and subsequently they gradually increased. On the 5th d, the levels increased to similar levels as the normal group (3.2 $\pm$ 0.49%) (*P*

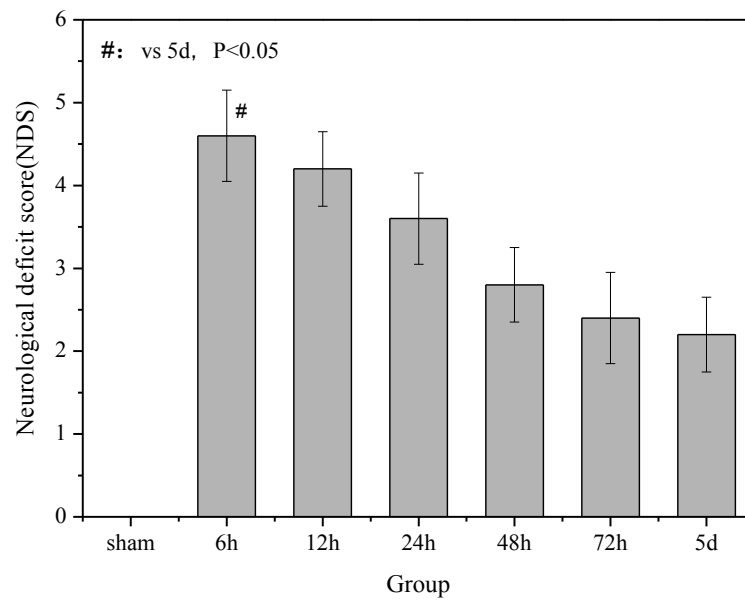


Fig. 1. Neurological function score.

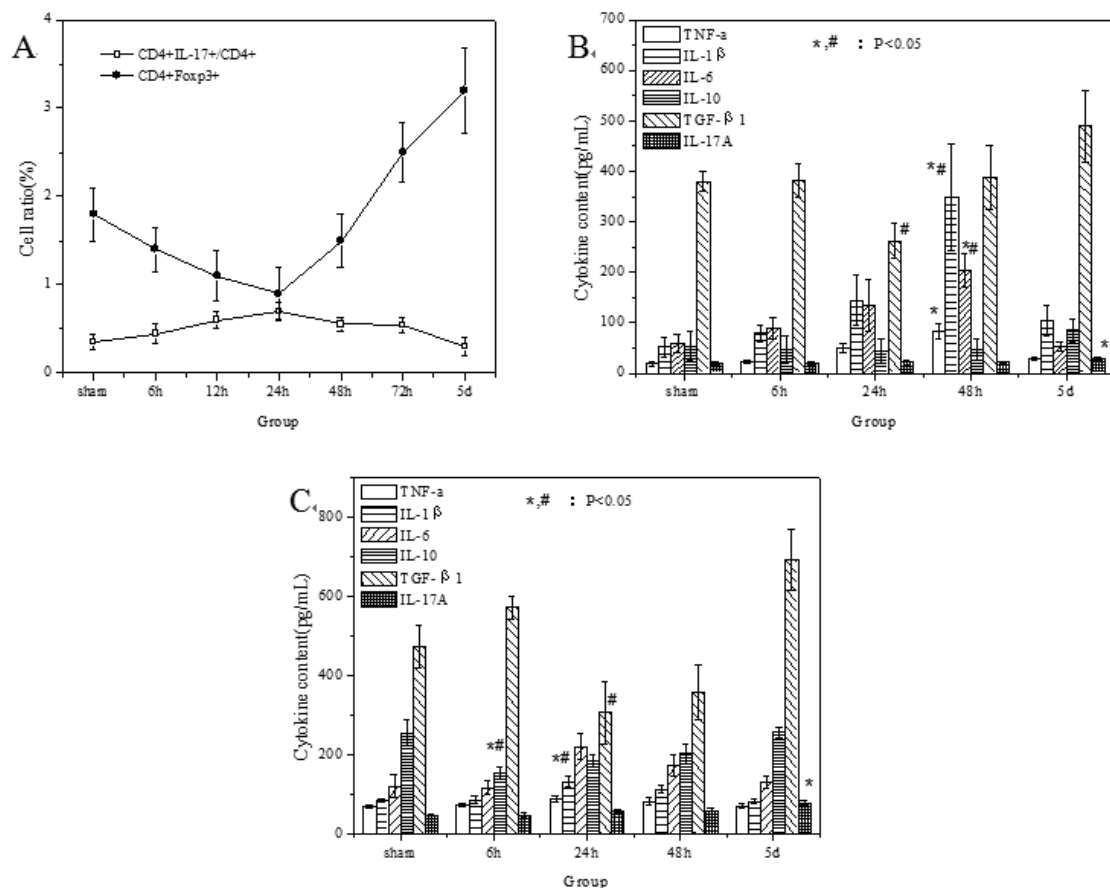


Fig. 2. Changes in immunological parameters following cerebral ischemia. **A)** Changes of the peripheral Th17 and Treg cell ratio following cerebral ischemia (*: CD4+IL-17+/CD4+ compared with CD4+Foxp3+, $p<0.05$; #: CD4+Foxp3+ compared with normal, $p<0.05$). **B)** The expression levels of Th17 and Treg-secreted cytokines in serum (*: compared with sham group, $p<0.05$; #: compared with 5 d group, $p<0.05$). **C)** The expression levels of Th17 and Treg-secreted cytokines in the brain tissue (*: compared with sham group, $p<0.05$; #: compared with 5 d group, $p<0.05$)

<0.05, Fig. 2A). This suggested that the balance in the ratio of Th17/Treg cells was affected following cerebral ischemia, and the early inflammatory response was apparent.

The differentiation and proliferation of Th17 and Treg cells were associated with cytokine secretion. Therefore, we examined the changes in the serum and brain-related cytokines, as shown in Fig. 2 B and C (* indicates $p < 0.05$ compared with the sham group; # indicates $p < 0.05$ compared with the 5 d group). The results indicated that the expression levels of the proinflammatory cytokines, namely TNF- α , IL-1 β and IL-6 that were associated with Th17 cells, were all increased in the early stage of cerebral ischemia. This may be related to the increase of brain injury that occurred in the acute stage, since the levels of the aforementioned cytokines were subsequently decreased. The levels of the inflammatory cytokines IL-10 and TGF- β 1 in Th17 cells were similar to those noted in Treg cells. In the early stage of cerebral ischemia, an initial decrease was noted, whereas subsequently the cytokine levels increased.

However, the expression of IL-17A demonstrated an inconsistent trend. Following cerebral ischemia, it gradually increased without decreasing. On the 5th d the levels of IL-17A peaked, which might be related to the secretion of IL-17A from other cells.

Foxp3 protein changes following cerebral ischemia

The changes in the expression levels of the Foxp3 protein were examined in the spleen tissues following cerebral ischemia. The results indicated a consistent trend with the Treg cells. The expression of Foxp3 was initially decreased at the 6 and 12 h time points and subsequently increased following 24, 48, 72 h and 5 d after cerebral ischemia (Fig. 3A). The results were significantly different only for the 12 h group compared with the control group (Fig. 3B). The expression of Foxp3 protein in the thymus was further examined. The expression of the ROR γ t protein was noted in the thymus, although the expression did not vary following the induction of cerebral ischemia.

Th17 invades the brain tissue

To further observe the direct relationship

between Th17 cells and brain injury, we analyzed the brain tissues by immunohistochemistry. Th17 cell invasion (IL-17⁺) was noted in the ischemic necrotic area, and no positive cells were present in the necrotic center. No positive cells were present in the contralateral normal brain tissue of the sham operated group. Concomitantly, IL-17A-positive astrocytes were noted in the ischemic side, which indicates that astrocytes may also secrete a small amount of IL-17A. The results are shown in Fig. 4.

DISCUSSION

Previous studies have shown that removal of Treg cells can promote and activate inflammatory T cells that results in increased expression of inflammatory factors IFN- γ (6). This protective effect is mainly mediated by IL-10. Recent studies have further confirmed this protective effect (7). In mice that were devoid of Treg cells, the area of cerebral infarction and neurological deficits were significantly increased, confirming that endogenous Treg cells play an important neuroprotective effect and this effect was more profound between 3 to 7 days following MCAO (8, 9). A lentiviral vector was used in order to interfere with the IL-23P19 gene expression in the model mice. The expression of Foxp3 protein in the subacute Treg cells was increased, whereas the volume of cerebral infarction was reduced and the neurological function was improved. Similar studies have further confirmed the increase in the number and/or function of Treg cells (10, 11). Notably, they have been shown to play a positive role in the treatment of cerebral ischemia and improve the prognosis of cerebral ischemia (10, 11).

We hypothesized that this is likely to be associated with the proinflammatory effects of Th17 cells and the anti-inflammatory effects of Treg cells (12). In the early stage of cerebral ischemia, the inflammatory response is activated. The Th17 subset is activated and the cells proliferate, and further activate additional inflammatory factors in order to participate in the inflammatory response. The induction of this process is confirmed by the increase in Th17 cells and the volume of cerebral infarction. In the later stages of cerebral ischemia, the number of Treg cells increases, indicating that

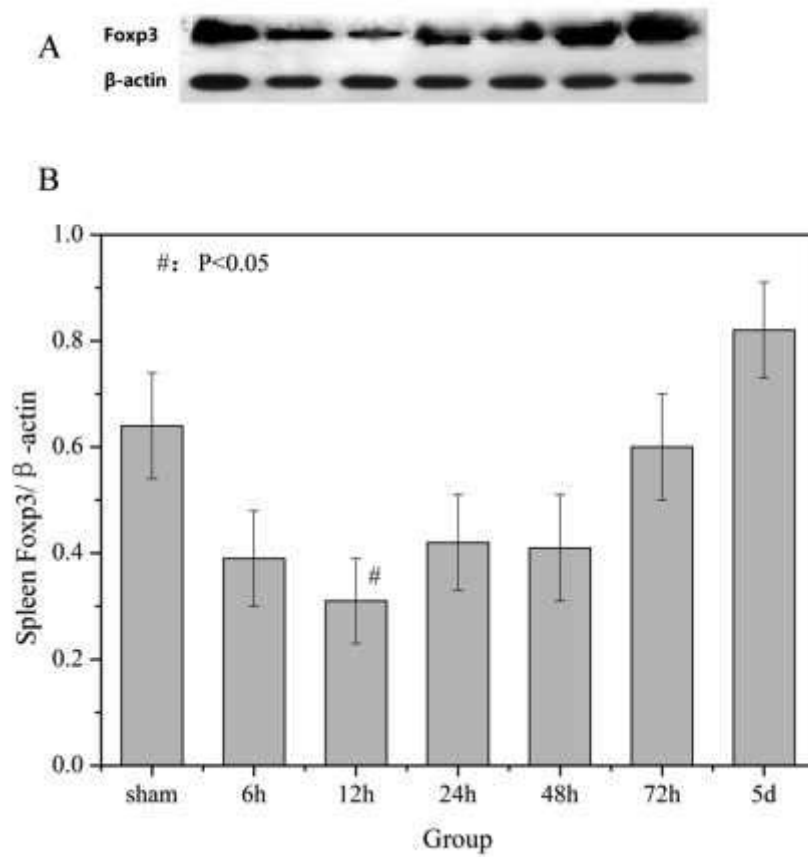


Fig. 3. The expression levels of the Foxp3 protein following the induction of cerebral ischemia. **A)** Western blot results of Foxp3 and β -actin. **B)** Quantitative analysis of Foxp3 in the spleen tissues.

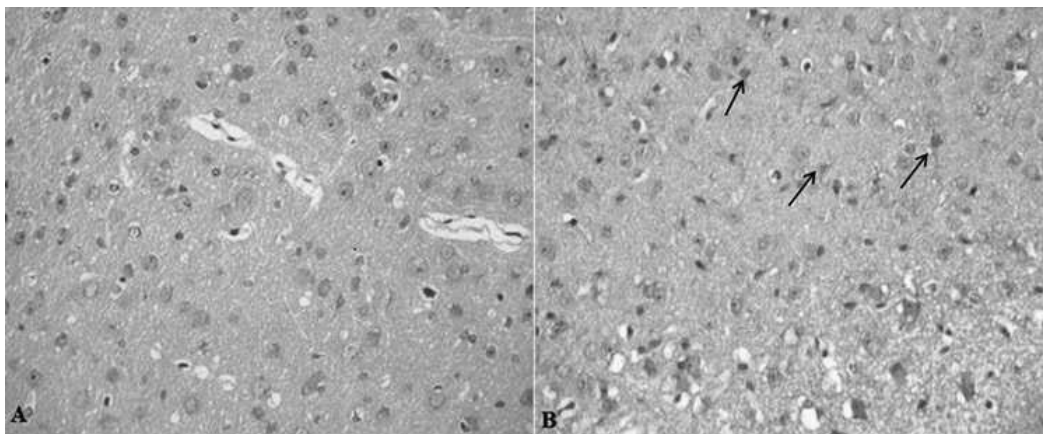


Fig. 4. Immunohistochemistry of IL-17+ cells in the brain tissue. **A)** Sham group. **B)** tMCAO group.

the primary T lymphocytes differentiate to Treg cells, while the stimulation of Th17 cells is inhibited. The inflammatory response is controlled and gradually weakened with the increase in the number of Treg cells. In the penumbra, the incomplete necrotic brain tissue was gradually restored, and the volume of cerebral infarction decreased (13, 14).

Immunohistochemistry revealed that Th17 cells infiltrated in the ischemic necrotic area of the brain tissues. Therefore, a disruption of the functional balance of the Th17/Treg cells may have occurred, following cerebral ischemia that was related to the extent of brain damage. This establishes an experimental basis for the next step of immunoregulation, and provides a theoretical basis for the further understanding of cerebral ischemic inflammatory injury.

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FOLLOW-UP OF PATIENTS WITH SYSTEMIC IMMUNOLOGICAL DISEASES UNDERGOING FATTY-DEGENERATIVE OSTEOLYSIS OF THE JAWBONE SURGERY AND TREATED WITH RANTES 27CH

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Regulated-on-activation, normal T cell expressed and secreted (also called RANTES, CCL5 or R/C) is a chemotactic cytokine that plays a key role in recruiting immune cells to inflammatory sites. R/C is involved in the pathogenesis of many systemic immune-mediated diseases (SIDs) and is upregulated in fatty-degenerative osteolysis jawbone (FDOJ) cavitations. Surgical cleaning of degenerative areas reduces the source of chronic R/C but might not be sufficient to reestablish the altered immunological patterns. The aim of the present study was to collect clinical data from patients suffering from SIDs who underwent dental surgery of FDOJ areas (n=46), by measuring R/C serum levels at the first visit (V0) prior to surgery, and at the second visit (V1). The majority of patients (n=41) were treated one month with ultra-low dose RANTES (27CH), a medicine used in micro-immunotherapy, while five patients were not. Mean and standard deviation of R/C serum levels at V0 in treated and untreated patients were respectively $48.5 \pm 25.8 \text{ ng/ml}$ and $42.48 \pm 22.22 \text{ ng/ml}$. Untreated patients had a tendency towards higher R/C levels at V1 ($68.36 \pm 30.7 \text{ ng/ml}$; $p=0.062$), while an opposite tendency was observed in treated patients ($40.9 \pm 20.3 \text{ ng/ml}$; $p=0.129$). Investigators observed that a cut-off set at 40 ng/ml at V0 seemed to be predictive of the efficacy of the dental surgery/treatment ($p=0.0013$, $n=26$) and that gender could influence R/C levels and patient's responsiveness. The Authors, being aware that this is a preliminary follow-up, wanted to lay the basis for forthcoming studies, in which a larger cohort of patients and well-defined inclusion/exclusion criteria will be established.

Regulated-on-activation, normal T cell expressed and secreted (also called RANTES, CCL5 or R/C) is a chemotactic cytokine that plays a key role in recruiting leukocytes, macrophages and eosinophils to inflammatory sites. Together with other inflammatory cytokines, R/C is implicated in the formation of inflammatory infiltrates. While most of the chemokines are expressed during the 'immediate early response', R/C is expressed 3–5

days after T-cell activation (1). R/C is involved in broad inflammatory features and in the pathogenesis of a variety of systemic immune-mediated diseases (SIDs), including cancer, atherosclerosis, neurological diseases, rheumatoid arthritis, diabetes, and Hashimoto's thyroiditis (2-4). SIDs cover a wide range of systemic disorders, directly and indirectly immune-related, in which immunological components, such as R/C and its receptors, can be

Key words: RANTES/CCL5, systemic immune-mediated diseases, fatty-degenerative osteolysis of jawbone surgery, micro-immunotherapy, ultra-low dose RANTES 27CH

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used as therapeutic targets. Indeed, therapeutic strategies aiming at decreasing R/C expression could be used to treat atherosclerosis (4), dysfunctions after transplantation (5) and autoimmune diseases (6, 7).

Jawbone cavitations (JCs) are jawbone areas characterized by dying or dead bone marrow. These fatty degenerative cavitations may be painful or, in some cases, can remain asymptomatic for years. JCs which are affected by fatty-degenerative osteolysis of the jawbone (FDOJ) may be distinguished by the overexpression of inflammatory mediators, and more specifically of R/C (8-10). The chronic R/C input might influence the immunological pattern and aggravate systemic immunological diseases (9, 10). Nowadays, the existence of FDOJ is largely ignored in mainstream dentistry. The reason is that conventional X-ray techniques have limited ability to visualize FDOJ areas (11) and, because of these diagnostic difficulties, jawbone disease is often underdiagnosed by dentists. To give the practitioner an aid to the diagnosis, a computer-assisted through-transmission alveolar ultrasound device was developed (12). Surgical cleaning of the FDOJ areas are currently aimed at reducing the source of chronic R/C input by removing the adipocytes from the inflamed JCs areas. However, the surgical removal of R/C source might not be sufficient to reestablish the altered immunological patterns, especially when patients suffer from immune-mediated diseases. In these patients, surgery could be improved by modulating the inflammatory pathways and, more specifically, by decreasing circulating R/C levels.

Immune system imbalances might be restored using a micro-immunotherapy (MI) approach. MI is a therapeutic strategy which uses immune regulators, including cytokines, in association with nucleic acids (plant-derived total DNA and RNA) and specific nucleic acids (SNA[®]). The active substances, prepared in ultra-low doses, are used in sequential formulas developed to treat different acute and chronic diseases. The use of RANTES 27CH, aims at reducing the altered levels of R/C and supports recovery after dental surgery of FDOJ areas. The goal of this follow-up is to evaluate the impact of RANTES 27CH in patients suffering from systemic immunological diseases, undergoing dental surgery

of FDOJ areas, by measuring R/C serum level before surgery (first visit, V0) and after one month of RANTES 27CH intake, at a second visit, V1.

MATERIALS AND METHODS

Patient characteristics

The research is based on data retrieved from 46 patients who underwent dental surgery, of whom 41 patients took the RANTES 27CH treatment for one month, while five did not. The patient characteristics are shown in Table I. All but three patients suffered from SID and chronic inflammation: chronic fatigue syndrome (CFS) (n=10), breast cancer (BC) (n=5), multiple sclerosis (MS) (n=5), rheumatoid arthritis (RA) (n=10), trigeminal neuralgia (TN) (n=8), etc. (Table I). Due to the different nature of SIDs and the treatment strategies, no exclusion criteria were listed. All patients gave their written informed consent to the surgery, the blood collection for further analysis and to medication intake. Blood samples were collected from all patients: before FDOJ surgery (first visit, V0) and after 20 to 526 days (second visit, V1).

Laboratory analysis

Blood samples were collected before surgery (V0) in S-Monovette 7.5-ml Z tubes, clot activator Silica (Fa. Sarsted AG & Co. Nümbrecht, Germany). Once collected, blood was swirled at least 2 times, then stored at 4°C and sent the same day to the laboratory that performed the analysis. The same procedure was carried out for treated and untreated patients at the second visit (V1). Measurement of RANTES/CCL5 in serum was performed using the Human Cytokine/Chemokine Panel I (MPXHCYTO-60K; Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions and analyzed using the Luminex[®] 200TM with xPonent[®] Software (Luminex Co, Austin, TX, USA). For measurement of RANTES/CCL5 in serum, samples were prediluted 1:100 in sample buffer according to manufacturer's instructions. To avoid dilution factor error, two control samples (low and high) were run in parallel and the two controls variance was calculated as well as the measurement fluctuations. The mean squared deviation for controls was below 10% (9.5% for low and 7.0% for high).

The analyses were carried out at the Institute for Medical Diagnostics, Berlin (inspected by DAKKS

[Deutsche Akkreditierungsstelle GmbH; accredited to DIN EN ISO/IEC 17025:2005 and DIN EN ISO 15189:2007]).

Treatment and dosing

RANTES 27CH is a micro-immunotherapy medicine manufactured by Labo'Life, and notified to the Belgian Federal Agency for Medicines and Health Product (FAMHP) under notification number 1507UH150F33. The medicine consists of lactose-saccharose globules impregnated with an ultra-diluted ethanolic preparation of RANTES, obtained by pharmacopeial dilution/succussion steps, reproduced 27 times. The globules, packed into capsules, are sublingually taken in the morning, on an empty stomach. The posology for the 41 patients was of one capsule per day, during one month.

Statistical analyses

Statistical analyses were performed using GraphPad Prism version 7.0. R/C serum levels at V0 were compared to post-treatment levels (V1) using paired 2-tailed Student's *t*-tests. If data were not normally distributed Wilcoxon matched pair test was preferred. The unpaired *t*-test was performed to analyze differences in RANTES

levels at V0 between men and women. 0.05 was considered as p-value threshold to assess statistical significance.

RESULTS

Of the five control patients who underwent FDOJ surgery without taking RANTES 27CH, all presented higher levels at V1 compared to V0. Due to the small number of patients, normality test failed and the difference came out as not statistically significant (Wilcoxon test, $p=0.062$, $n=5$). Mean and standard deviation were 42.48 ± 22.22 ng/ml at V0 and 68.36 ± 30.7 ng/ml at V1 (Fig. 1A). Analyzing R/C serum levels in patients treated with RANTES 27CH, an opposite effect was observed, with a tendency towards lower levels at V1 compared to V0 (Wilcoxon test, $p=0.129$, $n=41$). The mean and standard deviation at V0 and V1 were respectively $48.5 \text{ ng/ml} \pm 25.8$ and $40.9 \text{ ng/ml} \pm 20.3$ (Fig. 1B). In order to evaluate whether the time spent between the first and the second visit could influence results and conclusions of the follow-up, the patients were grouped in three windows of time, based on the number of days after the first visit (V0) as follow:

Table I. *Characteristics of patients.*

VARIABLE	FDOJ patients without RANTES 27CH (n=5)	FDOJ patients treated with RANTES 27CH (n=41)
Sex	M (n=2); F (n=3)	M (n=16); F (n=25)
Chronic fatigue syndrome (CFS)	M (n=0); F (n=0)	M (n=8); F (n=2)
Breast cancer (BC)	F (n=0)	F (n=5)
Multiple sclerosis (MS)	M (n=0); F (n=1)	M (n=1); F (n=3)
Rheumatoid arthritis (RA)	M (n=1); F (n=1)	M (n=4); F (n=4)
Allergy	M (n=1); F (n=0)	M (n=0); F (n=1)
Hashimoto's thyroiditis	M (n=0); F (n=0)	M (n=0); F (n=1)
Migraine	M (n=0); F (n=0)	M (n=0); F (n=1)
Trigeminal neuralgia (TN)	M (n=0); F (n=1)	M (n=3); F (n=4)
Osteoporosis	M (n=0); F (n=0)	M (n=0); F (n=1)
No SID	M (n=0); F (n=0)	M (n=1); F (n=2)
Days after V0 (V1)	35 - 160	20 - 526

between 20 to 50 days, between 51 and 80 days, and more than 80 days. Only in those patients having their follow-up visit (V1) within 50 days, a significant reduction of the levels of RANTES was observed at V0 compared to V1 (paired *t*-test, $p=0.034$, $n=15$) (Fig. 1C). When visit V1 occurred after 50 days, being between 51 and 80 days, and between 81 and 526, no significant changes were observed in serum R/C levels (data not shown). Findings suggest that FDOJ patients treated with RANTES 27CH, checked between 20 and 50 days post-surgery, responded as expected to the treatment and dental surgery.

Having listed the R/C concentration at V0 in ascending order, a cut-off was identified at 40 ng/ml; patients who had R/C serum values at V0 over 40 ng/ml responded as expected to the surgery and treatment with a reduction of their R/C levels at V1 (Wilcoxon test, $p=0.0013$, $n=26$). On the other hand, serum levels of the patients who had values at V0 below 40 ng/ml, increased (Fig. 2, A, B).

Considering that patients were treated for one month, they were further grouped into those having their R/C levels at V0 below and over 40 ng/ml,

based on the days passed after V0 to analyze the three windows of time: between 20 to 50 days, between 51 and 80 days, and more than 80 days. The increase of the R/C serum levels at V1, in patients with their levels at V0 <40 ng/ml, was not confirmed within the 50 days after V0, nor in the two other groups of patients (Fig. 2C). On the other hand, the responsiveness described in patients having R/C levels at V0 >40 ng/ml, was confirmed in the subgroup of patients who had their second visit within 50 days (paired *t*-test, $p=0.0057$, $n=10$) (Fig. 2D).

Because no significant differences exist between men and women in RANTES levels at V0 (unpaired *t*-test, $p=0.19$), nor in the days after the first visit (unpaired *t*-test, $p=0.23$), it was analyzed whether gender could influence the responsiveness to the treatment/dental surgery. As shown in Fig. 3A, the R/C levels in men at V1 were lower than levels at V0 (Wilcoxon test, $p=0.0083$, $n=16$), while no changes were observed in women. Again, the reduction of serum R/C levels was observed only in men having their V0 levels higher than 40 ng/ml, as shown in Fig. 3B (Wilcoxon test, $p=0.0002$, $n=13$). No changes

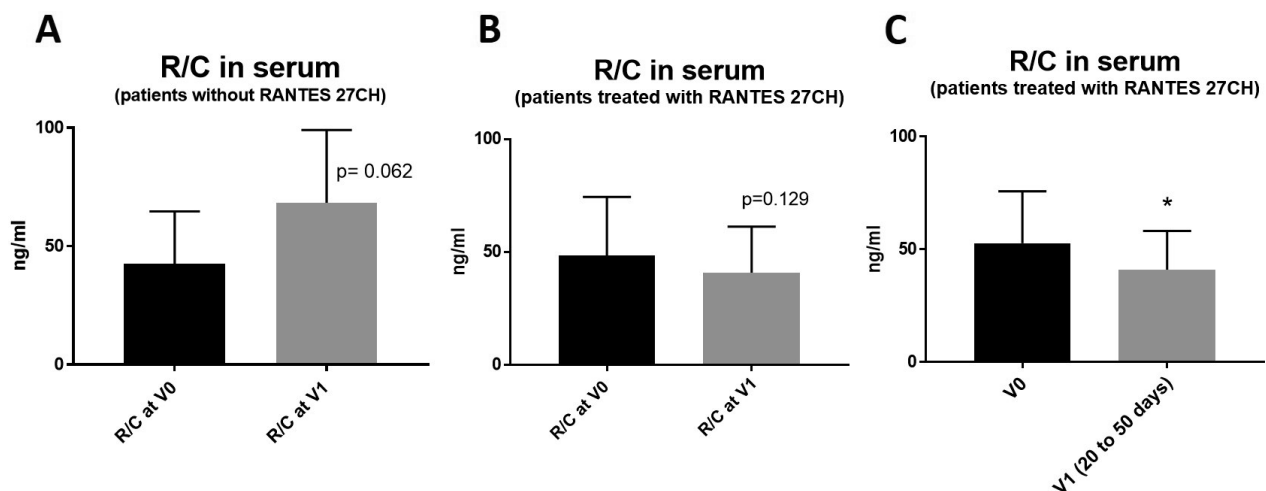


Fig. 1. RANTES levels in FDOJ patients treated or not with RANTES 27CH. **A)** R/C serum levels (ng/ml) measured in FDOJ patients at first visit V0, before surgery, and at second visit V1 ($N=5$). There is a trend towards higher R/C levels at V1 compared to V0. **B)** R/C serum levels (ng/ml) measured in FDOJ patients at first visit V0, before surgery, and at second visit V1, after one month of treatment with RANTES 27CH ($N=41$). There is a trend towards lower R/C levels at V1 compared to V0. **C)** R/C serum levels (ng/ml) measured in FDOJ patients at first visit V0, before surgery, and at second visit V1, performed within 50 days, after one month of treatment with RANTES 27CH. R/C levels decreased at V1 ($p=0.034$, $n=15$). Error bars represent standard deviation.

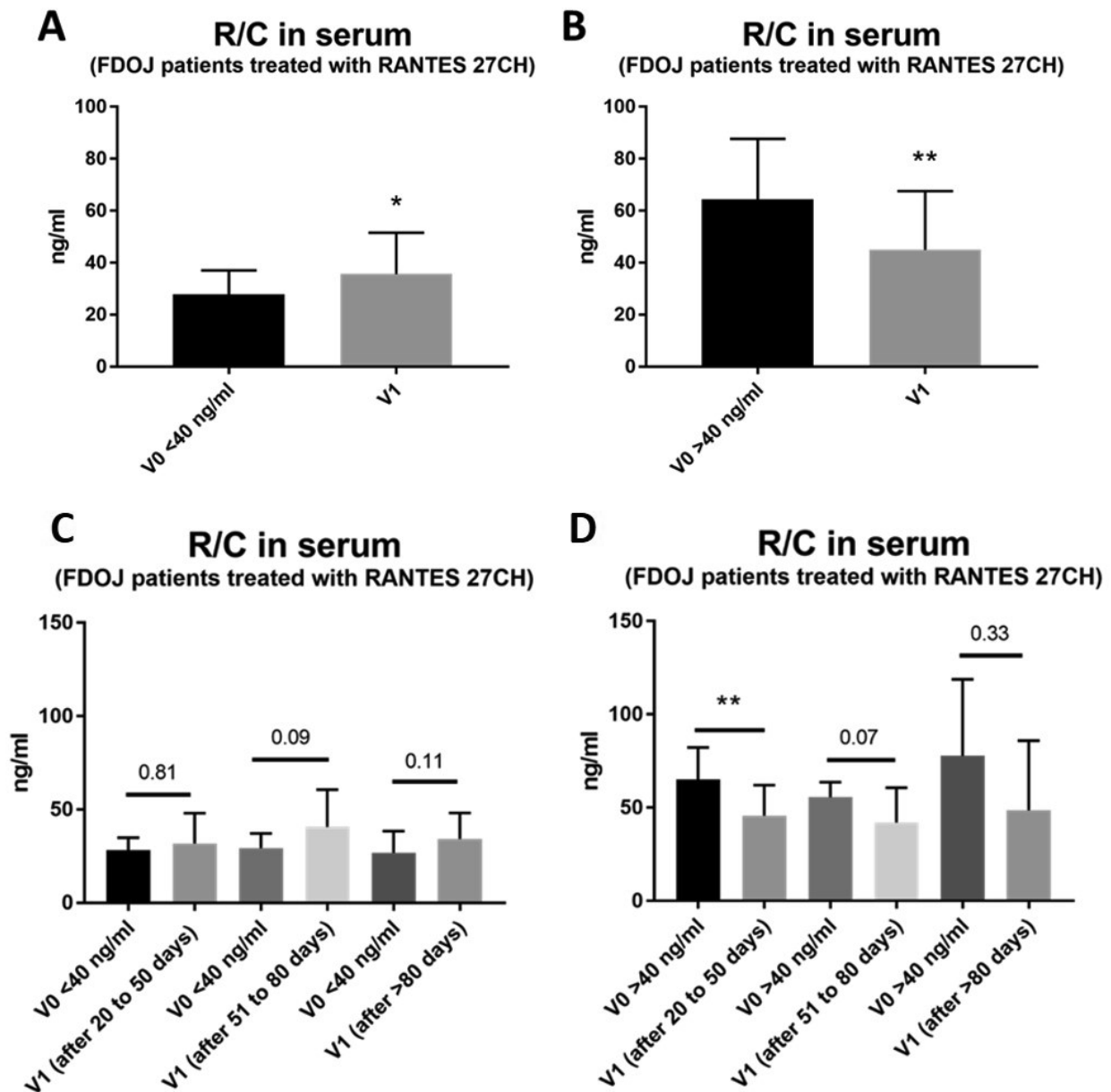


Fig. 2. Treatment response cut-off at 40 ng/ml at V0. **A)** R/C serum levels (ng/ml) measured in FDOJ patients having their levels below 40 ng/ml at first visit V0, and at second visit V1, after one month of treatment with RANTES 27CH. R/C levels increased at V1 compared to V0 ($p=0.017$, $n=15$). **B)** R/C serum levels (ng/ml) measured in FDOJ patients having their levels over 40 ng/ml at first visit V0, and at second visit V1, after one month of treatment with RANTES 27CH. Serum R/C levels at V1 decreased compared to V0 ($p=0.0013$, $n=26$). **C)** R/C serum levels (ng/ml) measured in FDOJ patients having their levels at V0 below 40 ng/ml treated with RANTES 27CH for one month, and having their second visit V1, within 50 days, between 51 and 80 days, and at more than 80 days. No significant changes were observed in R/C levels at V1 compared to V0. **D)** R/C serum levels (ng/ml) measured in FDOJ patients having their levels at V0 over 40 ng/ml, treated with RANTES 27CH for one month, and having their second visit V1, within 50 days, between 51 and 80 days, and at more than 80 days. Only in those patients having their V1 within 50 days, authors observed a reduction in serum R/C levels ($p=0.0057$, $n=10$). Error bars represent standard deviation.

were seen of circulating R/C levels between V0 and V1, in women patients grouped by R/C serum levels at V0: below or over 40 ng/ml (Fig. 3C).

Changes in R/C levels were also examined at V1 versus V0 by grouping the patients by gender and SID. As shown in Fig. 4, a tendency toward an R/C

level reduction at V1 compared to V0 was observed in BC patients (Wilcoxon test, $p=0.06$, $n=5$) and in male patients affected by CFS (paired t -test, $p=0.16$, $n=10$), TN (Wilcoxon test, $p=0.25$, $n=7$) and RA (Wilcoxon test, $p=0.21$, $n=8$). However, the small number and the heterogeneity of patients

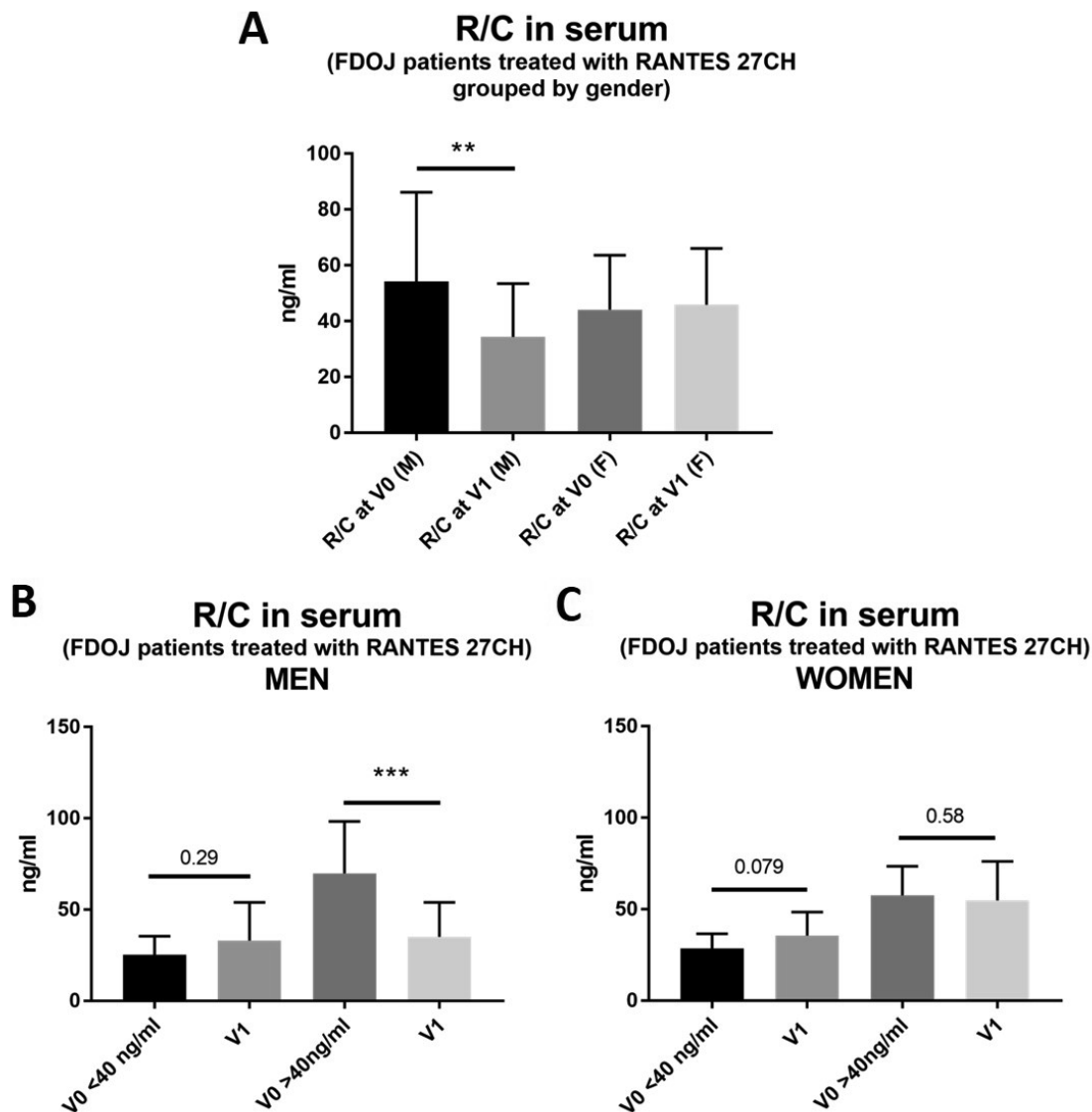


Fig. 3. Gender and cut off at 40 ng/ml at V0 were considered to group the treated patients. **A)** R/C serum levels (ng/ml) measured in FDOJ patients (men and women) treated with RANTES 27CH for one month. Men responded with lower levels at V1 compared to V0 ($p=0.0083$, $n=16$). No significant changes were observed in R/C levels at V1 compared to V0 in women. **B)** R/C serum levels (ng/ml) measured in FDOJ patients (men) treated with RANTES 27CH for one month, having their levels at V0 below or over 40 ng/ml. Only in those patients having levels >40 ng/ml, authors observed a reduction in serum R/C levels ($p=0.0002$, $n=13$). **C)** R/C serum levels (ng/ml) measured in FDOJ patients (women) treated with RANTES 27CH for one month, having their levels at V0 below or over 40 ng/ml. No significant changes were observed. Error bars represent standard deviation.

participating in the follow-up made it difficult to draw any conclusions.

DISCUSSION

Dental surgery allows the elimination of the local source of R/C, however, it may not be sufficient to reduce circulating levels of R/C, especially in those patients suffering from other pathologic conditions that can affect directly and/or indirectly chemokines and R/C expression.

In the described cohort of FDOJ patients, R/C levels were all higher than baseline levels reported by Biancotto et al. in healthy subjects. In the cited

study, the Authors analyzed serum levels of various cytokines, including RANTES, using a Bio-Rad 27-plex Luminex kit. Mean of RANTES in men and in women were respectively 9534.5 pg/ml (=9.5 ng/ml) and 11848.9 pg/ml (=11.8 ng/ml) (13). Quantification of cytokines varies significantly depending on sample collection procedures, commercial kit performance, other pre-analytical variables, and it is difficult to make a comparison among clinical studies. For those reasons, the present Authors are prudent in affirming that the circulating levels of RANTES found in FDOJ patients are higher than the healthy baseline levels.

In order to reduce the inflammation in FDOJ patients and, in particular, to decrease the circulating

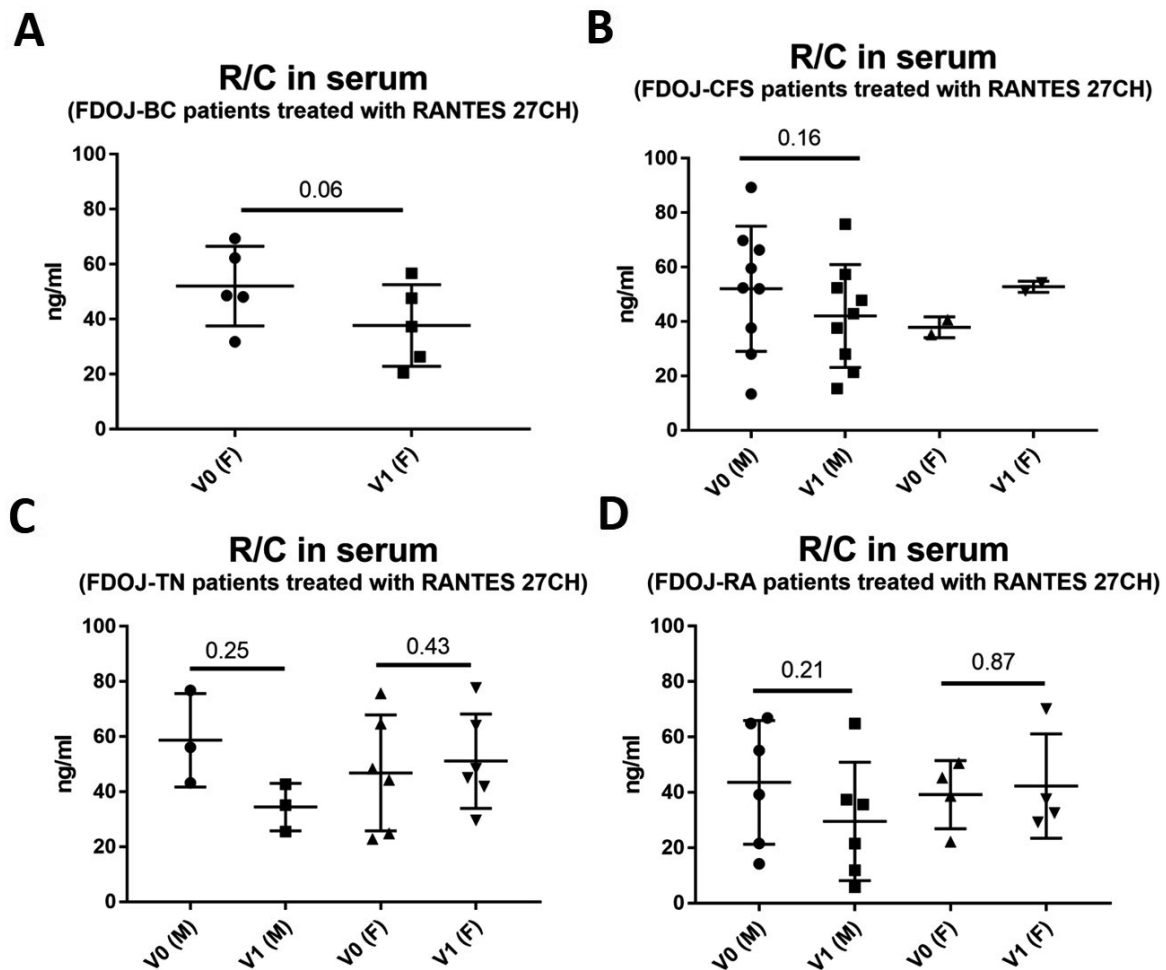


Fig. 4. Patients were grouped by gender and type of SID to check differences in treatment response. Scatter plot (mean and standard deviation) representing R/C serum levels (ng/ml) measured in FDOJ patients (men and women) treated with RANTES 27CH for one month, affected by (A) breast cancer (BC), (B) chronic fatigue syndrome (CFS), (C) trigeminal neuralgia (TN), and (D) rheumatoid arthritis (RA).

R/C level, most of the patients were treated during one month (n=41) with the ultra-low dose chemotactic cytokine RANTES 27CH, while five patients were not. The ultra-diluted medicine RANTES 27CH aims at reducing circulating levels of RANTES inducing a “hormetic response”. The term “hormesis” consists of the biphasic dose phenomenon, firstly observed by the pharmacologist and toxicologist Schulz, during his multiple chemical tests on yeast, and further studied by Calabrese (14). Hormesis is a cellular response that gives the ability to correct an altered homeostasis, adjusting to conditions that are optimal for cell survival; hormesis is the plausible mode of action of homeopathic medicines (15) and the possible mechanism of action of ultra-diluted medicines, including RANTES 27CH.

The five untreated FDOJ patients had their R/C serum levels increased at V1, while on the other hand, patients treated with RANTES 27CH tended to lower levels at V1 compared to V0. Analyzing differences in treatment responses, the Authors observed: i) a response cut-off at 40 ng/ml at V0; ii) that the patients having their second visit within 50 days responded as expected to the dental surgery/treatment with decreased R/C levels at V1 compared to V0 ($p=0.0057$, $n=10$); iii) that only men having their levels at V0 over 40 ng/ml had their levels decreased at V1 ($p=0.0002$, $n=13$). The gender difference could be attributed to the fact that in women, the difference in estrogen, progesterone, or both hormone levels, may have influenced R/C expression and, in turn, the surgery/treatment responsiveness.

In conclusion, this preliminary follow-up of SID patients who underwent FDOJ surgery, suggests that R/C serum level over 40ng/ml as well as gender could influence the responsiveness of the dental surgery followed by the anti-inflammatory medicine RANTES 27CH intake. These findings should be confirmed in a larger cohort of patients in which well-defined inclusion/exclusion criteria would be established.

Conflict of interest

JL has no conflicting commercial or financial interests. IF and BL work for Labo'Life, the pharmaceutical company that develops and commercialized micro-immunotherapy medicines,

including RANTES 27CH. This professional relationship does not influence the scientific neutrality in regard to this research.

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LETTER TO THE EDITOR

PROTECTIVE EFFECTS OF NANO-ELEMENTAL SELENIUM AGAINST CHROMIUM-VI-INDUCED OXIDATIVE STRESS IN BROILER LIVER

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The valuable role of selenium in mitigation of oxidative stress and heavy metal toxicity is well-known. Thus, the aim of the current study on broiler chickens was to examine whether nano elemental selenium (Nano-Se) supplementation can reduce the effects of chromium VI ($K_2Cr_2O_7$) toxicity. For this purpose, a total of 150, one-day-old broiler chickens were allotted to five groups with three replicates: control group (standard diet), poisoned group ($K_2Cr_2O_7$ via drinking water), protection group ($K_2Cr_2O_7$ + Nano-Se), cure group ($K_2Cr_2O_7$ for initial 2 weeks and then Nano-Se), and prevention group (opposite to the cure group). The broilers were detected by the activities of marker enzymes and oxidative stress markers including, aspartate aminotransferase (AST), alanine transaminase (ALT), gamma-glutamyl transferase (GGT) and superoxide dismutase (SOD), glutathione peroxidase (GSH-px), malondialdehyde (MDA), respectively. The $K_2Cr_2O_7$ administration caused histopathological damage in the liver of the chickens. Moreover, changes in serum biochemical indicators and oxidative stress parameters were also observed. Nano-Se supplementation increased the levels of GSH-px but reduced the activities of SOD, MDA, GGT, ALT and AST in the experimental groups ($P < 0.05$). Our results showed that Nano-Se plays a protective role by preventing the oxidative stress induced by the chromium VI in broiler chickens.

To the Editor,

Chromium (Cr) is considered as an essential trace element in animal and human bodies. It plays an active role in the metabolism of lipids, proteins, and carbohydrates. However, the two predominant forms, i.e. trivalent Cr and hexavalent Cr, of this mineral at high exposure can make it toxic metal (1, 2). Cr is also added to animal feed, in order to improve the meat quality and promote the development and growth of chickens (2). A series of *in vivo* and *in vitro* studies demonstrated that Cr can be poisonous and dangerous by inducing oxidative stress and

histopathological changes in the kidney and liver of chickens (2, 3). Nano-selenium (nano-Se) particles can provide an elevated effect of selenium compounds during biochemical activity and act as a co-factor that reduces the free radicals produced in metabolic processes by the antioxidant defense system with other antioxidants (4, 5). Dietary Se added to animals can restore the normal antioxidant capacity and decrease the inflammatory stress response through GPx (Se-containing enzyme), which is responsible for the reduction of organic and hydro peroxides in the presence of reduced glutathione (GSH) (2, 3, 6).

Key words: chromium, nano-selenium, oxidative stress, chickens

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Recently, nanoparticles have been extensively utilized in the livestock industry due to their exceptional characteristics, such as the large surface area with low cost depending on the type of particle (4), even though several studies have suggested the protective role of conventional Se and its antagonistic interactions against the toxic effect of different ions (3, 4). In addition, the effect of Se on brain oxidative stress induced by Cr (VI) has been studied in chickens (7). Despite the widespread use of nano-sized selenium (nano-Se) in broiler feed, very scarce information is available regarding its antioxidant mechanism. Therefore, we examined the biochemical and oxidative stress parameters including ALT, AST, GGT, SOD, MDA, and GSH-px to explore the effect of nano-Se on liver oxidative stress induced by $K_2Cr_2O_7$ (Cr, VI).

MATERIALS AND METHODS

Reagents

Potassium dichromate ($K_2Cr_2O_7$, purity $\geq 99\%$) and nano elemental selenium (purity $\geq 99\%$, an average size of 40 nm) were obtained from Macklin Biochemical Co. Ltd. Shanghai, China. Commercial assay kits and reagents including AST, ALT, GGT, SOD, MDA, and GSH-px and 2'-benzoyloxycinnamaldehyde (BCA) were bought from the Nanjing Institute of Biological Engineering, Inc. Jiangsu, China and Beyotime Institute of Biotechnology, China. All other chemical and reagents were provided by the Department of Clinical Veterinary Medicine, Huazhong Agricultural University Wuhan, China. Furthermore, all plastic and glassware materials were washed with distilled water and were rinsed by soaking them in diluted HNO_3 (1 %) prior to their use. All other reagents were of analytical grade.

Animal treatment and experimental design

All the experimental procedures were approved and conducted under the guidelines provided by the Institutional Animal Care and Ethics Committee of Huazhong Agricultural University, Wuhan, China.

A total of 150 Arbor Acres (AA) broiler chickens (1 day old) were purchased from a commercial poultry company (Wuhan, China). The broilers received standard

laboratory conditions (12:12 h light–dark period, relative humidity 50~60%, and temperature: $22\pm 1^\circ C$) with free access to commercial feed and water till the end of the experiment. The experimental room was cleaned regularly with disinfectant and all chickens were observed daily and their details were recorded. Notably, oral 50% lethal dose (LD_{50}) of $K_2Cr_2O_7$ was determined via acute toxicity experiments, as previously described by Horn (8) and the Nano-Se dose (0.5 mg/kg) used in the present study was utilized from previous research (9).

The birds were divided into five groups of 30 broilers (10 birds per cage): (i) the control group received standard diet and water (ii); the poisoned group received $K_2Cr_2O_7$ (8% LD_{50}) via drinking water and (iii) the protection group received both $K_2Cr_2O_7$ + nano-Se (nano-Se, 0.5 mg/kg via diet and $K_2Cr_2O_7$, 8% LD_{50}) till the end of experiment; (iv) The cure group received $K_2Cr_2O_7$ (8% LD_{50}) for the initial 2 weeks and then received nano-Se (0.5 mg/kg) till the end of experiment; and (v) the protection group received nano-Se (0.5 mg/kg) for the initial 2 weeks and then received $K_2Cr_2O_7$ (8% LD_{50}) till the end of experiment

The whole trial period was divided into three stages (1st stage~21 days, 2nd stage~28 days, and 3rd stage~35 days), and at the end of each period, 10 chickens per group were fasted overnight and placed on an electronic balance to record their body weight. The chickens were then euthanized (after intraperitoneal injection: 40 mg/kg body weight) by cervical dislocation and the livers were removed, cleaned, dissected, and weighed.

Histopathological analysis

The liver was extracted from the bodies of broilers following the necropsy and washed with ice-cold saline. Subsequently, the samples were embedded in paraffin, and cut into 4- μm thick sections, and stained with hematoxylin and eosin. Each sample tissue was examined by light microscopy.

Homogenates preparation and protein measurement

The broiler liver was removed, cooled in ice, and homogenized (Polytron homogenizer, Switzerland in a ratio of 1:9). To discard the cell debris, all homogenates were centrifuged for 10 min at 2500–3000 g. The achieved homogenate was instantly used for biochemical assays.

BCA protein assay kits (Beyotime Institute of

Biotechnology, China) were used to determine the protein content. For this purpose, 10 μL of the homogenate was mixed with 250 μL of the BCA working solution and incubated at 37°C for 30 min. Subsequently, a microplate reader was used to measure the protein content at 562 nm. The protein concentration was expressed as $\mu\text{g/mL}$.

Estimation of marker enzymes

Levels of different liver marker enzymes, such as AST, ALT, and GGT, in serum were determined by using IDEXX VET TEST 8008 Chemistry Analyzer (IDEXX Laboratories Inc., USA) with commercially available kits (Nanjing Institute of Biological Engineering, Inc. Jiangsu, China), as per the manufacturer's instructions.

Measurement of biomarkers of oxidative stress

The activities of SOD, GSH-px, and MDA assays were determined using commercially available kits according to the manufacturer's instructions (Nanjing Institute of Biological Engineering, Inc. Jiangsu, China). Briefly, the SOD activity was determined in the liver homogenate by following the inhibitory rate of nitrite. Superoxide radical ions were used to oxidize the nitrite to hydroxylamine at 550 nm. The SOD content was expressed as U/mg protein. Similarly, GSH-px activity was determined as described earlier (10). The absorbance was calculated at 420 nm on a spectrophotometer. The content of GSH-px was determined as u/mg protein. MDA activity was determined as previously described (10). The red substances at 532 nm were used to measure MDA activity and were expressed as nmol/mg protein.

Statistical analysis

Data was determined as mean $\pm$ standard deviation (SD). One-way ANOVA was used for the comparisons of mean values of control and experimental groups. All statistical differences were considered significant when $P < 0.05$. SPSS version 11.0 software (SPSS, Chicago, IL, USA) was used for data analysis.

RESULTS

Ratios of liver and body weight

The poisoned group ($\text{K}_2\text{Cr}_2\text{O}_7$) showed a persistent decrease in the ratios of the liver and body weight at

the three different periods, as compared to the control group ($P > 0.05$). Similarly, the protection group revealed almost constant ratios ($P > 0.05$) of the liver-body at all the three periods (days 21, 28 and 35). However, the prevention group showed a significant decrease in the ratios of the liver and body weight particularly at 35 days, when compared to poisoned group ($P < 0.05$). Lastly, Nano-Se supplementation in the cure group showed a non-significant increase at day 28 and day 35 when compared to the poisoned group ($P < 0.05$).

Histopathological analysis

H&E-stained sections of the liver tissue from the control group showed normal histological structures with nucleus, cytoplasm, and cell contour (Fig. 1a). In contrast, the morphology of the liver tissues was slightly changed and the liver was observed with nucleus fragmentation, hepatic blood sinus, pyknosis, and full of red blood cell mass in the hepatic sinus gap with an expanded centrilobular vein in the poisoned group (Fig. 1b). Moreover, in the protection group, the exposure to $\text{K}_2\text{Cr}_2\text{O}_7$ along with Nano-Se also caused some disorganization and reduction in liver cells (Fig. 1c). Conversely, in the cure (Fig. 1d) and prevention group (Fig. 1e), the damage was significantly reduced in liver organs with the supplementation of Nano-Se, especially at day 35 and day 28, respectively.

Analysis of biochemical indexes in broiler livers

To determine the effect of Nano-Se on $\text{K}_2\text{Cr}_2\text{O}_7$ toxicity in broiler chickens, we measured the enzymatic activities of ALT, AST, and GGT in serum samples (Fig. 2). Fig. 2a shows the results of the ALT contents in the control, poisoned, protection, cure, and prevention groups. In the poisoned group, the content of ALT was higher as compared to control group in all the three periods ($P < 0.05$). In addition, the protection group showed a slight decrease activity of ALT as compare to the poisoned group. Moreover, the prevention group showed a distinct decrease ALT activity within all the three periods as compared to the poisoned group ($P < 0.05$). Lastly, the activity of ALT abruptly dropped at days 28 and 35 in the cure group ($P < 0.05$).

Fig. 2b shows the results of the AST contents in the control, poisoned, protection, cure, and prevention groups. In the poisoned group, the content of AST was higher as compared to control group in all the three periods ($P < 0.05$). In addition, the protection group showed a slight decrease activity of AST as compared to the poisoned group ($P < 0.05$). Moreover, the prevention group showed a distinct decrease in AST activity with increasing pattern in all three periods as compared to the poisoned group. Lastly, the activity

of AST was higher on day 21 ($P > 0.05$) but a distinct decline was observed on days 28 ($P > 0.05$) and 35 in the cure group ($P < 0.05$).

Fig. 2c shows the results of the GGT contents in the control, poisoned, protection, cure, and prevention groups. In the poisoned group, the content of GGT increased with the time period and was higher as compared to the control group in all the three periods ($P < 0.05$). In addition, the protection group showed a decreased activity of GGT as compared to the

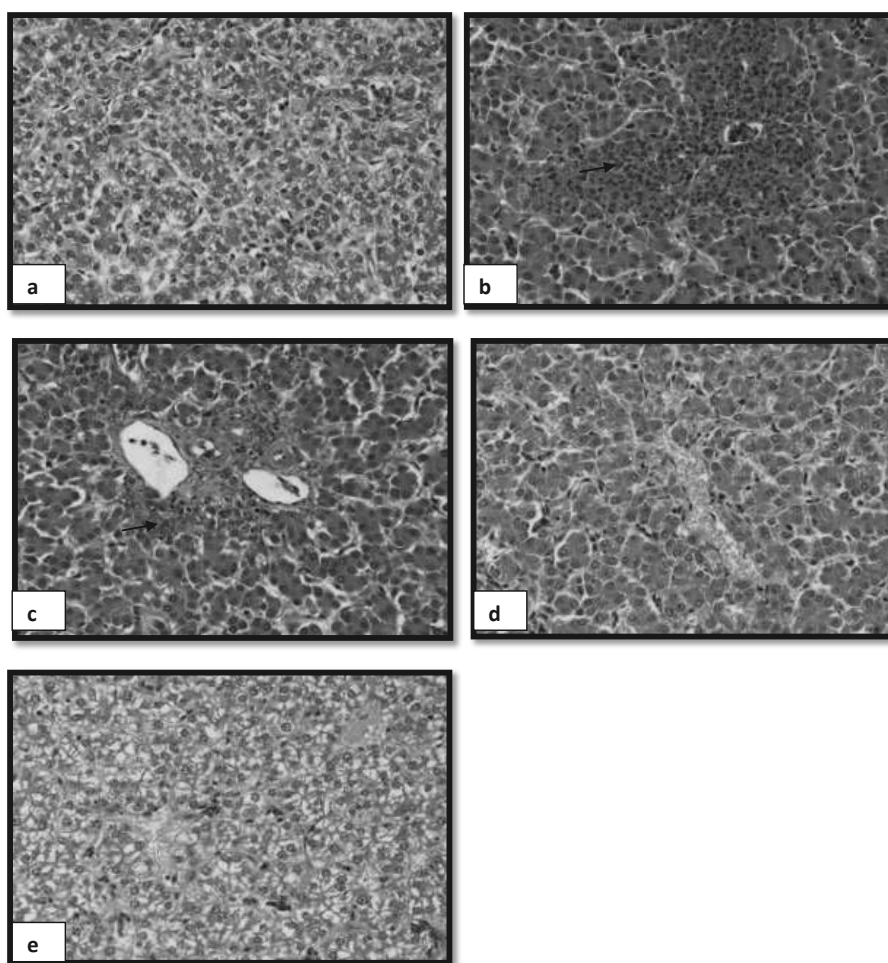


Fig. 1. The figure depicts mast cell activation pathway releasing inflammatory cytokines/chemokines. Inhibitory effect of **Fig. 1.** Photomicrograph of liver of chickens at 35 days. **a)** Control group (standard diet) -showing normal histological structures with nucleus, cytoplasm, and cell's contour. **b)** Poisoned group ($K_2Cr_2O_7$ through drinking water) showing vacuolar degeneration in liver of chickens with nucleus fragmentation, hepatic blood sinus, pyknosis, and full of red blood cells mass in the hepatic sinus. **c)** Liver of the chicken from protection group supplemented with $K_2Cr_2O_7$ + Nano-Se throughout the experiment. **d)** Liver of the chicken from cure group supplemented with $K_2Cr_2O_7$ for initial 21 days and then Nano-Se. **e)** Liver of the chicken from prevention group supplemented with Nano-Se for initial 21 days and then $K_2Cr_2O_7$; Bar = 50 μ m; H & E.

poisoned group ($P < 0.05$). Moreover, the prevention group showed a distinct decrease in GGT activity (in increasing pattern at 3 time periods) compared with in all three periods as compared to the poisoned group ($P < 0.05$). Lastly, decreased activity (except day 21) of GGT was observed in the cure group ($P < 0.05$).

Activities of biomarkers of oxidative stress in broiler livers
Fig. 3 shows the effect of Nano-Se supplementation

on liver SOD, GSH-px, and MDA activities in broilers treated with $K_2Cr_2O_7$.

Fig. 3a shows the results of the SOD assay in the control, poisoned, protection, cure, and prevention groups. In the poisoned group, the activity of SOD was higher as compared to the control group in all the three periods ($P > 0.05$). In addition, the protection group showed a slight increased activity of SOD as compared to the poisoned group. Conversely, the

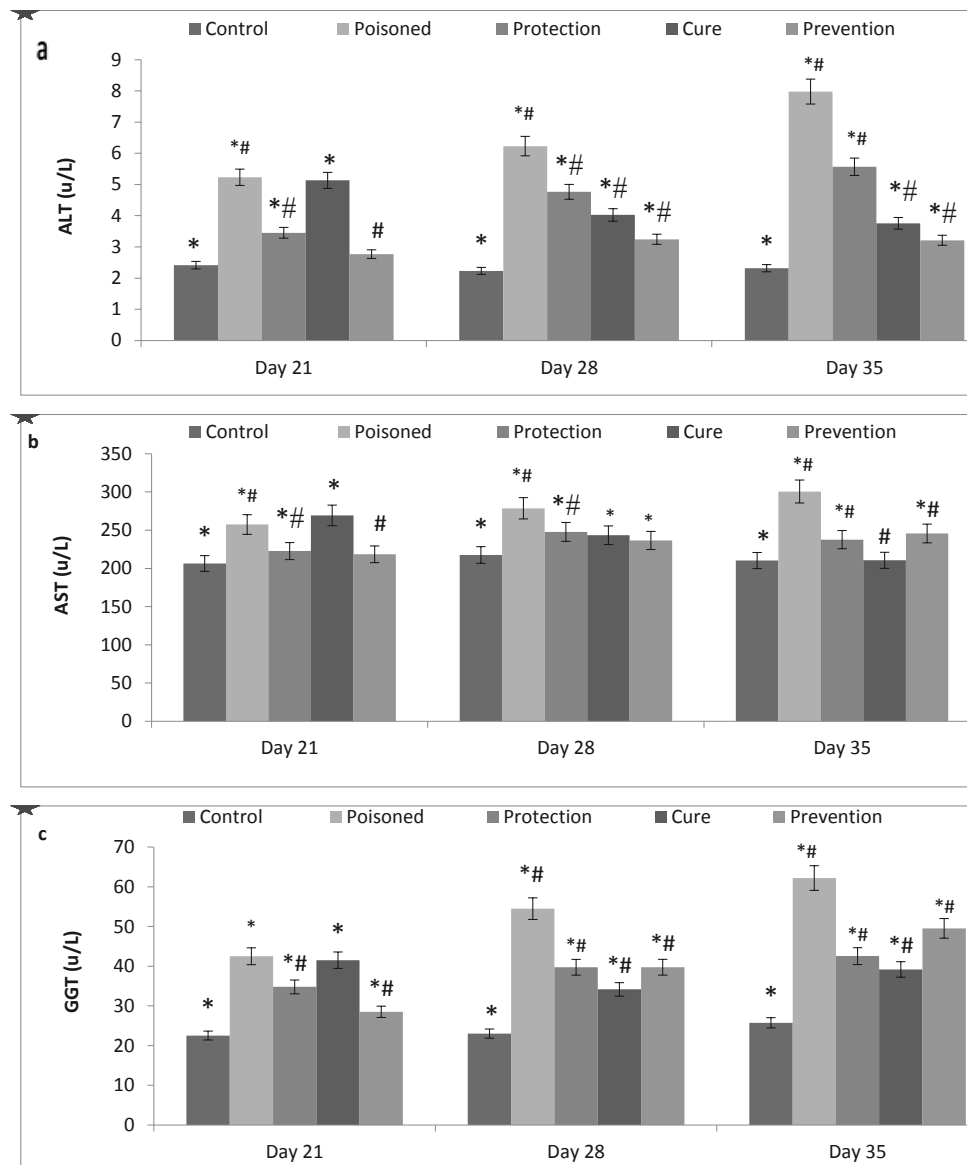


Fig. 2. Changes in the enzymatic activities of ALT, AST, and GGT in serum samples. **a)** ALT activity in the control and experimental group broiler chickens. **b)** AST activity in the control and experimental group broiler chickens. **c)** GGT activity in control and experimental group broiler chickens. Data are expressed as the means $\pm$ SD ($n = 10$). Bars with a shared common symbol are statistically significant ($P < 0.05$).

prevention and cure groups showed a distinct decrease in SOD activity in all three periods (except day 35 in prevention group) as compared to the poisoned group ($P < 0.05$).

Results of the MDA assay are shown in Fig. 3b. In the poisoned group, the activity of MDA was significantly higher as compared to the control group in all the three periods ($P < 0.05$). On the other hand, MDA level significantly decreased in the prevention group on day 28 and abruptly increased on day 35 when compared to the poisoned group ($P < 0.05$). Conversely, a slightly lower activity of MDA was

observed in both the protection and cure groups when compared with the poisoned group ($P > 0.05$).

Results of the GSH-px assay are shown in Fig. 3c. The level of GSH-px in the poisoned group was significantly decreased as compared with the control group at all three periods of the experiment ($P < 0.05$). The GSH level also decreased ($P < 0.05$) in the cure and prevention groups in the time periods except on days 21 and 35, respectively (approaching the levels of the poisoned group at these 2 time periods). Moreover, persistent increase in the level of GSH-px assay was observed in the protection group ($P < 0.05$).

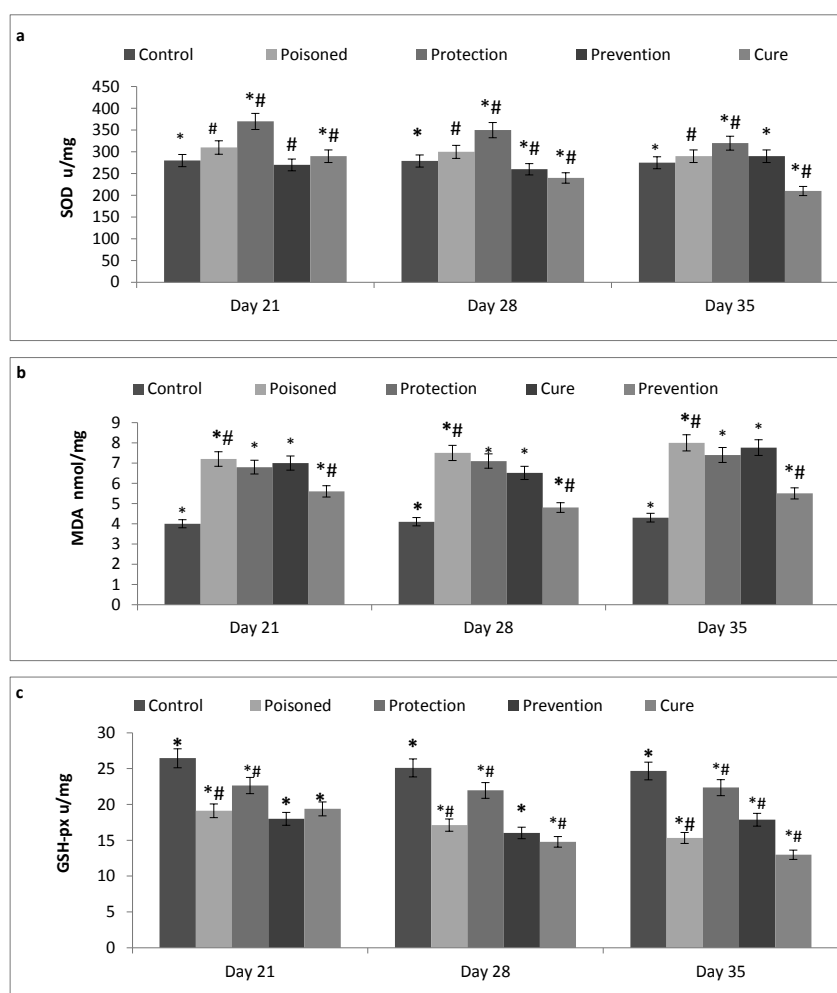


Fig. 3. Changes in the activities of biomarkers of oxidative stress. a) SOD activity in the liver of control and experimental group broilers. b) MDA activity in the liver of control and experimental group broilers. c) GSH-px activity in the liver of control and experimental group broilers. Data are expressed as the means $\pm$ SD ($n = 10$). Bars with a shared common symbol are statistically significant ($P < 0.05$).

DISCUSSION

In the present study, histological examination of the broiler liver showed severe histopathological changes in the poisoned group. However, Nano-Se supplementation visibly reduced the symptoms induced by the Cr (VI). Selenium can protect the animal vital organs by increasing the excretion of Cr and affecting its kinetics due to favoring the reorganization of elements, which clarify the alterations in histopathology (7, 11). The present study also revealed that the liver-body ratios were increased with the supplementation of Cr(VI) or Nano-Se, whether alone or in combination. Cell damage in any vital organ is generally followed by the discharge of various cytoplasmic enzymes into the blood, which can provide a clinical diagnosis (12). In the current study, the elevated levels of serum ALT, AST and GGT in the poisoned group broilers were in line with the findings of Uyanik (12), who suggested that Cr caused alterations in sheep. The increase in ALT, AST or GGT is mainly as a result of the outflow of these marker enzymes into the bloodstream from the liver cytosol (13), highlighting the hepatotoxic effect of Cr. In addition, various studies have reported diverse results (decrease, increase, or stable inactivity) of antioxidant enzyme activity in animal models (6, 14).

In conclusion, this study revealed that Cr (VI) exposure induces lipid peroxidation by inhibiting some of the enzymatic antioxidants and increasing hepatic enzymes such as ALT, AST and GGT. Accordingly, Cr(VI) exposure enhanced the concentrations of SOD and MDA and decreased the activity of GSH-px in the liver of broilers, probably influencing the regular metabolism and absorption of elements. On the whole, the current study obviously demonstrates the protective roles of Nano-Se against the deleterious effects of Cr toxicity.

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LETTER TO THE EDITOR

LAYERING POLY(LACTIC-CO-GLYCOLIC ACID)-BASED ELECTROSPUN MEMBRANES AND CO-CULTURE CELL SHEETS FOR ENGINEERING TEMPOROMANDIBULAR JOINT DISC

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The temporomandibular joint disk (TMJD) lacks blood vessels and is characterized by slow self-repair. Qualitative lesions in TMJD are difficult to repair. In this study, electrospun poly(lactic-co-glycolic acid) (PLGA) scaffolds were used to reconstruct temporomandibular joint discs by tissue engineering. Rabbit temporomandibular joint disc cells (TMJDCs) and rabbit synovium-derived mesenchymal stem cells (SMSCs) were co-cultured in 1:1 ratios. Cell sheets were induced by ascorbic acid incubated with electrospun PLGA scaffolds for 14 days in the presence (10 ng/ml in culture medium) or absence of TGF- β 3. Dimethylmethylene Blue Assay (DMMB) was used to determine the content of glycosaminoglycans in the extracellular matrix. The expression of Col1a1, Col2a1, Sox-9 and Runx-2 was quantified by RT-PCR, and the expression of type II collagen was observed by immunofluorescent staining. After 14 days of cultivation, the electrospun PLGA scaffold-loaded cell sheets could form an articular disc tissue with certain morphological characteristics. The expression of chondrogenic-related genes (Col2a1, Sox-9) and the secretion of extracellular matrix (GAG, type II collagen) in the co-culture group were close to those in the TMJDC group alone. The results suggest that PLGA electrospun scaffold-loaded co-cultured cell membrane could be used in the tissue engineering reconstruction of the temporomandibular joint disc.

To the Editor,

The temporomandibular joint disc (TMJD) is a fibrocartilaginous tissue located between the mandibular condyle and the temporomandibular joint. Due to lack of blood vessels, lymphatic vessels and nerves, the metabolism is slow in this tissue, leading to poor healing and repairing tissue damages (1). Bone tissue engineering offers the elimination of all the risks associated with regeneration of new functional bone by the synergistic combination of biomaterials

such as scaffolds (2). Temporomandibular joint disc cells (TMJDCs) play an important role in the generation, maintenance and remodeling of TMJD extracellular matrix (3). *In vitro*, the amplification of TMJDC layers causes a rapid de-differentiation phenomenon, changes in cell phenotype and decrease of their ability to synthesize extracellular matrix (4). In recent years, mesenchymal stem cells from various sources have been widely used in the field of tissue engineering. Among them, synovium-

Key words: temporomandibular joint disc, cell sheet, co-culture, electrospinning, tissue engineering

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derived mesenchymal stem cells (SMSCs) have higher colony forming efficiency and amplification and differentiation ability and can synthesize cartilage oligomeric matrix proteins, connexin, and glycosaminoglycan (GAG) (5). However, the growth of stem cells induced by growth factors leads to hypertrophy, which is manifested by up-regulation of expression of X-type collagen (COL-X), alkaline phosphatase (ALP) and metal matrix proteinase (MMP)-13. While the use of parathyroid hormone-related protein (PTHrP) and some other growth factors can inhibit the hypertrophy phenomenon (6). The co-culturing of chondrocytes and stem cells during cartilage tissue engineering may not only reduce the number of chondrocytes, but also inhibit cell hypertrophy and mineralization during the stem cell differentiation into chondrocytes (7). Therefore, the fiber scaffolds that better mimic the main structure of the TMJD extracellular matrix are more suitable for the tissue engineering approaches to TMJD repair. The electrospinning technology refers to a direct and continuous preparation of fibers with a large surface area. This is an advantage for transportation of nutrients, communication between cells and effective cell response (8) and also helps in increasing the tissue density (9). In this study, TMJDCs and SMSCs were used as seed cells, and TGF- β 3 was used as growth factor. The sandwich model was established to investigate whether TMJDCs co-cultured with SMSCs in a three-dimensional environment could be used as cell scaffolds and provide advantages for cartilage tissue engineering.

MATERIALS AND METHODS

Electrospinning

Poly(lactic-co-glycolic acid) (PLGA) (MW 150 kDa) was purchased from Shandong Academy of Pharmaceutical Sciences, China and its 12% (wt/v) solution was prepared using chloroform as a solvent. The polymer solution was transferred to a 1-ml syringe and positioned in the pump for the electrospinning process. All experiments were carried out at a flow rate of 0.8 ml/h and a distance between nozzle and collector was maintained at 15 cm. PLGA mats were electrospun for 30

min at 20 ± 0.4 kV. The electrospun fibres were collected into a water bath. After vacuum drying for 24 h, the mats were stored in a desiccator until further use.

Analysis of fiber diameter and pore size

PLGA mats were rinsed with phosphate-buffered saline (PBS) and fixed overnight in 0.05% glutaraldehyde at 4°C. After dehydration through a graded series of ethanol, the samples were subjected to critical point drying, sputter coated with gold (Desk IV, Denton Vacuum, Moorestown, NJ, USA), then examined with scanning electron microscope (JEOL-6700F, Japan). The obtained images were used to measure the diameter of 183–190 individual fibers and pores from each scaffold type using Image J software (<http://imagej.nih.gov/ij/>).

Cell isolation and culture

All cell culture reagents were obtained from Gibco (South Africa) or Sigma, unless otherwise specified. White New Zealand rabbits (2–3 weeks old) were purchased from Jilin University Experimental Animal Raising Centre (Changchun, China). The Animal Care and Experiment Committee of Jilin University School of Medicine approved the animal study protocols. SMSCs were isolated from the medial outer region of the knee joints according to the established protocol (5). Passage 3 and 4 cells were used for further experiments. TMJDCs were isolated and expanded as described earlier (5). Passage 1 cells were used for further experiments.

Cell sheet engineering

In both cases, the scaffolds were seeded with pure TMJDC and SMSC cell populations, as well as their combination with 1:1 TMJDCs to SMSCs ratio. Cells were seeded onto 24-well plates at a cell density of 2×10^5 cells/well and were cultured in DMEM high glucose (Hyclone, China) supplemented with 15% fetal bovine serum (FBS) (Gibco; Invitrogen Corp.) and 1% penicillin/streptomycin. The cells became confluent after 24 h in culture. The culture medium was then changed to DMEM (Sigma-Aldrich) high glucose supplemented with 1% ITS, 100 nM dexamethasone, 0.4 mM L-proline, 10 ng/ml TGF- β 3 (PeproTech) and 70 μ g/ml Vc. The medium was replenished three times a week. Confluent cells were cultured for 7–10 days until the cells at the edge of the dishes wrapped, which implied that cell sheets had formed and could be detached.

Multilayering

After electrospinning, the disks of 15 mm in diameter were punched from the mats. For sterilization, the disks were exposed to UV light for 30 min each side. The cell sheet detached from the dishes was attached to an electrospun membrane disk. The resulting unit blocks were left for 30 min at 37°C with 50 µl fresh medium. The constructs were then assembled by stacking together two unit blocks. Each multilayered assembly was left for 3 h in a 24-well plate with 50 µl medium to allow further cell attachment between the different layers. Finally, 1.5 ml serum-free chondrogenic medium (high-glucose DMEM, 1% ITS, 0.2 mM ascorbic acid, 100 nM dexamethasone, 0.4 mM L-proline) supplemented with 0 or 10 ng/ml TGF-β3 (PeproTech, Rocky Hill, NJ) was added. (The groups cultured in the medium supplemented with 10ng/ml TGF-β3 were labeled with (+) and those cultured in the absence of TGF-β3 were labeled with (-) further in the text) The medium was replenished three times a week. The tissue constructs were cultured for 14 days.

Quantification of glycosaminoglycan content

All chemical reagents were obtained from Sigma. The sulfated glycosaminoglycan (GAG) content of engineered tissues was quantified by dimethylmethylene blue chloride (DMMB, Sigma-Aldrich) assay as previously described (10). For normalization, the DNA content of samples was determined in a fluorescence plate reader using the Quant-iT Pico-Green dsDNA Assay Kit (Invitrogen, USA), as directed by the supplier. Both DMMB assay and Pico-Green assay were performed in triplicate, and three samples were assayed for each group.

Immunofluorescence staining

Fluorescent carbocyanine dye CM-Dil (Invitrogen, USA) was used to label the SMSC membranes before the cell sheet engineering, as directed by the supplier. After growing in culture for 14 days, the labeled SMSCs were analyzed by immunofluorescence staining for cartilage marker type II collagen, similar to the approach used in the previous report (11). Immunostaining was observed and analyzed using an inverted fluorescence microscope (Leica DMI3000B; Leica Microsystems, Wetzlar, Germany).

Quantitative real-time RT-PCR

Total RNA was extracted from each sample using

Trizol (Invitrogen, USA). cDNA was synthesized from the total RNA using Prime-Script™ RT reagent Kit with gDNA Eraser (Takara, Dalian) according to the manufacturer's instructions. RT-PCR (7300 Applied Biosystems, Rotkreuz, Switzerland) was performed as previously described (12) using SYBR®Premix Ex Taq™ (Takara) to quantify the expression levels of Col1a1, Col2a1, Sox-9 and Runx-2. For each sample, the Ct value of each target sequence was subtracted to the Ct value of the reference gene (rabbit GAPDH,) to derive ΔCt. The expression level of each target gene was calculated as 2^{ΔCt}, each sample was analyzed in triplicate, and for each group three samples were assayed. RT-PCR was performed using specific primers listed in Table I.

Statistical analysis

Data are reported as means ± SD and were analyzed statistically using the SPSS 13.0 software. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to determine significant differences ($p < 0.05$).

RESULTS

Electrospinning fiber characteristics

SEM observation (Fig. 1) shows that PLGA scaffolds had good fibers without nodes. The diameter of PLGA fibers was 2.17±0.55 µm, and the pore size was 17.86±7.5 µm, which was a large-diameter electrospun fiber stent.

Morphology and mechanical properties of layered constructs

The PLGA fibers were contracted with the cell sheets after loading. After 2 weeks of culture, the PLGA fibers became thicker and were contracted by 65±9% compared to the initial loading. The cell sheets and scaffolds in each group showed tight central union, the color was milky white or light yellow, and the texture was tough, similar to the cartilage tissue (Fig. 2).

GAG quantification

Compared with the cellular components, the secretion of GAG in TGF-β3-induced group was higher than that in the non-induced group (Fig. 3A). The same trend was observed after DNA

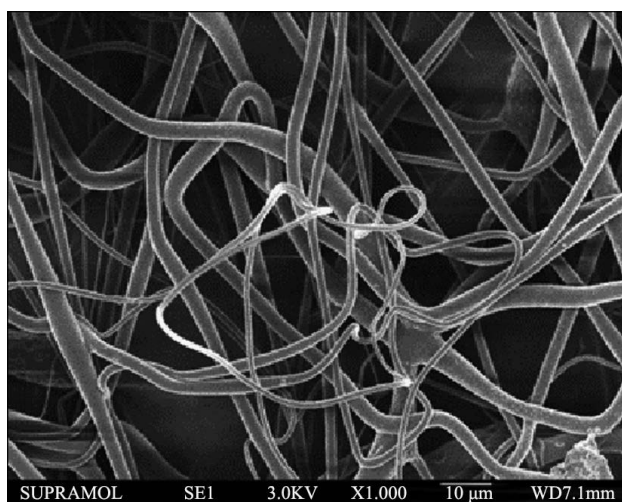


Fig. 1. Scanning electron microscope (SEM) images show the morphology of electrospun fibres obtained from a mixture of PLGA and chloroform solution.



Fig. 2. The tissue constructs were cultured for 14 days. From left to right: TMJDCs (+), Co-culture (+), SMSCs (+), TMJDCs (-), Co-culture (-), SMSCs (-). Size shown in centimeters.

quantification (Fig. 3B). The level of GAG secretion was the highest in TMJDCs (+) group, followed by Co-culture (+) and SMSCs (-) secretion in the TGF- β 3 group ($P < 0.05$). The GAG/DNA ratio in Co-culture (+) was close to TMJDCs (+). In the groups without TGF- β 3 treatment, the GAG of Co-culture (-) was close to TMJDCs (-), GAG/DNA Co-culture (-) was significantly higher than TMJDCs (-) ($P < 0.05$).

Immunofluorescent staining

As the PLGA scaffolds and cell membrane were contracted and formed wrinkles after the loading, the immunofluorescent staining demonstrated that the distribution of cells showed some non-homogeneity. Compared with the same cellular components, the collagen synthesis in each group after induction by

TGF- β 3 was higher than in the corresponding non-induction group. The SMSCs in the Co-culture (+) group showed chondrocyte properties, and there was a significant collagen expression around the cells (Fig. 4C, white arrows). The collagen secretion in the Co-culture (-) group was smaller than in the TMJDC (-) group, showing only a small level of expression at the edges of the tissue (Fig. 4C, white arrow). Comparing to the SMSC (-) group, chondrogenic differentiation trends were observed.

RT-PCR results

The expression levels of Sox9, Col2a1, Runx2 and Col1a1 mRNAs were detected by real-time RT-PCR. The housekeeping gene GAPDH was used as an internal reference gene. The expression of Col1a1 was decreased in TMJDCs (+) and Co-culture (+) compared to TMDCs (-) and Co-culture (-) ($P < 0.05$), while the expression of Col1a1 in SMSCs (+) was increased compared to SMSCs (-) ($P < 0.05$) (Fig. 5A). The expression level of Col2a1 was consistent and demonstrated the following order, regardless of whether TGF- β 3 was added or not: TMJDCs > Co-culture > SMSCs ($P < 0.05$) (Fig. 5B). The levels of SOX9 in TMJDCs and SMSCs were significantly increased after induction ($P < 0.05$), while the SOX9 level was decreased after induction of Co-culture (Fig. 5C). The expression of RUNX2 gene in the TMJDCs and Co-culture induced groups was significantly lower than that in the non-induced group ($P < 0.05$), and the expression level of RUNX2 in SMSCs was increased (Fig. 5D) ($P < 0.05$).

DISCUSSION

Electrospinning fibers can provide support for the cell sheets and in assisting their assembly and enhancing the strength of the nascent tissue. The wet-spinning electrospun PLGA fiber were improved in material porosity, low crystallinity, better hydrophilicity and slower degradation rate (13). We observed $70 \pm 3\%$ contraction 21 days after the cell sheets were loaded on the PLGA electrospun fiber membrane (9). The samples contracted by $65 \pm 9\%$ compared to the initial load after 14 days of load culture. The co-culture of chondrocytes and stem cells

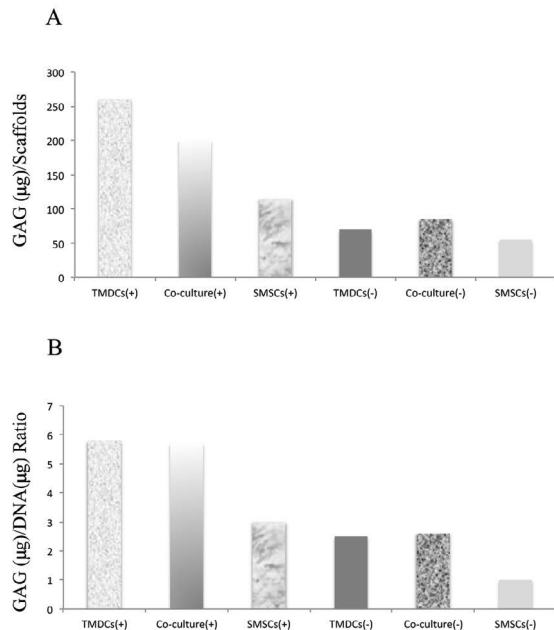


Fig. 3. GAG (A) and GAG/DNA (B) in constructs after two weeks of culture. The differences between groups connected by the same letter are statistically different ($p < 0.05$).

ratio 1:1 was superior (14), thus we chose TMJDCs/SMSCs ratio 1:1 for co-culture studies. The co-culturing of chondrocytes and stem cells showed that co-culture can promote the secretion of extracellular matrix and GAG to a high degree compared to mono-cultured chondrocytes. The GAG secretion in co-culture also increased and was a similar level to that observed in TMJDCs (Fig. 3). The collagen secretion in Co-culture (+) was similar to TMJDCs (+), as demonstrated by the immunofluorescence staining (Fig 4). The SMSCs were differentiating into chondrocytes in Co-culture (+) and involved in cartilage formation. The cytokines secreted by TMJDCs induced the induction of SMSCs in the co-culture environment, but the induction effect was not significant with TGF- β 3 supplementation. The TGF- β 3 signaling mainly depends on the activation of Smad, MAPK pathway and elevated expression of Col2a1 and SOX9 (15). In our results, SOX9 expression level was significantly high in TMJDCs

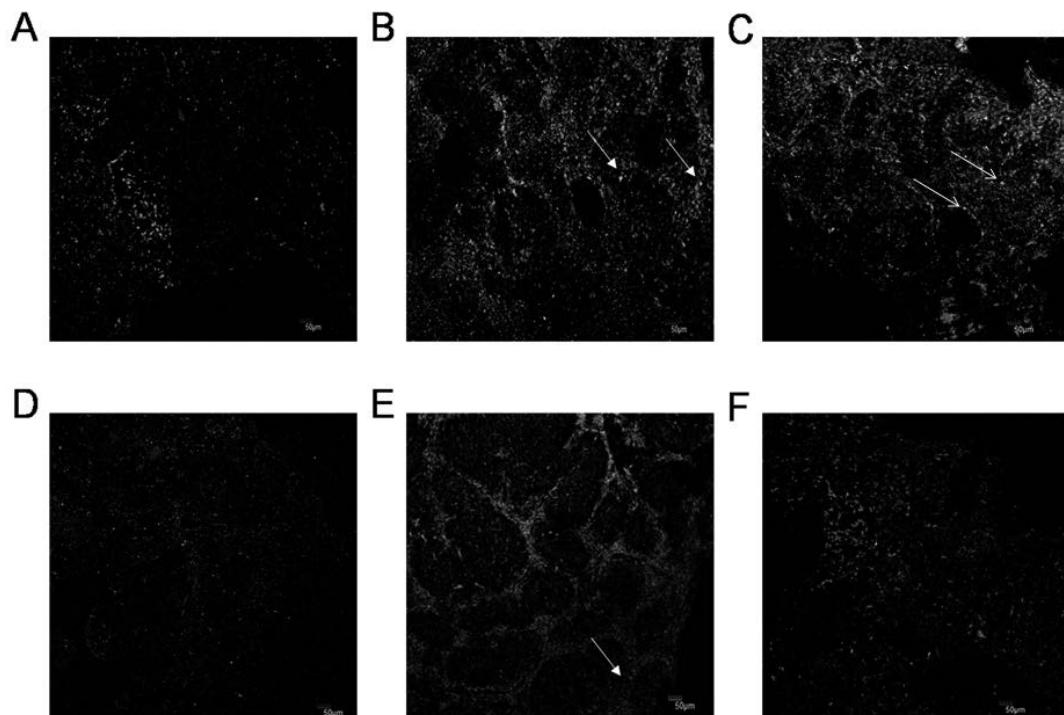


Fig. 4. Immunofluorescent staining of chondrogenic differentiation trends. A) TMJDCs (+); B) Co-culture (+); C) SMSCs (+); D) TMJDCs (-); E) Co-culture (-); F) SMSCs (-) under fluorescence microscopy and white arrow shows the collagen expression around the cells.

and SMSCs after induction. SOX9 expression in Co-culture (+) was lower than in Co-culture (-) due to the increased expression of vascular endothelial growth factor (VEGF) in the co-culture but Col2a1 expression remained up-regulated (16). TGF- β 3 can also inhibit cell hypertrophy by decreasing the RUNX2 expression in TMJDCs (+) and Co-culture (+) (Fig. 5). The expression of Col1a1

gene in TMJDCs (+) and Co-culture (+) groups was lower than in TMJDCs (-) and Co-culture (-) but Col1a1 expression in SMSCs was significantly up-regulated by TGF- β 3 (17), and cytokines in the chondrocytes extracellular matrix also induced the Col1a1 expression in SMSCs. In this study, we also found that in the non-induced groups the Col1a1 gene expression level in Co-culture was significantly

Table I. List of primers.

<i>Col1a1</i>	5'-GCAACAGTCGCTTCACCTACA-3'
	5'-CAATGTCCAAGGGAGCCACAT-3'
<i>Col2a1</i>	5'-CCTCCGTCTACTGTCCACTGA-3'
	5'-ATTGGAGCCCTGGATGAGCA-3'
<i>Sox9</i>	5'-CCTTCAACCTTCCTCACTACAGC-3'
	5'-GGTGGAGTAGAGCCCTGAGC-3'
<i>Runx2</i>	5'-AGGGACTATGGCGTCAAACA-3'
	5'-GGCTCACGTCGCTCATCTT-3'

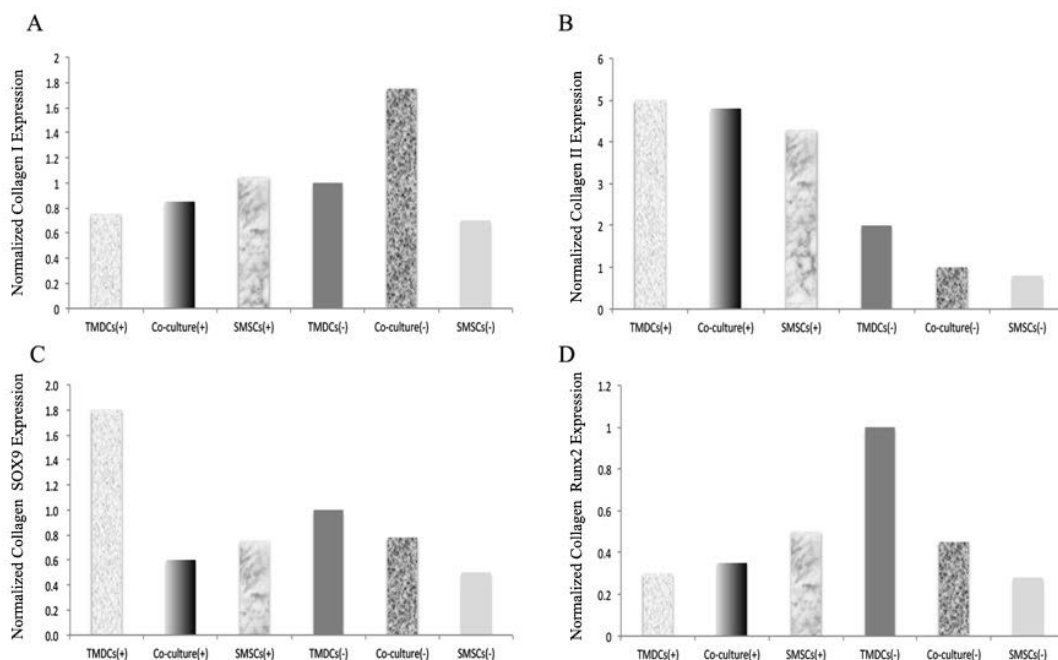


Fig. 5. Quantitative gene expression results for *Col1a1* (A), *Col2a1* (B), *SOX-9* (C) and *RUNX2* (D). Groups connected by the same letter show statistically significant difference ($p < 0.05$).

higher than that in the other two groups. The results showed that SMSCs could not only express the chondrocyte-associated genes and matrix secretion, but also up-regulate the expression of *Colla1* gene under induction by growth factors and in the co-culture environment. The data prove that SMSCs are suitable for TMJD tissue engineering and are a good source of cells.

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LETTER TO THE EDITOR

THE RELATIONSHIP BETWEEN IGF-2, IGFBP-2, AND IGFBP-3 LEVELS IN PATIENTS SUFFERING FROM PREDIABETES

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Many studies have shown that intervention in the early stages of diabetes may have a pivotal role in reducing the risk of cardiovascular diseases. This study was designed to assess possible relationships between insulin-like growth factor 2 (IGF-2), insulin-like growth factor binding proteins 2 and 3 (IGFBP-2, IGFBP-3) and prediabetes. A total of sixty clinically ascertained prediabetes cases and twenty-five healthy controls were included. Serum IGF-2 and binding proteins were estimated using commercially available ELISA kit. All groups had a positive correlation between all serum parameters. Multinomial logistic regression showed that all the study parameters directly affected each other. The results could not prove any correlation between IGF-2 and its binding proteins during prediabetes stage. Further assessments of these factors in larger groups of males and females in diabetic individuals could be useful to support our hypothesis that these factors change only in diabetes mellitus.

To the Editor,

Diabetes is a major worldwide health problem causing blindness, kidney failure, heart attacks, stroke and other health problems. The prediabetes stages are defined as fasting hyperglycemia and impaired glucose tolerance caused by dysfunctional insulin secretion and/or insulin activity. A growing body of evidence shows that prediabetes condition is a risk factor for type-2-diabetes and cardiovascular complications. Although IGT and IFG were described many years ago, the underlying causes of these disorders are still unclear. Recently, some studies have demonstrated the presence of insulin-like growth factor (IGF-2, IGFBP-2, and IGFBP-3), in diabetes mellitus (DM) patients (1-3).

IGF-2 is a 7.5 kDa peptide composed of 67 amino acid residues. IGFs usually need insulin receptors in order to show their hypoglycemic effects. It has been shown that insulin receptor is responsible for

both the mediation of cellular insulin effects and some of the mitogenic effects of IGF-2 (1-3). IGF-2 inhibits hepatic glucose production in liver and glycogen synthesis. It increases glucose uptake and oxidation in peripheral target tissues, and also lipid and protein synthesis. In the course of fat tissue, IGF-2 increases glucose uptake but this effect is weaker than insulin. The half-life of the IGFs which are chronically transported with free or specific binding proteins ranges from 15 to 20 minutes. IGFs bind to binding proteins and are therefore protected from proteolytic enzymes. It is known that IGFBPs inhibit typical insulin-like effects of IGFs, restrict its transport through capillaries, inhibit binding to membrane receptors, and prevent conversion to an active form. IGFBP-2 and IGFBP-3 are 36 kDa proteins with non-glycosylated low molecule weight which belong to the superfamily of IGF binding proteins (4). IGFBP-2 and IGFBP-3 bind

Key words: prediabetes state; insulin-like growth factor 2, insulin-like growth factor-binding protein

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to IGF-2 in the serum and antagonize its biological effects, which may cause tumor development and hypoglycemia by preventing extremely free IGF-2 in the serum. After IGFBP-3, IGFBP-2 is responsible for IGF-2 transportation in blood (5). IGFBP-3 has an important role in the binding of IGF-2 to the IGF receptors by controlling the concentration of IGF-2 (6).

The objectives of this study were to determine serum levels of these molecules in prediabetes patients and to examine the relationship between the parameters and highlight how these molecule levels change during prediabetes stage in circulation. It was found that all 3 of the proteins discussed above have not been fully assessed in prediabetes patients. Additionally, all these parameters are in close relationship with insulin and at least some of them take part in diabetes pathogenesis; on the other hand, we asked whether these parameters could be classified as components which take part in glucose metabolism in prediabetes patients.

MATERIALS AND METHODS

Study population

This study was conducted on sixty prediabetes patients and twenty-five healthy age-matched controls who visited the Cerraphasa medical laboratory based in Turkey between June 2014 and February 2016. The prediabetes patients were diagnosed according to the American Diabetes Association (ADA). Clinical data were carefully recorded, and a full medical history for all participants including previous evidence of diabetes, any associated systemic diseases, previous use of immunomodulatory, antilipidemic, antidiabetic, antihypertensive or antipsychotic medications was obtained. The patients were also tested and were free of chronic inflammatory diseases, coronary heart disease, liver, kidney disease and malignant disease history or connective tissue disease. The prediabetes patients were further classified into an Impaired Fasting Glucose (IFG) group (n:30, female: 23, male: 7; age average: 46 ± 14 years) and an Impaired Glucose Tolerance (IGT) group (IGT, n:30, female: 15, male: 15; mean ages: 48 ± 12 years) through Oral Glucose Tolerance Tests (OGTT). All the participants are from Turkey. Blood samples were obtained from all the

participants, collected into tubes without anticoagulant (for routine biochemical and hormonal assays and ELISA tests), with EDTA (for HbA1c analysis) and sodium fluoride (for glucose analysis) at both baselines and 2nd after glucose overloading between 08:00 and 10:00 A.M and were immediately transported to the laboratory for analysis. Body mass index (BMI) was calculated as the ratio of weight (Kg) to height squared (m^2).

Blood samples were centrifuged at 3000 rpm x 10 min. Serum and plasma samples were collected and frozen at -80°C in polypropylene tubes until assayed. Routine tests, plasma glucose, and HbA1c levels were assayed on the test day.

The study protocols were approved by the Ethics Committee of the Affiliated Hospital of Istanbul University. All the participants provided their written consent in order to participate in the study (Ethics Ref No: 83045809/604.01/02-124088).

Examination of regulatory parameters

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

ELISA of serum IGF-2, IGFBP-2, and IGFBP-3

Quantitative determinations of serum IGF-II, IGFBP-2 and IGFBP-3 were achieved using corresponding commercially available ELISA kits from Shanghai Sunred Biological Technology Co., (Sun Red, China) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was carried out using SPSS software version 15.0 (SPSS Inc., Chicago, IL). Normal distribution of continuous variables was examined by the Shapiro-Wilk test. Normal distribution of three variables

was calculated by using one One-way ANOVA. The unpaired student *t*-test was used to compare the meaningful variables in the 2-item subgroup. Kruskal-Wallis test was used for 3 groups to compare the variables with no normal distribution. Bonferroni-corrected Mann-Whitney U-test was used to compare the meaningful variables in the 2-item subgroup. Pearson correlation coefficient was used to examine the relationship between normal variables with continuous variables. Spearman correlation coefficient was used to examine the relationship between continuous variables without normal distribution. Comparison values (*p*) under 0.05 were considered statistically significant in all statistical comparisons in the study. Regression analysis was carried out in order to confirm our findings.

RESULTS

Metabolic parameters

The results of the current study showed no statistically significant difference between the three groups in terms of age, BMI, serum total cholesterol, HDL cholesterol, LDL cholesterol, and triglyceride. However, HbA1c levels were significantly elevated in both IFG and IGT groups compared to the healthy control group ($p < 0.01$ and $p < 0.01$). Also, Insulin and HOMA-IR serum levels showed a significant increase in IFG and IGT groups compared to the control group ($p < 0.05$ and $p < 0.001$; $p < 0.01$ and $p < 0.001$). Serum insulin and HOMA-IR levels were significantly higher in the IGT group compared to the IFG group ($p < 0.001$ and $p < 0.05$). This study showed that in the three-group comparison, there was no difference in terms of serum IGF-2, IGFBP-2, and IGFBP-3 (Table I).

Three group comparison in males and females separately

Our results showed there were no differences in serum IGF-2, IGFBP-2, and IGFBP-3 between the three study groups (Table II).

Correlation analysis in three assay groups

Correlation analysis of the study is given in Table III. The results showed a positive correlation between serum IGFBP-2 and serum IGF-2 ($p < 0.001$), and IGFBP-3 ($p < 0.001$) in all groups. In

addition, there was no correlation between serum parameters and age, BMI, serum total cholesterol, HDL cholesterol, LDL cholesterol, Insulin, HOMA-IR, and triglyceride (not shown in Table III).

Regression analysis between study parameters □

In the current study, multiple logistic regressions analysis was carried out to identify the variables associated with the other independent variables. All serum variables were considered as possible predictors of other independent variables. Backward elimination was performed. First of all, all independent variables were chosen and then they were gradually eliminated until no omitted variable would significantly contribute to the model. We set up three models; when IGFBP-3 was chosen as a dependent variable, serum IGF-2, and serum IGFBP-2 were chosen as explanatory variables with prediction ratio (R^2) of 91.5%. Likewise, when IGFBP-2 was chosen as a dependent variable, serum IGF-2 and serum IGFBP-3 were chosen as explanatory variables with prediction ratio (R^2) of 92.9%; when serum IGF-2 was chosen as a dependent variable, serum IGFBP-2, and serum IGFBP-3 were chosen as explanatory variables with prediction ratio (R^2) of 92.5%.

DISCUSSION

Individuals in the prediabetes stage are at high risk of developing T2DM since they already have insulin resistance and glucose tolerance and may have a large number of dysfunctional β -cells. In addition to these facts, IGT and IFG are strong predictors of metabolic syndrome and cardiovascular heart disease. There is increasing evidence suggesting that prediabetes stage must be the target of new therapeutic agents for prevention of type-2-diabetes (7). Here, we present the first study demonstrating the relationship between IGF-2, IGFBP-2, and IGFBP-3 in prediabetes patients. Changes in parameter levels and relationship between them in the current study may be important because changes in bioavailability of these parameters may contribute to diabetes incidence.

IGF-2 and its binding protein are metabolic factors that are fundamental to growth, development,

Table I. Clinical characteristics and parameters laboratory values of the study.

	Control	Impaired Fasting Glycemia	Impaired Glucose Tolerance	P value
n	25	30	30	-
Gender (F/M)	19/6	23/7	15/15	-
Age (Year)	41.1±12.9	46.3±14.3	48.6±12.1	-
BMI (kg/m ²)	24.4±5.6	28.8±7.0	33.03±5.8	0.397
Total cholestrol (mg/dL)	191.2±47.5	208.4±81.6	196.8±31.6	0.711
HDL cholestrol (mg/dL)	56.7±14.6	56.6±16.4	52.9±16.2	0.695
LDL cholestrol (mg/dL)	122.0±40.2	121.8±26.3	127.6±27.6	0.764
Triglyceride (mg/dL)	106.6±60.4	113.8±63.2	143.3±66.1	0.052
Fasting Plasma Glucose (mg/dL)	92.3±5.75	110.40±5.65	109.02±11.30	<0.001*
2nd Hour after Plasma Glucose Challenge (mg/dL)	98.8±18.65	110.92±20.2	157.07±24.10	<0.001*
HbA1c (%)	5.23±0.29	5.60±0.38	5.65±0.55	<0.01*
Insulin (mIU/L)	6.9 ± 2.5	11.6 ± 4.1	21.5 ± 2.6	<0.05*
HOMA-IR	1.97±1.24	3.21±1.18	5.03±1.37	<0.01*
Serum IGF-2	273.7 (179.4-781.0)	241.0 (90.1-712.8)	300.0 (172.3-807.8)	0.181
Serum IGFBP-2	581.5 (462.9-998.7)	550.7 (246.1-2051.2)	528.6 (381.9-1997.2)	0.987
Serum IGFBP-3	41.7 (32.0-122.5)	35.3 (17.5-123.8)	44.4 (23.3-118.1)	0.215

* $p < 0.05$ **Table II.** Values of working parameters according to gender in groups (ng/mL).

	Male	Female	P value
Control	n:6	n:19	
Serum IGF-2	220.9 (215.2-425.1)	291.4 (179.4-781.0)	0.283
Serum IGFBP-2	501.9 (476.4-778.8)	674.5 (462.9-1998.7)	0.080
Serum IGFBP-3	34.9 (33.8-60.4)	42.4 (31.9-122.5)	0.092
IFG	n:7	n:23	
Serum IGF-2	212.7 (143.9-628.6)	244.9 (90.1-712.8)	0.344
Serum IGFBP-2	526.3 (415.7-2051.2)	579.6 (246.1-2013.7)	0.320
Serum IGFBP-3	30.3 (24.5-117.6)	38.4 (17.5-123.8)	0.097
IGT	n:15	n:15	
Serum IGF-2	313.0 (188.8-807.8)	286.9 (172.3-800.4)	0.838
Serum IGFBP-2	531.2 (390.9-1997.2)	525.9 (381.9-1934.9)	0.744
Serum IGFBP-3	45.1 (23.2-118.1)	41.8 (26.9-118.1)	0.713

* $p < 0.05$

and metabolism (8), therefore, any disorder which affects these factors could cause diseases such as diabetes. Some studies have revealed the relationship between IGF-2 and insulin secretion (9, 10), obesity (11) and hypoglycemia (12). There is also evidence that obesity and plasma insulin levels contribute with IGFBP-2 (13, 14). Regarding IGFBP-3, some studies have proved its role in diabetes complications and insulin regulation (9, 10, 15, 16).

The most comprehensive clinical study we found in the literature was by Frystyk et al. (17), who compared fat in participants without DM to thin participants. It has been reported that free IGF-1 and IGF-2, total IGF-2 and IGFBP-3 levels increase, IGFBP-1 and 2 levels decrease and IGF-1 does not change. In obese participants with T2DM, total and free IGF-2 and IGFBP-3 levels keep increasing; however, except in obese participants, IGF-1 levels

decreased. In the same study, it was found that in participants with T1DM, IGF-2 and IGFBP-3 levels were normal, free IGF-1 and 2 decreased and IGFBP-2 levels increased. The results of that study support that regulation of these molecules in serum shows differentiation in T1DM and T2DM, IGF-IGFBP the correlation is different but some

differences come out on their own apart from the existence of T2DM.

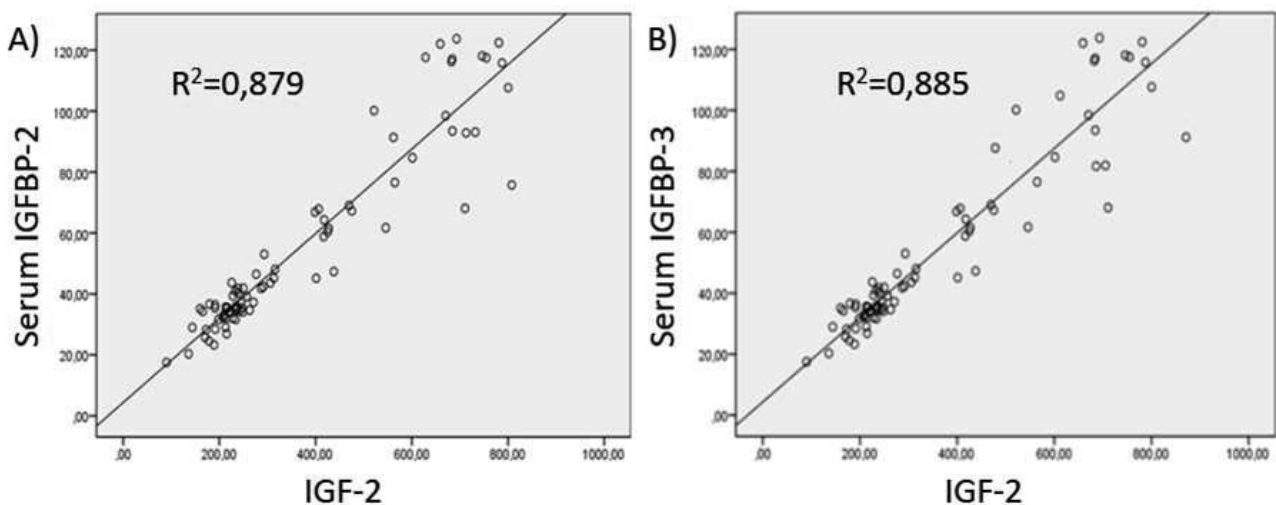
As mentioned above, in accordance with previous studies, the analysis of the factors in our study was carried out on diabetic patients. Our results suggest that the serum levels of these proteins in prediabetes stage do not show any significant change compared to the healthy control group. The most explicit feature of our prediabetes groups is that participants had insulin resistance. Although HOMA-IR levels increased in those groups, a significant change could not be seen in IGF-2, IGFBP-2 and IGFBP-3 levels in comparison to the control group. The fact that among IGF-2, IGFBP-2 and 3, there is a positive correlation in all subjects and that there is no significant correlation between BMI and metabolic indicators along with insulin, make us think that those proteins do not show a change in prediabetes stage. It is tempting to speculate that an adaptive response mechanism steps in and, apart from insulin and blood sugar, serum levels of molecules are protected in prediabetes stage. Another important result of our study is the positive correlation that was found in all participants in every group and between IGF-2 and binding proteins. Therefore, it could be understood that serum levels are kept relatively stable as IGF-2, IGFBP-2, and IGFBP-3 may have a direct relationship to each other even in the existence of hyperinsulinemia before apparent diabetes develops.

Table III. Correlation analysis results (Spearman Correlation).

All groups together	Serum IGF-2	Serum IGFBP-2	Serum IGFBP-3
Serum IGFBP-2	$r=0.813$ $p<0.001^*$	-	$r=0.854$ $p<0.001^*$
Serum IGFBP-3	$r=0.923$ $p<0.001^*$	$r=0.854$ $p<0.001^*$	-
Control			
Serum IGFBP-2	$r=0.896$ $p<0.001^*$	-	$r=0.864$ $p<0.001^*$
Serum IGFBP-3	$r=0.946$ $p<0.001^*$	$r=0.864$ $p<0.001^*$	-
IFG			
Serum IGFBP-2	$r=0.774$ $p<0.001^*$	-	$r=0.848$ $p<0.001^*$
Serum IGFBP-3	$r=0.890$ $p<0.001^*$	$r=0.848$ $p<0.001^*$	-
IGT			
Serum IGFBP-2	$r=0.859$ $p<0.001^*$	-	$r=0.889$ $p<0.001^*$
Serum IGFBP-3	$r=0.925$ $p<0.001^*$	$r=0.889$ $p<0.001^*$	-

* significant

Fig. 1. Correlation graphs. The correlation between serum IGF-2 and A) serum IGFBP-2 and B) serum IGFBP-3 in all study groups.



Also, it is worth noting that although all serum parameters have a correlation with each other, regression analysis showed that their serum concentration correlated with only some of these parameters. In other words, in regression models, only serum IGFBP-3 fits in all formula. These results are important as they showed which molecules were directly influenced by another in prediabetes and healthy control group subjects, and this will help researchers to use the current study results to conduct more comprehensive large-scale studies.

In conclusion, the outcomes of the current study showed that the serum levels of IGF-2, IGFBP-2, and IGFBP-3 do not change during prediabetes stage.

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LETTER TO THE EDITOR

THE ROLE OF OSTEOPONTIN AND ITS RECEPTOR IN MENINGIOMA DEVELOPMENT AND PROGRESSION

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Meningiomas are common in intracranial tumors, the majority of which are benign with slow growth and low recurrence rate. The aim of the present study was to assess the role of osteopontin and its receptor CD44 (Cluster of differentiation -44) in meningioma development and progression. A total of 2 groups were included, namely an experimental group with 54 meningioma patients and a control group with 30 patients with internal and external decompression. The disease group included subjects, from whom meningioma tissue specimens were collected during surgery for tumor excision, whereas the control group included subjects who were subjected to scalp laceration and provided normal meninx tissues. The expression of osteopontin and CD44 was determined by immunohistochemistry. Osteopontin expression was negative in normal meninx, and its expression was increased in meningioma tissues ($P<0.05$). The positive expression of osteopontin increased according to the histological grade of meningioma ($r=0.417$, $P<0.001$). A highly significant difference was noted between non-invasive and invasive meningiomas ($P<0.001$). The positive expression of CD44 correlated with the grade of meningioma and its invasiveness ($P<0.05$), although it exhibited no correlation with the degree of peritumoral edema ($r=0.033$, $P>0.05$). The increased expressions of osteopontin and CD44 were observed in all grades of meningioma, and correlated with its development ($r=0.961$, $P<0.001$). Osteopontin and CD44 play important roles in the development and progression of meningioma and can be used as prognostic markers for tumor recurrence and progression as well as therapeutic targets for the development of new drugs.

To the Editor,

Meningioma is a tumor that originates from the meningeal region and the meningeal spaces around that region. The majority of these tumors are mainly distributed to the wall of Vena cava sinus and near the vein branches of venous sinus, as well as the olfactory sulcus, saddle area and other parts of the basis cranii (1). Several meningiomas have intact envelopes with a slow growth rate that can cause clinical symptoms mainly through oppressing the surrounding tissues (2). The onset of this disease has

relatively few symptoms such as headache, epileptic seizures and psychoses that require differential diagnosis (3). Although most meningiomas are benign tumors, the tumor tissues cannot be totally excised. In addition, certain meningiomas exhibit malignant biological characteristics that are characterized by invasive growth in the surrounding tissues and obscure boundaries with surrounding tissues. In some cases the tumors invade the venous sinuses. The diagnosis of malignancy is conducted by histochemical and immunohistochemical

Key words: osteopontin, CD44, meningioma, invasiveness, peritumoral edema

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examination (4). The aforementioned factors render the total excision of the meningioma tissues particularly difficult (5). Consequently, the prognosis of the patients is influenced, notably if they are associated with vitamin K-dependent coagulopathies (6). The aforementioned studies suggest that further investigations are required that will aid the diagnosis of meningiomas.

Osteopontin is a glycoprotein that contains sialic acid, glutamic acid, aspartic acid and serine residues. Its main function is to establish cellular communication with matrix inorganic molecules (7). Due to its signal transduction activities, osteopontin interacts with the extracellular matrix that is related to various biological functions, including sclerotic calcification, inflammatory reaction, apoptosis and immunoreaction (8, 9). The majority of studies demonstrated that osteopontin contributed significantly in the development, progression and invasion of tumors. CD44 is a cell adhesion molecule that is widely distributed around the cell surface (10). Currently, a vast number of studies have indicated that CD44 played an important role in tumor progression and development (11, 12). The aim of the present study was to investigate the expression and the mechanism of action of osteopontin and its receptor CD44 in meningioma tissues in order to provide insight into the pathogenesis and prognosis of meningioma.

MATERIALS AND METHODS

Patients

A total of 54 patients who were diagnosed with meningioma and had undergone surgery for tumor excision in the neurosurgery department of the Affiliated HongQi Hospital of the MuDanJiang Medical University between February 2016 and March 2017, were included in the experimental group. The patients who were included in this group did not receive treatment prior to surgery. A total of 30 patients with internal and external decompression following scalp laceration were included in the control group. Normal meninx tissues were collected from these patients. The study was approved by the Ethics Committee of the Affiliated HongQi Hospital of MuDanJiang Medical University and all the patients

signed an informed consent. Meningioma specimens were graded as follows: grade I - non-invasive meningioma, grades II and III - invasive meningioma. Magnetic resonance imaging (MRI) T2 weighted image was used to detect the degree of peritumoral edema prior to surgery. According to this classification, grade 0 denoted no edema (no high signal around tumor), whereas grade 1 denoted mild edema (high signal $\leq 2\text{cm}$), grade 2 moderate edema (high signal $>2\text{cm} \sim 1/2$ hemisphere), and grade 3 severe edema (high signal $>1/2$ hemisphere).

Instruments and reagents

The experimental instruments included microtome (Qingzhoushi Xinhang Jixieshebei Co., Ltd, China), glass slide, cover glass (Beijing Huasheng Letter Spectrum Instrument Co., Ltd, China), drying machine (Guangzhou Junqi Experimental Instruments Co., Ltd, China), electro-heating standing temperature cultivator (Hangzhou Huier Instrument and Equipment Co., Ltd, China), general optical microscope (Shanghai Optical Instrument Factory, China) and ultrapure water machine (Beijing Haokai SCI-Tech Co., Ltd, China).

The reagents included the following: paraformaldehyde (Taiyuan Weixing Pharmaceutical Co., Ltd), ethyl alcohol (absolute ethyl alcohol with different concentrations like 95%, 85% and 70% Hubei Taichen Jianrui Pharmaceuticals Co., Ltd), dimethylbenzene (Xiamen Meishang Medicine Co., Ltd), hydrochloric acid (Synthetic Branch of Fujian Sanming Chemical Industry Limited Company), neutral balsam (Shanghai Y-Y Bio-Technique Co., Ltd), mouse anti-human OPN protein monoclonal antibody (Shanghai Tiyo Biotechnology Co., Ltd), mouse-anti-human CD44 monoclonal antibody (Shanghai Wolwo Pharma Co., Ltd) and SP kit (Shenzhen Baoankang Biotechnology Co., Ltd).

Operating steps

Continuous paraffin sections were made from all specimens. The specimens were stained with hematoxylin and eosin (H&E) in order to identify the pathological grading. Concomitantly, the immunohistochemical SP staining technique was employed in order to detect the expression levels of osteopontin and CD44. However, with regard to the normal meninx tissues, the expression levels of osteopontin and CD44 were detected only by immunohistochemistry SP. Prior to the experiment, several preliminary experiments were initially conducted

in order to optimize the reaction conditions.

The immunohistochemistry methodology for the detection of osteopontin was conducted as follows: The tissue sections were initially prepared, using routine dimethylbenzene in order to dewax the samples, whereas gradient ethyl alcohol (from high to low) was used for tissue hydration and phosphate buffered saline (PBS) for tissue washing (5 min every time). The sections were immersed into citric acid buffer solution and anti-retrieval was conducted using high pressure conditions for 15 min (95°C, PBS washes twice, for 5 min each time). An H_2O_2 solution (3%) was used and endogenous peroxidase and biotin were added to the sections for incubation for 10 min at 7°C. The serum of the non-immunized animal was added to every section for sealing with an indoor temperature of 20°C. The mouse anti-human OPN antibody was diluted at 1:100, and 50 μ L anti-I were added for overnight incubation at 4°C. The slices were washed with PBS 3 times, for 5 min each time. Anti-II was added to the samples and allowed to stand for 30 min at room temperature. The samples were subsequently washed 3 times with PBS for 5 min each time. The reagent diaminobenzidine (DAB) was used to develop the color for the detection of the proteins. The degree of tissue staining was continuously observed under a microscope and the cells were washed by tap water for 10 min and placed into a hematoxylin solution. The samples were removed from the solution following 5 min of incubation and were washed with tap water for 10 min. The tissues were dehydrated and mounting solution was added. The detection for CD44 was conducted according to the methodology described above.

Immunohistochemistry

The positive expression of osteopontin in the cells was noted in endochylema, while the positive expression of CD44 was mainly noted in the cell membrane. A double-blind method was used in order to observe every section independently. Every section was randomly observed under 5 views with a high power lens. The scores were measured according to the degree of cell staining. The staining intensity was the major determinant of the evaluation of pathology noted for the majority of the cells. The scoring system was as follows: 0 indicated no positive cells, yellow staining was denoted as 1, claybank staining as 2, sepia staining as 3. The percentage of total

tumor cells that were positive and included less than 5% staining were classified as 0, the percentage of tumor cells that were positive for 5-25% of staining as 1, for 26-50% as 2, and for higher than 51% as 3. The sections were classified into 4 grades according to the two scores: 0 denoted negative, 1-3 weakly positive (+), 4-6 positive (++) and 7-9 strongly positive (+++).

Statistical analysis

The SPSS 19.0 software (SPSS, Chicago, IL, USA) was used for data analysis. Mann-Whitney rank-sum test was used to analyze the differences between groups. Spearman's rank correlation was used to analyze the variables. A P value less than 0.05 ($P < 0.05$) was considered statistically significant.

RESULTS

Expression of osteopontin

The positive expression of osteopontin was mainly noted in the endochylema of the meningioma tissue. Osteopontin expression was negative in normal meningioma, while its expression in meningioma tissues was positive and in some cases was associated with strong staining. The difference noted between these two groups was significant ($P < 0.001$).

The expression levels of osteopontin in grades I and II meningioma tissues exhibited significant differences ($P < 0.001$), whereas the same result was noted between grades I and III tissues ($P < 0.001$). In contrast to these groups, the difference between grade II and III with regard to osteopontin expression was not significant (> 0.05). The expression levels of osteopontin were positively correlated with the grade of meningioma ($r = 0.417$, $P < 0.001$).

The positive expression levels of osteopontin between non-invasive and invasive meningioma tissues exhibited significant differences ($P < 0.001$) in the experimental group, while the expression levels in the invasive meningioma were mainly positive and strongly positive.

In the experimental group, the positive expression of osteopontin correlated with peritumoral edema of meningioma ($r = 0.587$, $P < 0.001$). The severity of the peritumoral edema was associated with the positive expression of osteopontin (Table I)

Table I. Osteopontin expression in cell tissues.

Category		Expression of osteopontin (number of cases N)				P
		-	+	++	+++	
Group	Control group	22	5	3	1	<0.001
	Experimental group	9	8	14	23	
Grade	I	7	5	3	3	-
	II	1	2	4	10	-
	III	1	1	6	11	-
Non-invasive		7	5	4	2	<0.001
Invasive		2	3	10	21	
Edema degree	0	4	3	2	4	-
	1	3	3	2	3	-
	2	1	1	3	4	-
	3	1	1	6	13	-

Comparison of I with II, $P<0.001$; comparison of I with III, $P<0.001$; comparison of II with III, $P>0.05$; comparison of 0 with 1, $P=0.793>0.05$; comparison of 0 with 2, $P=0.115>0.05$; comparison of 0 with 3, $P=0.001<0.05$; comparison of 1 with 2, $P=0.172>0.05$; comparison of 1 with 3, $P=0.002<0.05$; comparison of 2 with 3, $P=0.158>0.05$.

Table II. Expression of CD44 in tissues.

Category		Expression of CD44 (number of cases N)				P
		-	+	++	+++	
Group	Control group	19	6	2	3	<0.001
	Experimental group	9	9	13	23	
Grade	I	6	6	4	2	-
	II	2	2	4	9	-
	III	1	1	5	12	-
Non-invasive		7	6	4	2	<0.001
Invasive		3	3	9	20	
Edema degree	0	2	1	4	6	>0.05
	1	1	2	4	4	
	2	1	4	3	2	
	3	4	3	2	11	

Comparison of I with II, $P<0.001$; comparison of I with III, $P<0.001$; comparison of II with III, $P=0.879>0.05$.

Expression levels of CD44

The positive expression of CD44 exhibited intense staining of the cell membrane. In normal meninx tissues, the staining indicated negative expression, whereas in meningioma tissues, positive and strongly positive staining was noted. The expression levels between these two groups were significant ($P<0.001$).

The positive staining of CD44 was increased according to the grade of meningioma in the experimental group, and its expression levels in meningioma correlated with the grade of meningioma ($r=0.503$, $P<0.001$).

The expression levels of CD44 between non-invasive and invasive meningiomas exhibited significant differences ($P<0.001$) in the experimental

group, whereas positive and strongly positive staining in invasive meningioma corresponded to the expression levels of CD44.

In the experimental group, the positive staining of CD44 that indicated its expression exhibited no correlation with the degree of peritumoral edema and meningioma ($r=0.033$, $P=0.732>0.05$). The data are shown in Table II.

Correlation between the expression levels of osteopontin and CD44 in meningioma tissue

The positive expression levels of osteopontin and CD44 exhibited no significant differences in meningioma tissue ($P=0.752>0.05$).

Table IV indicates that the expression levels of osteopontin and CD44 in meningiomas correlated with the grade of meningioma ($r=0.961$, $P<0.001$).

DISCUSSION

The present study indicates that the expression levels of osteopontin were stained negatively in the normal meninx tissue, while its expression in meningioma tissue was stained positively and strongly positive ($P<0.001$). Takano et al. (13) demonstrated that the expression of osteopontin

in glioma was apparently higher than that noted in normal brain tissue. Wong et al. (14) indicated that the expression levels of the osteopontin gene were higher in the cancerous compared with the normal cervical tissue. The aforementioned studies indicate that the high expression of osteopontin exhibits a correlation with the development of the tumor, which is consistent with the data presented in the current study. In addition, the mechanism of the formation of peritumoral edema of meningioma has not been fully discovered, although it is deduced that it may have a correlation with high levels of osteopontin expression. Taken collectively, the results suggest that osteopontin plays an important role in the occurrence, development and evolution of meningioma.

Pham et al. (15), using gene knockout experiments, demonstrated that breast cancer stem cells lose CD44 immunophenotyping following CD44 knockout. Moreover, the cells became devoid of the original stem cell features and the tumorigenic characteristics thus leading to the formation of normal non-cancer stem cells. Based on this study, the positive expression of CD44 may correlate with the occurrence of meningioma, which is consistent with the data of the present study. Taken collectively,

Table III. Expression levels of osteopontin and CD44 in meningioma tissue.

Category	Expression (number of cases N)				p
	-	+	++	+++	
Osteopontin	9	8	14	23	0.752
CD44	10	9	13	22	

Table IV. Expression levels of osteopontin and CD44 in meningiomas.

Grade	Expression (number of cases N)							
	Osteopontin				CD44			
	-	+	++	+++	-	+	++	+++
I	7	5	4	2	6	6	5	1
II	2	1	4	10	2	2	4	9
III	1	1	6	11	1	1	5	12

the data suggest that the positive expression of CD44 is increased with pathological grading, the grade of malignancy and the extent of invasiveness, although it may exhibit no correlation with the presence of peritumoral edema.

Furthermore, the expression levels of osteopontin and CD44 in all meningioma grades were similar, and the positive staining rate that corresponded to the expression levels of these two proteins correlated with each other in meningioma tissues ($r=0.961$, $P<0.001$). The data suggest that both proteins exert synergistic effects on the occurrence, development and evolution of meningioma. In conclusion, osteopontin and CD44 play an important role in the occurrence, development and evolution of meningioma.

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LETTER TO THE EDITOR

THE IMPLICATION OF DIABETES METABOLOMICS IN THE EARLY DIAGNOSIS AND PATHOGENESIS OF PANCREATIC CANCER

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The aim of this study was to analyze metabolite differences in pancreatic cancer and diabetic patients, to better diagnose these diseases. Gas chromatography-mass spectrometry was used to evaluate the metabolomic differences in blood samples of 50 pancreatic patients, 50 diabetic patients and 50 healthy people. Metabonomic data was analyzed with Primary component analysis and discriminant analysis. The results show that pancreatic cancer patients, diabetic patients and healthy people can have significantly distinct metabolite profiles. Upregulated metabolites in the serum of the diabetic group included sugars (glucose, fructose), cholesterol, tyrosine and phosphoric acid and other substances, and down-regulation was observed in lactic acid, glycine, alanine, glutamine, proline, citric acid and other substances. It is indicated that identification of the most common changes in specific markers between the two diseases, can provide a new perspective and experimental basis for a better understanding of the metabolic differences and the pathogenesis of the two diseases in future. The present study sheds new light on the diagnosis of pancreatic cancer and diabetes.

To the Editor,

Pancreatic cancer is an aggressive type of cancer with high incidence rates, and symptoms usually only evident in the advanced stage (1). Novel methods for early diagnosis and prognosis are needed for a better management of these patients. Recently, metabolomic approaches have been used to identify new markers for diagnosis and prognosis in different types of chronic diseases and cancer (2). The serum contains a large number of amino acids, organic acids, amines, carbohydrates and steroids, with strong polarity and low volatility, which usually need to be derivatized before analysis (3). Derivatization not only extends the gas chromatographic range but also helps to discriminate between compounds with similar structure. At the same time, issues

related to carrier absorption and column wall in the sample with high polarity and low volatility can be eliminated and peak shape can be improved (4). Carbohydrates, steroids and amino acids may not be easily detected with liquid chromatography-mass spectrometry (LC/MS) because of the non-retention of the chromatographic system or the hard ionization of the structure, though good results can be obtained with gascromatografia-spettrometria di massa (GC/MS) after derivatization (2). Thus, GC/MS and LC/MS together can allow the evaluation of a complete metabolic spectrum and, in particular, associations between different metabolic pathways (4).

In this study, N,O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) was used as a derivatization reagent to characterize and identify

Key words: pancreatic cancer, metabolomics, diabetes

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endogenous metabolites in serum of pancreatic cancer patients, diabetic patients and normal subjects by using metabolomics based on GC/MS. Our aim was to find new biomarkers for the two diseases and evaluate changes in endogenous substances levels, in patients with pancreatic cancer or diabetic background.

MATERIALS AND METHODS

Experimental equipment

The experimental equipment included Thermo DSQ single-quadrupole rod gas chromatography, spectrum 5417R ultra-speed low-temperature centrifuge (Eppendorf, Germany), XW-80A whirlpool mixer (Shanghai Qingpu Puxi Instruments, China), TGL-16G low-speed centrifuge (Shanghai Anting Science Instruments, China), BT224S one ten thousandth analytical balance (Sartorius AG Co., Ltd., Germany) and KS2200B Ultrasonic Cleaner (Kunshan City Ultrasonic Instrument Co., Ltd., China).

Experimental reagents

The main experimental reagents used were amino acid standards, free fatty acid standards and sugar standards (ANPEL Laboratory Technologies Co., Ltd., Shanghai), L-2-chlorophenylalanine (Sigma-Aldrich, USA), bis-trimethylsilyl-trifluoroacetamide (BSTFA) (Sigma-Aldrich, USA), pyridine, methoxyamine (Sigma-Aldrich, USA), Meth-Q deionized water (Millipore, USA), acetonitrile (ACN), methanol (MeOH) chromatographically. (Merck, Germany).

Sample collection

Blood samples of 50 patients with pancreatic cancer (PC group) were provided by the fourth Affiliated Hospital, School of Medicine, Zhejiang University. The patient's age, gender and other basic information and medical history were obtained from medical records. All samples included in the study were confirmed with pathological findings.

Fifty diabetic patients (MD group) in the hospital were enrolled in the study and blood samples were collected. In cases where blood glucose levels were higher than 7mmol/L before dinner or higher than 11.1mmol/L after dinner, diabetes was considered as a factor affecting glucose levels.

Blood samples from 50 volunteers, considered as the control group of healthy participants, were also provided by the university medical college affiliated hospital. The basic information of all healthy subjects such as age (range 41-70 years) and sex was recorded. Subjects with previous medical history of metabolic diseases such as cancer, diabetes and heart disease were excluded. The PC group consisted of 30 male and 20 female patients while the DM group consisted of 28 male and 22 female patients. Healthy volunteers were equally distributed according to sex. All the subjects (ages: 41-70 years) enrolled in this study signed an informed consent and the study was approved by the ethics committee of the fourth Affiliated Hospital, School of Medicine, Zhejiang University. The prior to sample collection, the patients were recommended to consume light food, avoid seafood and spicy products. Smoking and consuming alcohol were recommended to be avoided to reduce fluctuations in metabolism caused by these factors. To reduce the metabolic error caused by factors other than the disease, age- and gender-matched patients and volunteers were selected for the study. Blood was collected before breakfast and the serum was separated and immediately frozen at -80.

Treatment and derivatization of serum metabolites

A mixture of 280 μ L cold acetonitrile/methanol (MeOH: ACN = 2: 1; v/v) was added into 70 μ L serum, then 50 μ L of L-2 chlorophenylalanine methanol solution 50 μ g/mL (internal standard) was added. The mixture was kept at 4°C for 10 min after vortex mixing for 2 min, and then it was centrifuged at 14000 rpm for 15 min. 250 μ L of the supernatant was added to the GC vial and the solvent was evaporated under reduced pressure. 80 μ L of the methoxyamine pyridine solution (15 mg/mL) was added to the GC vial, vortexed and oxidized at 80°C for 30 min. 100 μ L of BSTFA (containing 1% TMCS as catalyst) was added to the sample, vortexed for 1 min, and subjected to trimethylsilylation at 80°C for 30 min and then injected into the analyzer. The QC2 sample was handled in the same way as the experimental sample

Chromatography mass spectrometry conditions

Derivatized extracts were determined by ThermoDSQ single quadrupole gas chromatography mass spectrometry. Chromatographic column: ColumnThermoTR5-MS. 1 μ L derivatized sample was injected without split. The

temperature of the injection port was 260°C, and the ion source temperature was 200°C. The programmed temperature rise mode was used, the column temperature was initially set at 80°C for 2 min and then the temperature increased from 80°C to 300°C with a speed of 10°C/min for 5 min. The total analysis time was 30 min, and the solvent removal time was 5 min. The carrier gas was helium; the carrier gas flow rate was 1 mL/min. The ionization mode EI was the mass spectrometry condition. The ionization voltage was 70 eV, and the mass spectrometry scanning range was m/z 50-800.

To avoid experimental bias or random errors, samples of patients and healthy volunteers were analyzed in random order. In addition, two kinds of QC samples were analyzed between runs of 10 experimental samples.

Identification of metabolomic differences

The retention time and mass-to-charge ratio of the difference variables were compared with the retrieved result of the NIST library (matching rate > 70%) for preliminary identification. Then, the identified organic acids, carbohydrates and amino acids were identified by standard. The GC/MS system automatically acquires the total ion chromatogram (TIC) and the fragmentation patterns (5). Each compound was individually cleaved by MS into a series of molecular ion fragments to form a characteristic ion fragment of the compound. The GC/MS system software automatically compares the mass-to-charge ratio and abundance of each of the ionic fragments with the chemical standard ion fragmentation patterns library of NIST library, to analyze the matching degree of the detection substances and standard substances. In general, values of the matching degree > 70% for the detection compound can be considered as the reference substance.

Statistical analysis

Unclassified Principal Component Analysis (PCA) was used to compare the three sets of data to analyse multivariate data and to increase statistical significance, thus a PCA model with four components was obtained.

To eliminate the impact of factors other than the disease, the three sets of data were further analyzed by OPLS-DA model to obtain a model with one predicted component and two orthogonal components.

In order to compare the differences between the serum metabolic profiles in groups of pancreatic cancer patients and healthy participants, pancreatic cancer patients and diabetic patients, diabetic patients and healthy subjects, OPLS-DA model was established using data of two groups.

Student's *t*-test was used to verify the differences in variables selected by the above three multidimensional statistical models. Differences $p < 0.05$ were considered statistically significant.

RESULTS

Comparison of serum GC/MS fingerprints between patients and healthy subjects

Serum samples were collected 3 months to 1 year post-diagnosis. The serum of pancreatic cancer patients, diabetic and healthy subjects was analyzed by GC/MS, and chromatographic peak separation was good as shown in the representative total ion chromatograms, in Fig. 1A. Differences in the composition and proportion of serum metabolites among the three groups are clearly seen in the total ion chromatogram of each group. Differences in the response intensity of glucose (16.99 min) and lactic acid (5.94 min) were the most significant. Changes

Table I. OPLS-DA model evaluation parameters.

Model Group		PC number	R2X(cum)	R2Y(cum)	Q2Y(cum)	Intercept R2	(RPT) Q2
PC Health	vs	1+2	0.640	0.983	0.976	0.035	-0.301
DM Health	vs	1+1	0.670	0.978	0.972	0.359	-0.219
DM vs PC		1+2	0.688	0.992	0.988	0.410	-0.359

PC: primary component. R2X, R2Y and Q2Y are OPLS-DA model parameters

in metabolite concentrations reflect differences in the physiological status of the three groups of subjects.

Multidimensional statistical analysis

The cumulative explanation rate (R2Xcum) of the PCA model was 69.4%, but the prediction rate (Q2cum) of the model was only 16.2%.

As can be seen from the three-dimensional model (Fig. 1B) composed by PC1, PC2 and PC3, the serum metabolic spectrum varied greatly among the pancreatic cancer patients, diabetic patients and healthy volunteers.

The results obtained from the OPLS-DA model

showed that pancreatic cancer patients and diabetic patients were completely differentiated from healthy subjects. The OPLS-DA model using data of two groups (Fig. 2) showed that the two groups are clearly distinguished. The explanatory rate R2Ycum of the model obtained after 7 times, the circulation interactive validation was higher than 0.960, and the prediction rate Q2Ycum was higher than 0.90. The results showed that the model was stable and reliable. The results of the response sequencing test showed that the intercepts of R2 and Q2 regression line meet the requirements, proving that the model has not been fitted (Table I, Fig. 2)

Table II. Serum metabolic differences based on GC-MS.

N	Metabolites	DM patients vs healthy controls		PCF patient vs healthy controls		DM patients vs PC patients		Pathway
		P^a	FC_b	P^a	FC_b	P^a	FC_b	
1	Lactate	3.49×10^{-5}	-2.48	2.69×10^{-4}	-1.72	2.8×10^{-4}	-1.45	Glycolysis
2	Fructose	1.49×10^{-7}	2.59	2.25×10^{-5}	2.20	3.49×10^{-5}	1.19	Sucrose metabolism
3	Glucose	2.2×10^{-3}	3.63	3.2×10^{-3}	1.80	3.49×10^{-5}	2.10	Sucrose metabolism
4	Glycine	7.7×10^{-5}	-1.55	—	—	3.49×10^{-5}	-2.06	Gly/Ser/Thr metabolism
5	L-alanine	0.030	-1.2	0.040	1.15	3.49×10^{-5}	-1.40	Alamine metabolism
6	L-leucine	—	—	1.19×10^{-4}	3.90	3.49×10^{-5}	-2.39	Val/Leu/Iso metabolism
7	Cholestrol	4.47×10^{-6}	1.57	1.2×10^{-4}	1.29	3.49×10^{-5}	1.20	
8	Hexadecaniocacid	—	—	6.4×10^{-5}	2.10	3.49×10^{-5}	-2.15	Fatty acid metabolism
9	Glutamine	3.59×10^{-6}	-2.70	—	—	3.49×10^{-5}	-3.15	Amino acid metabolism
10	L-Serine	9.48×10^{-7}	-1.79	—	—	3.49×10^{-5}	-2.29	Amino acid metabolism
11	L-Proline	3.49×10^{-4}	—	—	—	3.49×10^{-5}	-1.44	Amino acid metabolism
12	L-Tyrosine	8.3×10^{-4}	2.89	1.45×10^{-4}	1.49	3.49×10^{-5}	1.98	Amino acid metabolism
13	L-Phenylalanine	—	—	7.99×10^{-5}	1.60	3.49×10^{-5}	1.20	Amino acid metabolism
14	Phosphoric acid	4.39×10^{-7}	2.39	—	—	3.49×10^{-5}	1.80	
15	Critic acid	8.55×10^{-7}	-2.89	—	—	3.49×10^{-5}	-3.94	
16	1,2-benzenedicarboxylic	6.9×10^{-6}	1.60	—	—	—	—	
17	Prpanoic acid	9.5×10^{-5}	1.57	—	—	—	—	

Analysis of differences

According to the OPLS-DA supervised multidimensional model established by the above groups, the variables with $VIP > 1$ were selected as factors separating the two groups.

Finally, variables with significant contribution and statistical significance to the distinction of the groups are listed in Table II. In this study, about 44 compounds, including organic acids, amines, sugars, amino acids, fatty acids and other small molecular metabolites, were identified from the GC/MS spectra of each group according to the NIST library and standard.

As depicted in Table II, metabolites that were up-regulated in the serum of the diabetic group included sugars (glucose, fructose), cholesterol, tyrosine and phosphoric acid and other substances, while down-regulated metabolites were lactic acid, glycine, alanine, glutamine, proline, citric

acid and other substances. Compared to the control group, serum metabolites in patients with pancreatic cancer including carbohydrates (fructose, glucose), cholesterol, alanine, leucine, tyrosine and phenylalanine were upregulated, whereas lactic acid levels were decreased.

Glucose, fructose, lactate, alanine, cholesterol and tyrosine levels were significantly different between the two groups, and variation trends of lactic acid, glucose and tyrosine levels are shown in Fig. 3. Analysis in metabolites of glucose metabolism, glycolysis and some amino acid metabolism revealed various changes in diabetic and pancreatic cancer patients. Compared to healthy volunteers, there were no significant changes in levels of leucine, hexadecanoic acid and phenyl alanine in the MD group, however, leucine, hexadecanoic acid and phenylalanine were significantly upregulated in the PC group. Moreover, these three metabolites

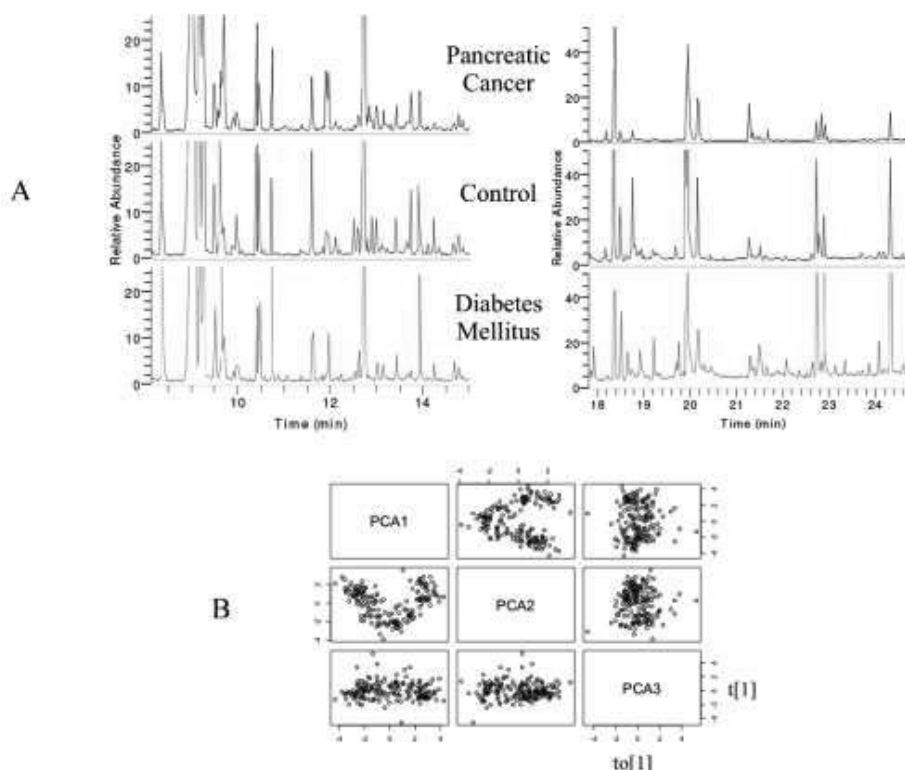


Fig. 1. Comparison of serum GC/MS fingerprints between patients and normal subjects **A)** Typical chromatogram of BSTFA derivatization. **B)** Scores of GC/MS spectral data from serum samples of pancreatic cancer patients, diabetic patients and healthy volunteers (PCA1: the first primary component, PCA2: the second primary component, PCA3: the third primary component)

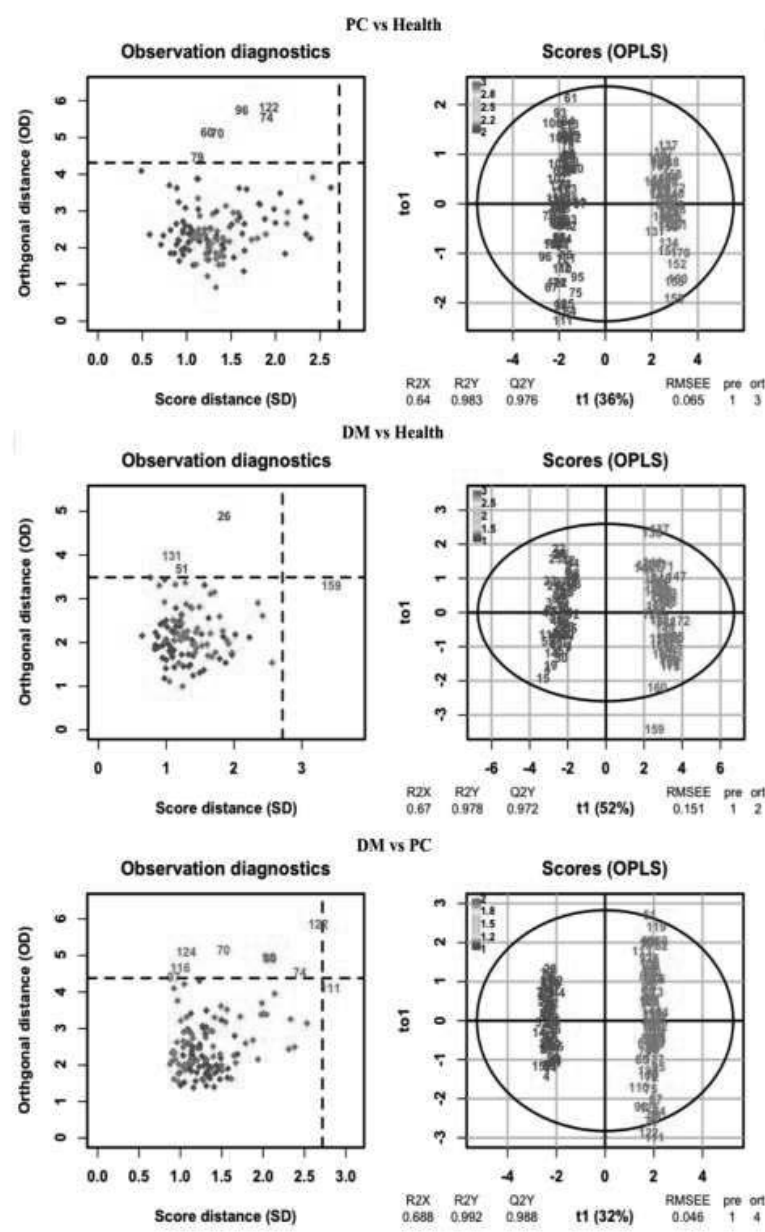


Fig. 2. OPLS-DA observation diagnostics and scores after pairwise comparison.

were significantly different among diabetic and PC patients. Therefore, changes in leucine, hexadecenoic acid and phenylalanine were distinctive for the pancreatic cancer group. Accordingly, glycine, glutamine, serine, proline, citric acid and phosphoric acid can be considered as distinctive metabolites for the MD group.

DISCUSSION

In this study, application of metabolomics in diabetic patients, patients with pancreatic cancer, diagnosed with histochemical and immunohistochemical techniques (6), and healthy subjects resulted in the distinction of these groups

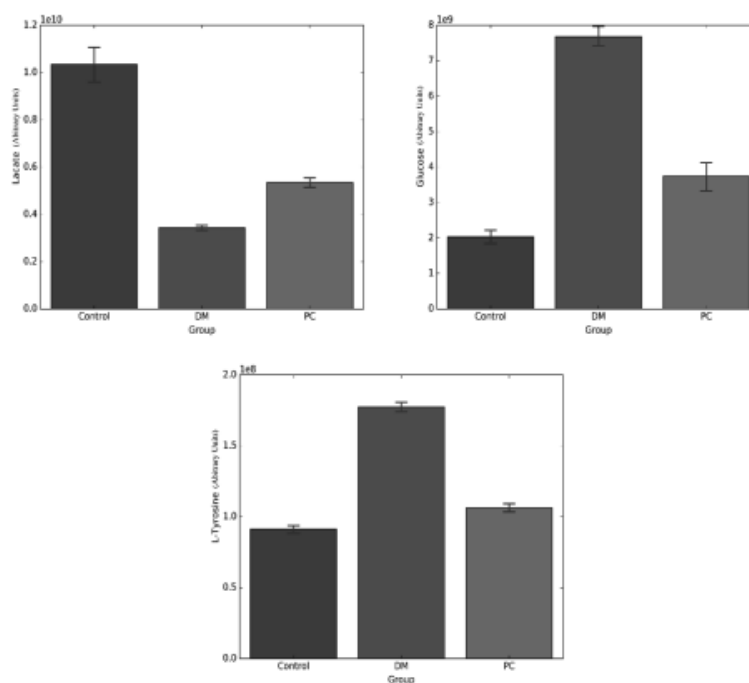


Fig. 3. Barplots of lactate, glucose, and L-tyrosine from different groups (mean area $\pm$ standard deviation).

based on the structure of endogenous metabolites, indicating that pancreatic cancer and diabetic patients are characterized by alterations in specific metabolic pathways. Such metabolic disorders may also occur in other liver disorders such as nonalcoholic fatty liver and vitamin K-dependent coagulation disorders or obesity and related diseases determined by exposure to endocrine disorders (7-9).

Of note, significant increase in glucose and fructose levels in the serum of patients with diabetes and pancreatic cancer were identified, which can be attributed to the insulin resistance and reduced sugar tolerance present in these patients.

Lactic acid is the end-product of glycolysis. Patients with pancreatic cancer and diabetes had significantly decreased levels of this metabolite. Other studies also suggest that glycolysis increases in cancer cells, leading to a down-regulation of tricarboxylic acid cycle and to an increased lactic acid level in serum (10). Detecting changes in Cys-C

levels can contribute to early diagnosis and treatment of diabetic retinopathy. Elderly patients with high Cys C level and long course diabetes are more predisposed to develop diabetic retinopathy (11). Citric acid, an important intermediate metabolite of tricarboxylic acid cycle (TCA), was reduced in diabetic patients, suggesting that TCA is disturbed, in line with other studies from literature (12).

Compared to healthy subjects, serum cholesterol levels of diabetic patients and pancreatic cancer patients were significantly higher. It was shown that cholesterol is closely related to the occurrence of diabetes and pancreatic cancer because increased cholesterol level damages the capillary circulation and weakens the vasodilatation function and can determine insulin resistance (13).

Phenylalanine was significantly increased in serum of patients with pancreatic cancer compared to diabetic patients and healthy people, whereas, levels of tyrosine were significantly increased in

patients with diabetes mellitus and pancreatic cancer were significantly increased compared to controls. The increase of tyrosine may be associated with the activation of catecholamine metabolic pathways, while the increase of phenylalanine may produce toxic substances that could affect the activity of some metabolic enzymes (14).

Compared to healthy volunteers, pancreatic cancer and diabetic patients shared common changes in sugar levels, amino acid metabolism and cholesterol. However, alterations in distinct metabolic pathways can distinguish patients with pancreatic cancer from diabetic patients, suggesting the importance of metabolism in these two diseases.

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LETTER TO THE EDITOR

HOUSE DUST MITE SUBLINGUAL-SWALLOW IMMUNOTHERAPY IN PERENNIAL RHINITIS: A DOUBLE-BLIND, PLACEBO-CONTROLLED IRANIAN STUDY

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Sensitivity to house dust mite allergens in the development of allergic rhinitis has a key role. In this study, the clinical and immunological effects of high dose *Dermatophagoides farinae* sublingual immunotherapy (SLIT) versus placebo were compared. Forty poly-sensitized patients, ages 6-33 years, with allergic rhinitis and positive allergic reaction to the mites were enrolled in the study. Twenty-one patients were placed in the SLIT group and 19 in the placebo group. Expression levels of IL-10, TGF- β , FOXP3 and IL-17 were measured by using real-time PCR before and after the administration of sublingual immunotherapy. Clinical efficacy was estimated by the reduction rate of symptom/medication scores in the SLIT group compared with placebo treatment. After 6 months of SLIT, TGF- β expression levels were increased compared to pretreatment ($P < 0.05$). SLIT with *D. farinae* extract is an effective treatment for poly-sensitized patients with allergic rhinitis. TGF- β mediated T-cell suppression may be an important mechanism in the first 6 months of SLIT.

To the Editor,

Allergic rhinitis can be effectively controlled in many patients with oral antihistamines and nasal corticosteroids but the allergy reoccurs after cessation of these drugs (1, 2). It is believed that allergen immunotherapy is the main successful approach to change the natural history of the disease (3-5). In this respect, subcutaneous immunotherapy is widely applied in the world for the treatment of allergic respiratory disease. Due to safety concerns and repeated injections which have led to serious complaints, particularly among children,

alternative forms of administration have been studied (6). During the two last decades, sublingual immunotherapy (SLIT) was gradually introduced in clinical practice, with the primary aim of improving safety and convenience (7). The efficacy of SLIT has been demonstrated in numerous clinical trials and confirmed by several meta-analyses (8). In addition, SLIT provides long-term clinical benefits, including long-term remedy, prevention of new sensitizations, and reduction in disease progression from rhinitis to asthma which rarely takes place in pharmacotherapy (5-7).

Key words: house dust mite allergens, SLIT, allergic rhinitis

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Sensitivity to house dust mite allergens is very common in some regions of Iran (9) therefore we conducted a double-blind, placebo-controlled trial of high-dose SLIT in patients with allergic rhinitis sensitized to the house dust mite *Dermatophagoides farinae*. The clinical symptoms and immunological factors were evaluated in these patients.

MATERIALS AND METHODS

Patients

Forty-seven patients, ages 6-33 years, with a history of perennial allergic rhinitis agreed to take part in this clinical trial. IgE-mediated allergic rhinitis was proved by positive skin prick tests to *D. farinae* standardized extract in all patients (Dome Hollister, USA). All patients had positive skin prick tests to at least two other inhalant allergens and underwent a spirometry test. The patients who showed symptoms of persistent asthma or chronic obstructive pulmonary disease, used oral steroids or had previously received immunotherapy, kept a pet at home, or had any other significant illness, such as autoimmune diseases or cancers, were excluded from the study. The study protocol, with approval Code IRCT2017042323235N9, was approved by the Ethics Committee of the Mashhad University Medical Sciences, Iran, and all patients signed informed consent forms before starting the treatment.

Allergen preparation

The standardized allergen and placebo of mite, *D. farinae* for immunotherapy was kindly provided by Zhejiang Wolwo Bio-Pharmaceutical, China. The drops were prepared in five different concentrations and labelled as No. 1, No. 2, No. 3, No. 4 and No. 5. The placebo was similar to the "*D. farinae* drops" in the smell, packaging, volume, storage conditions and labeling.

Study design

This was a single-center, randomized, double-blind, placebo-controlled study. Subjects were divided into two age- and sex-matched groups, one with 21 patients and the other 19. Then the groups were randomly placed into SLIT or placebo treatment, respectively.

The drops were prepared in 5 concentration, No. 1 had 1 µg/ml protein concentration and No. 5, which was the highest concentration, had 1000 µg/ml. The dose gradually

increased and the maintenance daily adult dose in was 2 drops of 1000 µg/ml protein concentration and the pediatric dose was 3 drops of 333 µg/ml (No. 4). Subjects were instructed to hold the drops under the tongue for two minutes and not to eat or drink for 15 minutes.

The length of the study was six months, initiated in June 2010 and completed in January 2011. Subjects filled weekly cards recording their symptoms and medication use. They were visited weekly in the build-up phase and monthly in the maintenance phase. The symptom scores were recorded weekly.

Rescue medication score

The following were values for each time of use:

Oral Loratadine, 10 mg, 0.5 point

Oral Telefast 120 mg, 1 point

Nasal corticosteroids, Flixonase, 2 points

The expression levels of IL-10, TGF-β, FoxP3, and IL-17 were also evaluated before and after treatment. Quantitative PCR (qPCR) for human FOXP3, GITR, and β-2-microglobulin (β₂M) was carried out on cDNA samples using the TaqMan® Master Mix (Takara, Japan) on Rotor-Gene System (Q-6000 Machine; Qiagen, Germany). qPCR for human IL-17, IL-10, TGF-β and β₂M on cDNA samples were carried out using SYBR Green I Master Mix (Takara, Japan).

Statistical analysis

All data analysis was performed using SPSS 11.5 for windows TM version software package (SPSS Inc. Chicago, Illinois, USA). Data were expressed as mean ±SD or as a proportion of sample size. All data were checked for normality by Kolmogorov–Smirnov test. Differences in proportions of quality variables were judged by the χ^2 test. Spearman correlation test was used to compare the correlation of laboratory assessments including IL-10, IL-17, FOXP3, and TGF-β before and after the treatment procedure. A two-sided P value <0.05 was considered statistically significant.

RESULTS

Clinical outcome

Of all patients included for evaluation of clinical response in this study, only thirty-four completed the course of treatment (19 in the HDM SLIT

group and 15 in the placebo group). Demographic information of the studied patients is presented in Table I. By examining the degree of symptoms and drug consumption during the 6 months, it was noted that this trend was declining over 6 months in both groups but it was more stable and prominent in the SLIT group. In our study, clinical efficacy was evaluated by comparison of the reduction rate in symptom/medication score between the two groups. There was a significant reduction rate of symptom/medication scores in the SLIT group compared with the placebo group after five months of treatment. For more precise analysis of clinical response according to mailing grading (11), the patients, based on the percentage reduction in the symptom/medication scores, were graded in four groups:

i) no efficacy or poor response (symptom/medication scores improved by <30%);

- ii) low efficacy or fair response (improvement 30-44%);
- iii) moderate efficacy or good response (improvement 45-59%);
- iv) high efficacy or excellent response (improvement of >60%).

Our study showed that all patients with severe rhinitis in the placebo group had a poor response to treatment and the converse majority of patients with severe rhinitis in the SLIT group demonstrated an excellent response to treatment. Details are shown in Table II.

Adverse events

Seven patients (36.8%) in SLIT and 5 (33%) in the placebo group reported adverse events and local reactions in mouth and throat. No severe systemic adverse events, such as anaphylaxis or urticaria, were

Table I. *Anthropometric and baseline data for all patients who completed the course of study.*

Variables (%)		Placebo n (%)	HDM SLIT n (%)
Age	1-18 years	10 (52.6)	9 (60)
	>18 years	9 (47.4)	6 (40)
Sex	Female	10 (52.6)	8 (53.3)
	Male	9 (47.4)	7 (46.7)
Severity	Mild	2 (10.5)	2 (13.3)
	Moderate	8 (42.1)	8 (53.3)
	Sever	9 (47.4)	5 (33.3)

Table II. *Relationship of response and disease severity in SLIT and placebo groups.*

Severity		Response			
		Poor	Fair	Good	Excellent
SLIT	Mild	1	0	1	0
	Moderate	1	1	0	6
	Severe	1	2	0	6
Placebo	Mild	0	0	0	2
	Moderate	1	1	1	5
	Severe	5	0	0	0

observed. None of the patients dropped out from the study due to an adverse event-related condition.

Immunologic responses

Cytokine expression level analyses in both groups revealed a significant increase of TGF- β expression after 6 months of immunotherapy compared with baseline in the SLIT group, while the changes were not significant in the placebo group (Fig. 1).

There was a significant reduction in IL-10 expression levels in the placebo group compared with pretreatment levels. In the SLIT group also there was a reduction in IL-10 expression but it was not significant.

In the SLIT group, there was an increment of

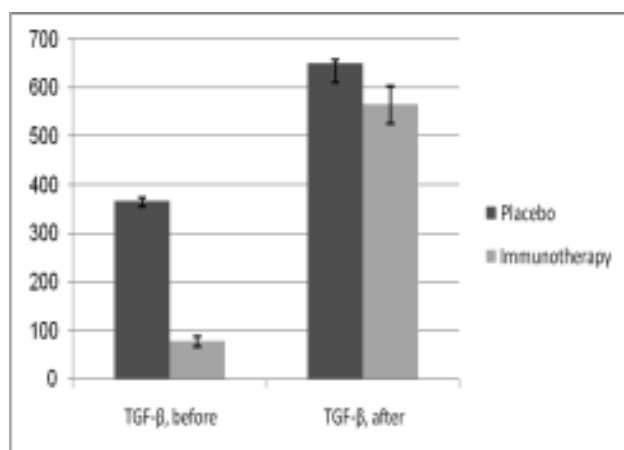


Fig. 1. TGF- β expression after intervention compared with baseline in SLIT and placebo groups.

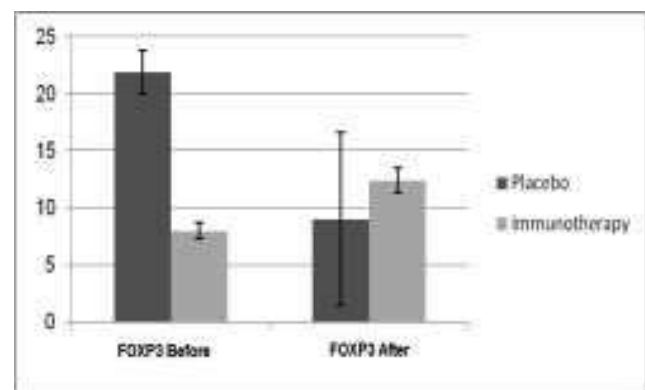


Fig. 2. FOXP3 expression in comparison to pretreatment in SLIT and placebo groups.

FOXP3 expression in comparison with pretreatment. Conversely, in the placebo group, FOXP3 expression was reduced compared with baseline level. There was a significant increment in FOXP3 expression in the SLIT group compared with the placebo group (Fig. 2). At baseline, there was a significant relationship between severity of clinical symptoms and IL-17 levels in both groups; after treatment IL-17 expression levels showed a significant reduction only in the placebo group but not in the SLIT group. There was no significant relationship between other cytokines and the clinical outcome.

There was also a significant correlation between expression of IL-17 and TGF- β in the whole group at the baseline ($P=0.008$, $r=0.67$) and after immunotherapy ($P=0.023$, $r=0.7$). Our results showed no significant correlation between other cytokines except for IL-10 and IL-17 before starting therapy ($p=0.043$, $r=0.54$) in the whole group.

DISCUSSION

The result of our study indicated that there was a significant direct relationship between disease severity and its response to treatment; more severe patients showed better responses. In contrast, in the placebo group there was an inverse relationship between severity and response to treatment. Accordingly, it seems the effect of SLIT in cases of severe rhinitis is more prominent. According to some similar clinical trials, the most common adverse reactions of SLIT include local reactions in the mouth, such as pruritus and a burning sensation of lips and tongue (7). In summation, according to our data and other reports, SLIT appears to be generally safe and tolerable and improves safety and convenience of immunotherapy (10). The investigations on different mechanisms of immunotherapy resulted in finding new forms of immunotherapy to improve the efficacy, increase safety and shorten treatment courses (11). It appears that there are several possible mechanisms which may explain the efficacy of SLIT. Oral tolerance is definitely not the only mechanism and there is some evidence of Treg involvement (12). Some researchers showed that SLIT had the ability

to increase the expression of FoxP3 and IL-10 after 4 weeks in comparisons with our work that showed TGF- β did not have any change during the course of SLIT after 52 weeks (13).

The study by O'Hehir reached the same conclusion as our research (14). We found that expression of TGF- β was increased in the SLIT group compared with pretreatment, unlike the placebo group. Similar to their work, our results indicated a decline in IL-10 and an increase in Foxp3 levels after immunotherapy. There was a significant increment in Foxp3 expression in the SLIT group compared with the placebo group after 6 months of treatment. Accordingly, our study was aligned with their results which indicated that during the first 6 months of high-dose SLIT there was an increment in FoxP3+Tregs in parallel with TGF- β level. It was hypothesized that the source of TGF- β may be FOXP3+Tregs, and it can also be suggested that TGF- β -mediated immunological changes may be an important mechanism in the first 6 months of SLIT. In another study, the authors observed a significant relationship between TGF- β and severity that we could not demonstrate (15, 16). Our investigation revealed that, although at first there was a significant relationship between disease severity and IL-17 levels, with the initiation of therapy the trend was changed. It was clearly observed that a significant decline in IL-17 expression could occur only in the placebo group. At that time, the IL-17 expression was related neither to the severity of symptoms nor to response to treatment. Therefore, it seems that another factor, apart from disease severity, is involved in the expression of this gene during immunotherapy. Since we know TGF- β is central to the differentiation of TH17 (17), one hypothesis is that the failure in the reduction of IL-17 following a clinically successful SLIT causes a significant increase in TGF- β .

SLIT with *D. farina* extracts is an effective treatment for sensitized patients with allergic rhinitis and its effect is more prominent in severe cases. TGF- β may have an important role in inducing tolerance in the first 6 months of SLIT but it can also induce IL-17 expression.

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LETTER TO THE EDITOR

FK228 RECOVERS THIRAM-INDUCED TIBIAL DYSCHONDROPLASIA IN CHICKEN VIA HYPOXIA INDUCIBLE FACTOR-1 α

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Tibial dyschondroplasia (TD) is a disease of many avian species characterized by an enlarged and avascular lesion in the proximal tibiotarsal bone. The aim of present study was to evaluate the effects of hypoxia-inducible factor-1 α (HIF-1 α) inhibition on thiram- induced TD using synthetic medicine FK228 and the association between HIF-1 α and heat-shock protein 90 (Hsp90). One hundred and fifty broiler chicks were equally divided into 3 groups: control; thiram fed; and FK228 treatment. Expressions of HIF-1 α and Hsp90 genes were analyzed by real-time quantitative polymerase chain reaction (RT-qPCR) on day 10 and 14 post-hatch. Western blot analysis of HIF-1 α and Hsp90 gene was performed to measure the protein levels at the end of the experiment. Results showed that HIF-1 α and Hsp90 levels were significantly ($P<0.05$) up-regulated in the thiram group as compared to the control group. Meanwhile, FK228 (HIF-1 α inhibitor) significantly ($P<0.05$) down-regulated the mRNA and protein levels of HIF-1 α and Hsp90, restored the size of growth plate and diminished lameness. In conclusion, HIF-1 α and Hsp90 play an important role in the formation of avascular growth plate and there is a direct relationship between HIF-1 α and Hsp90 for the progression of TD pathogenesis. Therefore, HIF-1 α may prevent and control TD in broiler chickens.

To the Editor,

Tibial dyschondroplasia (TD) is a skeletal abnormality of broiler chickens in which growth plate cartilage fails to form the bone and a white avascular cartilage plug is formed that causes lameness. Avian growth plates have randomly oriented longer columns of cells and there is a deep penetration of metaphyseal blood supply as compared to mammalian growth plate, which ultimately lead to a more vascular growth plate in chickens (1).

Hypoxia-inducible factor-1 α (HIF-1 α) is a subunit of HIF-1, which is very sensitive to oxygen among other subunits. During hypoxia, highly conserved heat shock proteins (Hsps) are regulated through the HIF-1 α pathway and they act as cellular chaperons for proteins (2). In TD lesions, due to lack of vascularization, hypoxia and low oxygen tension induce inadequate cellular energy production. However, in mammals hypoxia is necessary for differentiation of cartilage and endochondral bone

Key words: bone, FK228, hypoxia-inducible factor-1 α , heat shock protein 90, tibial dyschondroplasia

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development (3). The role of hypoxia and HIF-1 α has been considered in TD incidence in the past few years (4, 5). Hsp90 play an important role in the viability of eukaryotic cells and has been recognized as being highly expressed in avian TD (6). Hsp90 inhibitors have previously been evaluated in TD-affected birds (1).

FK228 (Istodax, Romedepsin, FR901228) is a natural cyclic depsipeptide, a well known antitumor agent and specifically inhibits the HIF-1 α stabilization in hypoxic conditions (7). This is the first study which was conducted using FK228 as an HIF-1 α inhibitor in broiler chicken, and an association between HIF-1 α and Hsp90 was studied in unvascularized avian growth plates for the control and treatment of TD.

MATERIALS AND METHODS

Study design

One hundred and fifty one-day-old broiler chicks were bought from a commercial hatchery (Chia Tai Animal Husbandry Co. Ltd, Wuhan, China) and raised under standard temperature and hygienic conditions. Chicks were randomly and equally divided into two groups initially, a control group (n=50) and a thiram-fed group (n=100). Both groups were fed standard normal diet and the thiram group received an addition of tetramethyl thiuram disulphide (thiram) (Hebei Zanfeng Bioengineering Co., Ltd. Hengshui, China) 50 mg/kg for the induction of disease after 4 days post-hatch (8). At day 8, half of the birds from the thiram fed group were separated and allocated as FK228 treatment group and treated with FK228 (Shanghai Biochempartner Co., Ltd. Shanghai, China) 0.5 mg/kg/d intraperitoneally from day 8 till the end of the experiment. Normal saline was administered to all the control birds. The dose of FK228 was designated based on studies using FK228 to prevent tumor development in mice (7). All three groups were raised for 14 days and the number of lame birds were recorded on a daily basis in each group. Twenty-five birds from each group were euthanized by cervical dislocation on days 10 and 14. The cartilage growth plate of tibial bones were dissected and were fixed in 4% paraformaldehyde and some of the bones were immediately frozen in liquid nitrogen and stored at -70°C for further analysis. TD was scored according to Pines et al. (2005) (8).

All animal experiments were planned and conducted according to the national legislations for animal welfare and were approved by the Institution Animal Care and Use Committee of Huazhong Agricultural University Wuhan, China.

Hematoxylin & eosin (H&E) staining

Tibial bone tissues (n = 6 from each group) were fixed in 10% formalin in PBS overnight at 4°C and then decalcified in 10% EDTA. Bone tissues were processed in a series of graded ethanol, clearing and embedding was carried out in xylene and paraffin, respectively. By using the routine procedures, three sections of 5 μ m tissue were stained with H&E for histological analysis.

RNA extraction and cDNA synthesis

For total RNA extraction, the growth plates of tibial bone were homogenized in TRIzol reagent (Invitrogen, Carlsbad, California, USA) and a final volume of 20 μ L total RNA was transcribed reversely into cDNA using TransScript First-Strand cDNA synthesis kit (TransGen Biotech Co. Ltd, Beijing, China) as specified by the manufacturer. Synthesis of cDNA was performed in a total 20 μ L reaction mixture containing oligo (dT)18, 2xTS reaction Mix and 5 μ L RNA through reverse transcription in a thermocycler (Applied Biosystems CA, USA) at reaction temperature of 42°C for 30 min and 85°C for 5 min.

Reverse transcription qPCR (RT-qPCR)

Quantitative real-time PCR was performed using specific primers designated through Primer Express Software v2.0 (Applied Biosystems), based on published *Gallus gallus* sequences (Table I). Total volume of reaction was 20 μ L consisting of 2 μ L cDNA, specific forward and reverse primers (1 μ L of each), 10 μ L SYBR Premix E Taq and ROX reference dye. Gene and primer specificity and the absence of primer dimer formation was confirmed by performing melting curve analysis. All PCR reactions were run with the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in quadruplex by using SYBR Premix Ex Taq™ kit (Takara, Dalian, China) with the following protocol: initiation with denaturing at 95°C for 30 s, 40 cycles of amplification at 95°C for 8 s, annealing for 30 s and extension at 72°C for 30 s. There were four biological replicates used for RT-qPCR for each group (n = 6). Relative expression of gene

level was standardized according to the glyceraldehyde 3-Phosphate dehydrogenase (GAPDH) gene expression. Relative quantification of each gene was performed using delta Ct (Δ Ct) method and differences between gene expression levels were analyzed by *t*-test and differences were considered significant at $P < 0.05$.

Western blot analysis

The bone tissue samples ($n = 6$ from each group) were homogenized in ice-cold PBS and incubated at 4°C for 2 h. Samples were centrifuged at 13,000 rpm for 10 min to collect the supernatant. Protein concentrations were measure by BCA method (Pierce, Rockford, USA) and were stored at -80°C for further use. Protein samples (40 μg) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gel at 100 mV and were transferred (130 mV for 2 h and 20 min) to polyvinylidene fluoride (PVDF) membranes (Millipore, BioSharp, Anhui, China) which were incubated in 5% skimmed milk at room temperature for 1 h. The membranes were incubated overnight at 4°C with primary anti-Hsp90 mouse monoclonal (1:500 ab 13492; Abcam)

and primary anti-HIF-1 α mouse monoclonal (1:500 ab 6489; Abcam) antibodies. The membranes were washed with PBS (0.01 mol/L, pH 7.4) for 10 min each, then the membranes were incubated with HRP labeled goat anti-mouse secondary antibody (1:4000 dilution) for 1 h at room temperature. Bands were visualized by Chemiluminescence (Beyotime Shanghai, China) and image was obtained with luminescent Image Analyzer (ImageQuant, USA).

Statistical analysis

The data was analyzed by one way ANOVA following the Tukey's test and presented as means $\pm$ SEM. The delta CT method was used to quantify the expressions and the differences between gene expression levels were analyzed by student's *t*-test. The differences were considered statistically significant at $P < 0.05$.

RESULTS

Effect of HIF-1 α inhibition in TD-affected chickens and growth plate morphology

After thiram induction, the birds started showing

Table I. Primers used for qPCR analysis.

Gene	Gene Description	Primer Sequence ('5-3')	Temperature $^{\circ}\text{C}$
HIF-1 α	NM204297	TGAGAGAAATGCTTACACACAG TGATGGGTGAGGAATTGGTTCAC	56
Hsp90	NM_001109785.1	CCTCCTCCATACGTGATGTGTCA GCCTGGGCATTGATGAAGATG	59
GAPDH	NM_204305.1	GCCCAGAACATCATCCCA CGGCAGGTCAGGTCAACA	58

Table II. Severity scores and body weights of broilers in control, thiram and FK228-treated groups.

Parameters	Control	Thiram	FK228
Severity score ¹	0	2	1
Average body weight (g)	270 $\pm$ 5 ^a	160 $\pm$ 7 ^b	212 $\pm$ 9 ^a

^{a-b}Means within the same row with no common superscript differ ($P < 0.05$).

¹Chicks with scores 0 were designated as normal, 1 as mild pathology, while those with scores 2 were regarded as severe pathology. The results are expressed as mean $\pm$ SE ($n=25$).

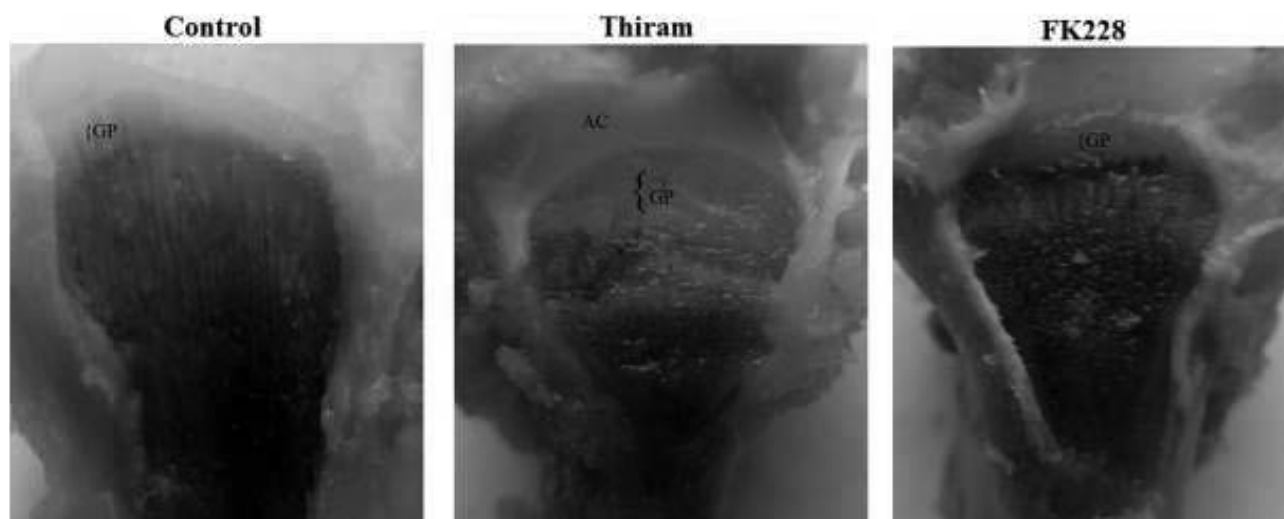


Fig. 1. Effect of inhibition of HIF-1 α activity on growth-plate width and morphology. Control group with normal growth plate size, thiram-induced tibial dyschondroplasia have increased growth plate lesion width while FK228 administration averts growth-plate enlargement. AC: articular cartilage; GP: growth plate.

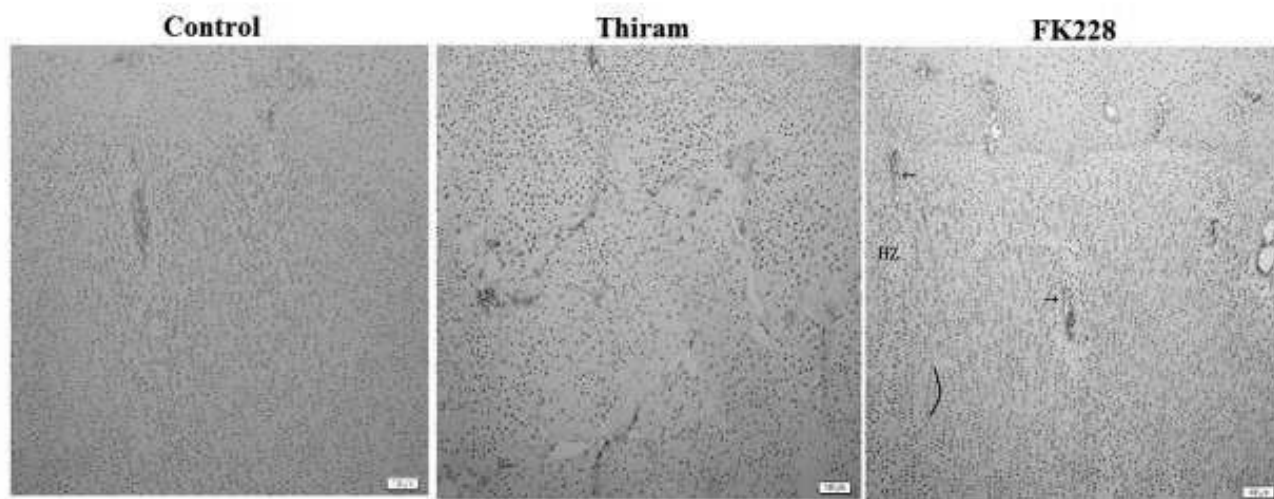


Fig. 2. Growth plate sections stained with hematoxylin and eosin staining (bar = 100 μ m). After FK228 treatment, appropriate chondrocyte disclosure and new blood vessels formation. Arrow indicates blood vessels in the hypertrophic zone. Columnar arrangements of chondrocytes can be seen in parenthesis. (n = 6 for each group). HZ: Hypertrophic Zone.

lameness with reduced feed and water intake, while severity of lameness was increased by day 7 as compared to the control group. At the end of the experiment all the birds were found lame in the thiram group, whereas in response to HIF-1 α inhibition in FK228 treatment group, improvements were found regarding lameness, body weight and

their ability to stand and walk properly as shown in Table II.

The enlarged growth plate section with opaque mass cartilage was found at the proximal side of tibia in the thiram-fed group as compared to the control group. Whereas in the FK228 treatment group, growth plate width was restored by healing,

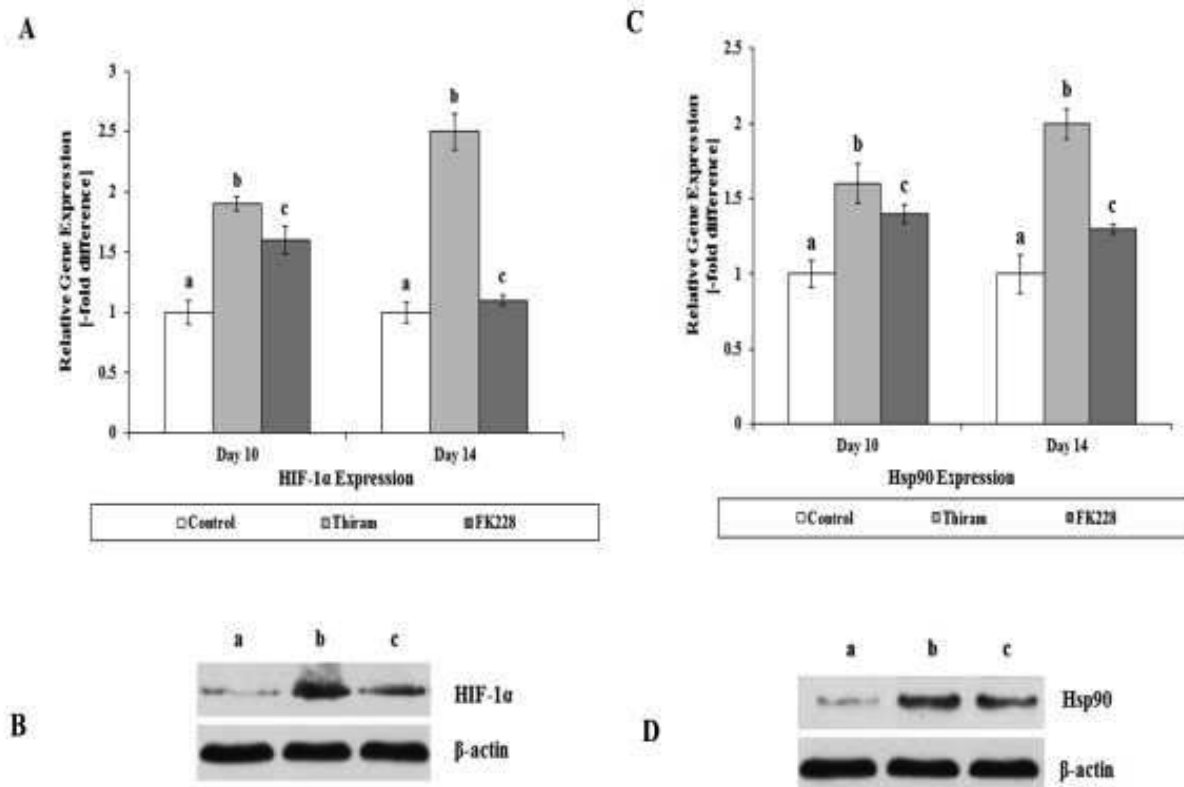


Fig. 3. A) Effects of inhibition of hypoxia-inducible factor-1 α (HIF-1 α) in TD-affected growth-plate. HIF-1 α gene expression levels were evaluated at day 10 and 14 after FK228 administration. The results are expressed in arbitrary units as the means $\pm$ SE ($n = 6$ for each group). Control group set to one thus equivalent to the N-fold difference. ^{abc} letters show significant difference ($P < 0.05$). **B)** Western Blot analysis of HIF-1 α in normal, thiram-fed and FK228-treated growth plates at the end of the experiment (day 14) after the FK228 administration, membranes were also probed with β -actin antibody ($n = 6$ for each group). Band a, control; band b, thiram-induced; band c, FK228 treatment. **C)** Hsp90 mRNA expression was analyzed in chicken epiphyseal growth plates of proximal tibiae isolated from normal, TD-induced, and Fk228-treated chicks. Data expressed in arbitrary units as the means $\pm$ SE ($n = 6$ for each group). Control group set to one thus equivalent to the N-fold difference. ^{abc} letters designate significant difference ($P < 0.05$). **D)** WB analysis of Hsp90 in normal, Thiram-fed and FK228-treated avian growth plates. Protein bands standard the Hsp90 probed with an Hsp90 antibody and membranes were also against β -actin antibody as a loading control ($n = 6$ for each group).

proper calcification and vascularization was found in most of the area on day 14 post-hatch (Fig. 1).

Histological analysis of growth plate

In the control group, chondrocytes were found in the normal columnar arrangement, flattened packed cells, whereas in the thiram-fed TD group chondrocytes were found disorganized, round in morphology, less vascularized and showed improper distinctions between the proliferative and hypertrophic zone. In the Fk228 treatment group, chondrocytes restored their morphology and columnar arrangement with sprouting blood vessels on day 14 (Fig. 2).

Gene expressions of HIF-1 α and Hsp90

To explain the involvement of HIF-1 α and Hsp90 in normal and TD-affected birds, mRNA level of the above genes were measured before and after the administration of FK228. The expression of HIF-1 α and Hsp90 was significantly up-regulated ($P < 0.05$) in the thiram group as compared to controls, however in the FK228 treatment group, HIF-1 α and Hsp90 levels were significantly down-regulated ($P < 0.05$) on day 10 and 14 (Fig. 3, A and C).

Protein analysis of HIF-1 α and Hsp90

Western blotting analysis further confirmed the

protein level of HIF-1 α and Hsp90 in the control, thiram-induced and FK228 groups. WB results revealed an increased protein level of HIF-1 α and Hsp90 in TD-afflicted birds as compared to the control group. The FK228 treatment of TD-induced birds markedly decreased the protein level of HIF-1 α and Hsp90 significantly on day 14 (Fig. 3, B and D).

DISCUSSION

During long bone development, chondrocytes of growth plate starts proliferation and during proliferation they stack together to form a columnar layer of cells. Most distal cells of this columnar layer stop proliferation, leave the cell cycle and differentiate into hypertrophic chondrocytes. These hypertrophic chondrocytes start mineralization of surrounding matrix through the invasion of blood vessels. Apoptosis of hypertrophic chondrocytes allows blood vessel invasion and finally trabecular bone formation occurs by invading cartilaginous matrix (10, 11).

Development of HIF-1 α inhibitors through clinical trials intended for cancer therapy has been studied previously (12). Among the various HIF-1 α inhibitors, FK228 was used to inhibit and bind the activity of HIF-1 α and hypoxia-induced angiogenesis for combating tumor development (7). This study examined the treatment of TD by HIF-1 α inhibitor medicine FK228 and the association between Hsp90 and HIF-1 α in the normal and TD-induced birds. In our experiment, thiram was used for the induction of TD due to its high accuracy in producing this disease (13).

Hypoxia is the major stimulus for the expression of HIF-1 α . TD lesions were hypoxic and up-regulation of HIF-1 α gene expression was reliant on the severity of lesion, while no hypoxia was observed in normal chicken growth plate (4). HIF-1 α is the major client of Hsp90 and regulates angiogenesis. A significant up-regulation of Hsp90 mRNA expressions in TD-afflicted birds in the current study is concomitant with earlier studies (1, 14). Genin et al. (2008) found the increased level of Hsp90 in the TD growth plate was caused by an increase in transcription factor HIF-1 α in response to hypoxia (4). Previously, the Hsp90 inhibition by employing Hsp90 inhibitor

medicine brought the angiogenesis in TD lesion in avian growth plate and finally ameliorated the lameness (14-15).

In conclusion, this is the first study to highlight the role of synthetic medicine FK228 as inhibiting HIF-1 α activity, which restores the morphology of growth plate, and mitigated lameness in broiler chicks.

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LETTER TO THE EDITOR

EYES IN PITUITARY DISORDERS

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The eye is a vital sense organ related to vision, conveying the underlying physical and mental state of well-being of an individual. Eye signs are often associated with endocrinal disorders such as exophthalmos in thyrotoxicosis. However, a thorough eye evaluation may lead to the identification of the early features that help in the diagnosis of various endocrine disorders. This is of vital importance especially in the central nervous system lesions. This is observed more in cases of pituitary mass lesions, which often present with functional hormonal alterations rather than visual symptoms. The definitive therapy has to be provided before it reaches the late stage of the disease which might lead to permanent visual disabilities. Hence, ophthalmologists, endocrinologists, neuro-radiologists and neurosurgeons need to intervene with combined efforts. In this review, we highlight the eye signs in pituitary disorders, along with a brief description of uncommon ocular-pituitary syndromes.

To the Editor,

The pituitary gland is the master gland of all endocrine glands exerting control over all hormonal axes. The gland is located deep inside the cranium in a bony cavity called the sella turcica or pituitary fossa. The pituitary fossa is surrounded by vital structures that are relevant in the visual pathway. Disorders of the pituitary gland present with either hormonal dysfunction or mass effect (1). Previously, there was a prolonged duration between the hormonal dysfunction and the diagnosis of pituitary mass lesions (2). The problem is compounded further in patients with non-functional pituitary adenomas who present only with neuro-ophthalmic features due to the mass effect (3). The delay in diagnosis could also be due to lack of neuro-ophthalmic knowledge of the attending doctors and the cursory examination of the patients, resulting in missing some important initial clues in the presentation (4).

Eyes and pituitary – developmental links

The pituitary gland is developed from the diencephalon portion of the primitive brain. The diencephalon also gives rise to the posterior structures of the forebrain including the thalamus, hypothalamus and the pineal gland. The optic nerve is attached to the diencephalon and the retina is derived from the optic cup, which itself is a derivative of the diencephalon (5). Most of the circadian rhythms of the human body are controlled by the regulated release of the hormones. Melanopsin in the retinal ganglion cells could activate the neuro-endocrine system, leading to alteration in the secretion of the melanocyte stimulating hormone (6). Pituitary adenylate cyclase activating polypeptide (PACAP) is a peptide with various functions that has been isolated from the pituitary and hypothalamus. In addition to the neurological tissues, the PACAP is seen in the eye, ophthalmic artery and other structures and has a

Key words: eye, endocrinology, pituitary disorders, macroadenoma, ophthalmopathy

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potential role in the regulation of the blood supply to the underlying tissues (7).

Eyes and pituitary – anatomical links

The ophthalmic defect which results from pituitary enlargement depends on which surrounding structures become compressed. The pituitary fossa is bound by optic chiasm superiorly, cavernous sinuses on both sides, and sphenoid sinus inferiorly (8). The optic chiasm is the structure where the optic nerves decussate and continue as the optic tracts. Normally the optic chiasm overlies the pituitary fossa directly. The anatomical variations in their relation include the prefixed (fossa overlying the posterior chiasmal notch) and postfixed (fossa overlying the anterior chiasmal notch) positions. The importance in identifying these variations lies in the fact that the enlarged pituitary may compress the optic nerve, chiasm or tract depending on their location and the size of the enlargement. The inferior nasal fibres cross anteriorly, forming the anterior knee of von Willebrand, and superior nasal fibres cross posteriorly. The anterior notch of the optic chiasm lies more inferiorly, making it more liable for compression by the growing pituitary tumour. Cavernous sinuses contain the 3rd, 4th and 6th cranial nerves along with the maxillary and the ophthalmic branch of the 5th cranial nerve.

Eye signs in pituitary mass lesions

Endocrine symptoms often precede the neuro-ophthalmic symptoms in patients with pituitary mass lesions (9). These symptoms include alterations in body weight, stature, growth velocity and progression of puberty. Overlooking these subtle manifestations could also contribute to delay in the correct diagnosis of the underlying endocrine disorder. Visual symptoms that are experienced by the patients with pituitary tumors vary with the tumor size and the resulting compression. The microadenomas (less than 1 cm in diameter) usually do not produce any ocular symptoms. The presence of visual symptoms in pituitary microadenomas should prompt the treating clinician to look for an alternate etiology. The visual signs could be due to the raised intracranial pressure, compression of the

structures in the visual pathway and the involvement of the cranial nerves in the cavernous sinus.

Macroadenomas usually result in visual symptoms including the field defects, eye pain, blurring of vision, double vision and progressive visual loss (10). Upper quadrantic bitemporal hemianopia is the classical field defect observed in the early stages of chiasmal compression. This is explained by the location and crossing of nasal fibers, which carry the images of the temporal field. Diplopia could be the presenting feature in an elderly patient of bronchial carcinoma with pituitary metastases (11).

Pituitary apoplexy is a condition characterized by the sudden bleeding into the enlarged pituitary gland. This is presented as sudden loss of vision and ophthalmoplegia, due to the involvement of the 3rd and 4th cranial nerves and also pressure on the optic nerve. Pituitary apoplexy should be considered in a patient with an abrupt neuro-ophthalmological presentation with the background of long standing headache (12). Early surgery for apoplexy results in a better chance of recovery of vision. Ophthalmologists have to be extra cautious in approaching the patients with atypical features and identify the underlying the space occupying lesion, leading to early detection of the tumors.

Syndromes involving the eyes and pituitary gland

The structures of the eye and pituitary gland are derived from the primitive neuroectoderm giving rise to a possibility of some congenital syndromes. Jung syndrome and Peters-plus syndrome have overlapping features and are reported from the Arab nations (13). The short stature could develop due to the growth hormone deficiency or associated malnutrition. The Cat-eye syndrome is also known as Schmid Fraccaro syndrome, which has a characteristic facial appearance (14).

Miscellaneous features

Optic atrophy at the time of presentation is very difficult to recover. There is only less than one third recovery. Most pituitary mass lesions progress to result in optic atrophy if the compression is not relieved early (15). Most of the ophthalmic structures lie in the radiation field and have adverse

consequences. They include an increased risk of cataract formation and cranial nerve damage leading to diplopia (16).

Clinical approach

In view of the above-mentioned facts, an endocrine screening is warranted in all patients with anterior segment anomalies. Vision-related quality of life is affected by the presence of pituitary tumours, and transsphenoidal surgery significantly improves this aspect of life quality (17). A close interaction between ophthalmologist and endocrinologist helps in the rapid diagnosis and accurate assessment of the condition and in identifying the correct therapeutic approach as well as minimizing post-surgical ophthalmic morbidity. All patients with unexplained pituitary hormonal dysfunction should be evaluated in detail by an ophthalmologist for the identification of a syndromic diagnosis.

In conclusion, eye signs are very important in the early identification of the pituitary lesions. Ophthalmologists need to be aware of the anatomy of the eye and possible lesions which may be the result of the association with the various surrounding structures. The subtle nature of the findings emphasizes the importance of having a team approach involving endocrinologist, neurosurgeon, neuro-ophthalmologist and neuro-radiologist. Such concentrated efforts would lead to early diagnosis and intervention for the better prognosis of the patient.

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LETTER TO THE EDITOR

IN-VITRO ANTIBACTERIAL AND ANTIOXIDANT POTENTIAL OF WINGED PRICKLY ASH, GREEN TEA AND THYMEI. HAFIZ¹, H.N. BHATTI¹, M.A. HANIF¹ and M. SHAHID²¹Department of Chemistry, University of Agriculture, Faisalabad, Pakistan; ²Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan

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Herbs and plants are mostly used as antimicrobials and antioxidants owing to the harmfulness and linked side-effects of synthetic chemical constituents. Plants and spices produce various metabolites with antibacterial and antioxidant potential. These metabolites are principally revealed as encouraging healing components or mediators which control ailments in human beings. The present study was aimed to characterize the extracts from selected medicinal plants through *in-vitro* activities. Winged prickly ash, green tea and thyme were selected and extracted through ethanol and methanol solutions. The extracts were assessed for antibacterial and antioxidant activities. The antibacterial potential of extracts showed the significant extent of the activity against *Bacillus subtilis* and *E. coli*. The maximum activity was noted in 80% methanolic fraction of *Thymus vulgaris* (15.20±0.64 mm) against *Bacillus subtilis*. Antioxidant potential exhibited the highest phenolic and flavonoid content in *Camellia sinensis*. The total phenolic content was significantly higher (1456.26±12.05 mg gallic acid) in 80% ethanolic fraction of *Camellia sinensis*. The flavonoid content in different plant extracts ranged from 8.17±2.02 to 376.29±7.11 mg/g. The radical scavenging DPPH assay also showed the significant antioxidant capacity of selected plants with the methanolic (50%) extract of *Camellia sinensis* found to be the most potent (78.95±7.12%). It was concluded that the alcoholic extracts of selected medicinal plants revealed the effective antibacterial and antioxidant activity, showing protective prospective against oxidative injury.

To the Editor,

One of the main world health concerns is the claim of active and reasonable novel medicines to treat infections, predominantly among developing nations where half of the deaths are due to infectious ailments. Herbal plants are primarily used as antimicrobials, not due to toxicity and unwanted side-effects of chemical drugs but also owing to growing comparison to chemical-based drugs and high prices (1).

Various edible and medicinal plants yield a variety of metabolites with anti-bacterial and antioxidant activities. These are largely reflected as

promising healing components in human beings. Such metabolites have substantial benefits. The antioxidant and antimicrobial actions of a single plant component make them more effective and applicable as preservatives in food processing. Plants show better inhibition properties against gram-positive bacteria than gram-negative. However, their efficacy against both types of bacteria designates the existence of a wide-range of antibiotic compounds (2).

Plant metabolites are an essential source of natural antioxidants with no safety issues. Antioxidant molecules have the ability to decrease

Key words: anti-bacterial, antioxidant, phenolics, flavonoids, plant extracts

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the oxidation process within substrate. Secondary bioactive metabolites control the development of heart problems, cancer, and neurodegenerative ailments through scavenging the free radicals. Commonly found phyto-constituents in herbs and plants are flavonoids and phenolic compounds. Phenolic compounds are vital antimicrobial components (3).

The antimicrobial effects of plant-based compounds are impacted by their species and origin, harvesting practices and time, their growth and extraction process, besides chemical profiling and structures. Additionally, the relationship of food constituents with natural antimicrobial compounds plays a vital role in the use and efficacy of these compounds as preservatives (4).

Keeping the potential sources of medicinal plants in mind, the aim of the present study was to characterize the *in-vitro* antibacterial and antioxidant activities of the extracts from selected plants.

MATERIALS AND METHODS

Raw materials

Winged prickly ash (*Zanthoxylum alatum* L.), green tea (*Camellia sinensis* L.) and thyme (*Thymus vulgaris* L.) were procured from a local market. The selection of plants was based on the anticipated antimicrobial and antioxidant potentials, as reported in literature.

Sample preparation

Ground plants were extracted separately with methanol and ethanol (30%, 50% and 80% solutions) using an orbital shaker. The extracts were filtered and residue was re-extracted. The solvent-free extracts were obtained using a rotary evaporator and stored at -4°C (5).

Antibacterial activity

The antibacterial assays were performed through the well diffusion method and evaluated by measuring the inhibition zones against the selected microbial strains (5). Antibacterial properties were tested against two Gram positive (*Bacillus subtilis* and *Staphylococcus aureus*) and two Gram negative (*Escherichia coli*, and *Pasteurella multocida*) strains. The antibiotic used in the study was ampicillin

Minimum inhibition concentration

The plant extracts were evaluated for minimum

inhibition concentration (MIC) by micro dilution in culture broth. The extracts that showed the inhibition zone in the well diffusion were dissolved for measuring MIC. The absorbance was measured at 500 nm by a micro plate reader. The lowest concentration at which color changes occurred was taken as MIC value. The results were expressed in $\mu\text{g/mL}$ (6).

Antioxidant potential

The antioxidant potential of the plant and spice extracts was estimated by using different antioxidant assays.

Determination of total phenolic content

Total phenolic content (TPC) of the plants was determined by the Folin-Ciocalteu method. The calibration curve was prepared with different concentrations of gallic acid. One mL of extract was mixed with the reagent and absorbance of the resulting blue color complex was measured at 765 nm. The quantification was made in respect to gallic acid (5).

Total flavonoids content

Total flavonoid content (TFC) of selected plant extracts were determined by preparing the reaction mixture from extracts and reagents. Absorbance of the reaction mixture was taken at 510 nm. TFC of the extracts were expressed as catechin equivalents from the linear regression curve of catechin (5).

DPPH scavenging activity

Freshly prepared methanol solution was added to plant extract at different concentrations and then the mixed solution was kept in the dark. Absorbance was noted at 517 nm. The low absorbance of reaction mixture indicates the high radical scavenging activity. The antioxidant activity of BHT and vitamin C as standard was also analyzed (5).

Statistical analysis

The data obtained was analyzed by calculating means and standard deviation using statistical software (SPSS Inc, Chicago, IL, USA).

RESULTS

The results of antibacterial activity showed that the extract of all selected plants produced zones of

inhibition against *B. subtilis*, *E. coli*, *S. aureus* and *P. multocida*. The maximum activity was observed in 80% methanolic fraction of *Thymus vulgaris* (15.20 ± 0.64 mm) against *B. subtilis* followed by 80% ethanolic fractions of *Camellia sinensis* (14.03 ± 1.36 mm). The order of antibacterial activities of different plants were observed as *Thymus vulgaris* > *Camellia sinensis* > *Zanthoxylum alatum* (Table I).

In general, extracts exhibited low to moderate antibacterial activity (zone of inhibition as 5-17 mm) with minor variation against gram positive and gram negative bacteria. The significant activity was shown against *B. subtilis* and *E. coli* followed by *S. aureus* and *P. multocida*. Very clear inhibition zones were observed around the positive control (ampicillin) against all the tested microbes.

The Minimum Inhibitory Concentration (MIC) assay was determined by two-fold serial broth dilutions method in sterile tubes. The highest minimal inhibitory concentration was shown by the ethanolic extract of *C. sinensis*, followed by *T. vulgaris* and *Z. alatum* (Fig. 1).

In the present study, total phenolic and flavonoid

content, and DPPH radical scavenging assays were used to quantify various antioxidant activities of selected medicinal plant extracts.

The results of TPC are expressed as mg of gallic acid equivalents per g of dry weight of plant material. Of all the plants, the TPC were markedly higher in 80% ethanolic fraction of *Camellia sinensis* (1456.26 ± 12.05) which signifies its highest antioxidant activity (Table II). The overall trend for TPC content was *Camellia sinensis* > *Thymus vulgaris* > *Zanthoxylum alatum*.

The total flavonoid content of plant extracts are expressed as mg Catechin equivalent per gram of plant extract fractions. The content in ethanolic extracts of different plants ranged from 8.17 ± 2.02 to 376.29 ± 7.11 mg/g. These phytochemical compounds revealed significant differences in their total content studied in various medicinal plants. The highest amount of flavonoids was found in *C. sinensis*. Depending upon flavonoid content, the order of different extracts was *C. sinensis* > *T. vulgaris* > *Z. alatum*. In methanolic extract, *C. sinensis* showed 557.68 ± 9.50 mg/g flavonoid content (Table II).

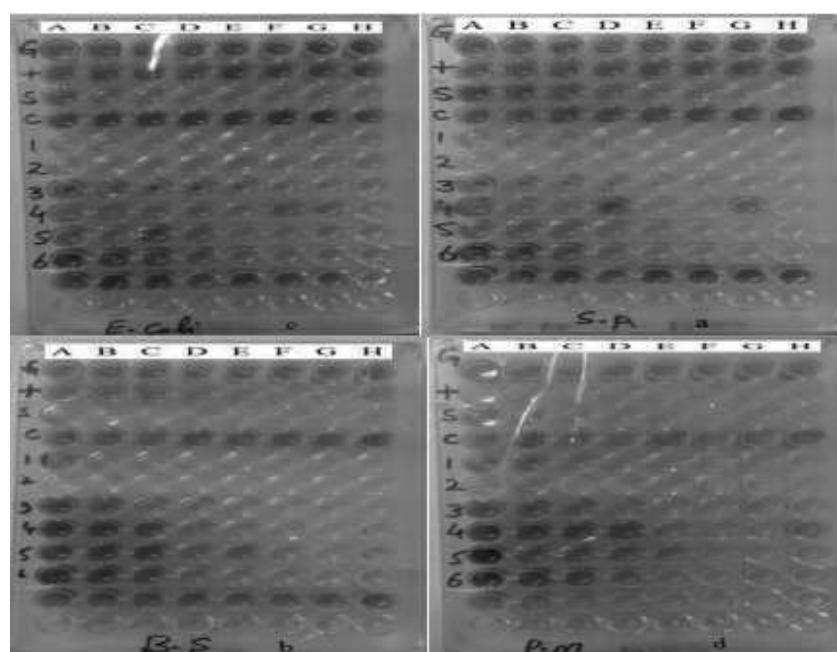


Fig. 1. Minimum inhibitory concentration (MIC) of selected plant extracts against bacterial strains. (1-6) are Samples; (+): positive control; C: negative control; G: growth control; S: sterility control. A

Table I. Antibacterial activity (Means $\pm$ SD) of selected plant extracts against target bacteria. (Inhibition zones in mm).

Plant extracts	<i>B. subtilis</i>			<i>S. aureus</i>			<i>P. multocida</i>			<i>E. coli</i>		
	<i>C. sinensis</i>	<i>T. vulgaris</i>	<i>Z. alatum</i>	<i>C. sinensis</i>	<i>T. vulgaris</i>	<i>Z. alatum</i>	<i>C. sinensis</i>	<i>T. vulgaris</i>	<i>Z. alatum</i>	<i>C. sinensis</i>	<i>T. vulgaris</i>	<i>Z. alatum</i>
Ethanol 30%	8.63 $\pm$ 0.31	7.30 $\pm$ 0.47	7.20 $\pm$ 0.61	8.49 $\pm$ 0.40	5.8 $\pm$ 0.59	6.8 $\pm$ 0.52	9.1 $\pm$ 0.54	6.8 $\pm$ 0.06	7.3 $\pm$ 0.43	8.63 $\pm$ 0.54	7.40 $\pm$ 1.91	8.99 $\pm$ 0.33
Ethanol 50%	9.57 $\pm$ 0.42	8.77 $\pm$ 1.91	8.53 $\pm$ 0.28	8.53 $\pm$ 1.31	6.26 $\pm$ 1.21	8.3 $\pm$ 0.25	8.2 $\pm$ 0.15	9.5 $\pm$ 0.21	6.9 $\pm$ 0.87	7.67 $\pm$ 0.21	10.23 $\pm$ 0.42	8.10 $\pm$ 0.42
Ethanol 80%	14.03 $\pm$ 1.36	10.10 $\pm$ 0.60	8.03 $\pm$ 0.17	13.12 $\pm$ 0.36	6.9 $\pm$ 0.54	9.0 $\pm$ 1.01	13.6 $\pm$ 0.23	7.2 $\pm$ 0.53	7.8 $\pm$ 0.69	16.53 $\pm$ 1.01	8.23 $\pm$ 0.31	8.73 $\pm$ 1.09
Methanol 30%	10.10 $\pm$ 0.27	8.60 $\pm$ 2.43	9.23 $\pm$ 1.30	8.44 $\pm$ 0.58	5.01 $\pm$ 0.23	7.1 $\pm$ 0.46	8.9 $\pm$ 1.04	7.5 $\pm$ 0.67	6.9 $\pm$ 1.05	8.77 $\pm$ 0.42	8.37 $\pm$ 0.22	7.13 $\pm$ 0.91
Methanol 50%	11.13 $\pm$ 2.56	9.60 $\pm$ 0.39	8.50 $\pm$ 0.35	11.32 $\pm$ 0.29	7.3 $\pm$ 0.64	8.5 $\pm$ 0.30	12.5 $\pm$ 2.32	7.1 $\pm$ 1.30	7.1 $\pm$ 3.61	9.67 $\pm$ 0.56	7.93 $\pm$ 0.70	7.07 $\pm$ 0.23
Methanol 80%	11.00 $\pm$ 0.51	15.20 $\pm$ 0.64	8.10 $\pm$ 0.24	10.98 $\pm$ 0.42	7.9 $\pm$ 1.02	9.7 $\pm$ 0.21	17.1 $\pm$ 1.01	10.7 $\pm$ 0.34	6.8 $\pm$ 0.09	14.93 $\pm$ 0.40	13.53 $\pm$ 0.40	7.07 $\pm$ 0.46

Table II. Antioxidant activities (Means $\pm$ SD) of selected plant extracts.

Plant extracts	Total phenolic content (mg GAE/g dry plants material)			Total flavonoid content (mg CE/g dry plant material)			Scavenging free radical activity (%) Vitamin C (+ control) = 92.76 $\pm$ 1.02%		
	<i>C. sinensis</i>	<i>T. vulgaris</i>	<i>Z. alatum</i>	<i>C. sinensis</i>	<i>T. vulgaris</i>	<i>Z. alatum</i>	<i>C. sinensis</i>	<i>T. vulgaris</i>	<i>Z. alatum</i>
Ethanol 30%	1378.90 $\pm$ 14.50	244.78 $\pm$ 5.52	86.08 $\pm$ 3.00	351.38 $\pm$ 4.14	107.92 $\pm$ 6.00	47.25 $\pm$ 3.03	73.21 $\pm$ 1.45	68.44 $\pm$ 4.00	48.26 $\pm$ 3.65
Ethanol 50%	1398.35 $\pm$ 3.51	145.96 $\pm$ 16.00	161.81 $\pm$ 3.54	180.82 $\pm$ 3.54	99.25 $\pm$ 3.03	98.45 $\pm$ 5.50	75.46 $\pm$ 6.41	69.29 $\pm$ 3.05	51.72 $\pm$ 1.98
Ethanol 80%	1456.26 $\pm$ 12.05	138.35 $\pm$ 3.51	195.50 $\pm$ 4.50	376.29 $\pm$ 7.11	102.85 $\pm$ 4.01	102.92 $\pm$ 4.00	76.18 $\pm$ 3.13	74.29 $\pm$ 9.50	54.75 $\pm$ 5.31
Methanol 30%	969.50 $\pm$ 5.50	179.14 $\pm$ 6.59	100.75 $\pm$ 7.01	288.52 $\pm$ 5.50	35.99 $\pm$ 3.60	56.57 $\pm$ 3.50	75.56 $\pm$ 0.09	65.63 $\pm$ 5.34	37.39 $\pm$ 6.02
Methanol 50%	1090.68 $\pm$ 9.02	100.65 $\pm$ 13.51	102.32 $\pm$ 4.04	171.38 $\pm$ 7.08	56.66 $\pm$ 6.51	67.52 $\pm$ 3.13	78.95 $\pm$ 7.12	77.47 $\pm$ 7.90	69.07 $\pm$ 8.91
Methanol 80%	1154.59 $\pm$ 14.50	86.89 $\pm$ 18.36	121.23 $\pm$ 5.02	557.68 $\pm$ 9.50	26.75 $\pm$ 2.54	65.80 $\pm$ 4.02	77.21 $\pm$ 2.56	63.98 $\pm$ 1.68	59.88 $\pm$ 0.07

The scavenging activity of the DPPH radical due to its reduction by different plant extracts at various concentrations is expressed in percentages. Vitamin C was used as positive control that showed 92.76 $\pm$ 1.02% radical scavenging activity. The methanolic (50%) extract of *C. sinensis* was found to be the most potent (78.95 $\pm$ 7.12%) while methanolic (30%) extract of *Z. alatum* (37.39 $\pm$ 6.02%) was the least effective (Table II). The ranking of the selected ethanolic plant extracts in order of their scavenging

capacity, based on absolute numbers was *C. sinensis* > *T. vulgaris* > *Z. alatum*.

DISCUSSION

Although all the fractions of methanol and ethanol extracts of the plants produced inhibitory actions against selected bacterial strains, 50% and 80% fractions of methanol presented more inhibitory effects than that of 30%. This tends to show that the

active antibacterial ingredients in the plants and spices were better extracted with methanol and ethanol as compared to water. Similar results were observed during work on stem bark of *Kigelia pinnata* (7).

The Gram-positive bacteria are more susceptible to spices and herbal extracts and essential oils from plants than Gram-negative, which have lipopolysaccharides as cell wall component that avert the active constituents to reach membrane (8). Hence the present findings are in line with reported facts.

Several researchers used thyme plant against many strains of bacteria and microbes and found high antibacterial potential. Another study reported that *Camellia sinensis* has anti-bacterial activity against resistant bacterial strains, such as vancomycin-resistant enterococci, MRSA, and MDR- *P. aeruginosa* (9).

The green tea extract showed inhibitory effects against several bacteria including *Salmonella* and *Shigella* species, *Vibrio cholerae*, *Campylobacter coli*, *Helicobacter pylori*, *Clostridium* and *Pseudomonas* (10). This extract can also exhibit a useful effect against viral infections since tea polyphenols considerably inhibit influenza-A virus and rotavirus in animals cell culture (11).

The findings of MIC are in-line with Gupta et al. (12), who investigated the antibacterial activity of some ethanolic and aqueous plant extracts against *S. aureus*, *Pseudomonas aeruginosa* and *Bacillus subtilis* and noted that the ethanolic extracts of four plants (*Achyranthes aspera*, *Cynodon dactylon*, *Lantana camara* and *Tagetes patula*) were effective against all tested bacteria with MIC's ranging from 25 to 125 mg/ml.

The antioxidant potential shows direct correlation with total phenolic content. Koksala et al. (13) reported the antioxidant and polyphenolic content as 158 and 256 µg GAE/mg dried extracts in ethanolic and water extracts of *T. vulgaris*, respectively. The total phenolic content of water extract was higher than that of ethanolic. The high level of phenolic compounds indicates the elevated antioxidant capacity of thyme.

The variation in phenolic content of plants described in several studies might be owing to variation in environmental conditions like climate, location, temperature, illnesses and exposure to pests, parts tested, time and technique of assessment. The

total flavonoid content is not associated with total phenolic content and variation depends upon the plant species and other factors. *C. sinensis* leaf extract is important as a functional food in the diet because of its high poly-phenol content. The active anti-oxidant and antimicrobial compounds in its extracts promote health by preventing lipid oxidation and providing antibacterial activity (14).

The basic structure of the phenols and other structural factors play a fundamental role in the mechanism by which these compounds are able to scavenge free radicals. Scavenging activity is attributed to the presence of phenolic hydroxyl groups, particularly in the 3'OH and 4'OH of the three-carbon chain (15).

In the present study, the alcoholic extracts of selected plants accounted for effective antioxidant activity, indicating their protective potential against oxidative damage. Hence, it is recommended to characterize the compounds present in these plant extracts, with potential bioactivities.

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LETTER TO THE EDITOR

AN INSIGHT INTO NUTRITIONAL PROFILE OF SELECTED *PLEUROTUS* SPECIESA. IRSHAD¹, S. SHARIF², M. RIAZ³ and F. ANJUM⁴¹Department of Biochemistry, Faculty of Sciences, University of Agriculture, Faisalabad-Pakistan;²College of Biosystem Engineering and Food Science, Zhejiang University-China; ³Department of Allied Health Sciences, Sargodha Medical College, Faculty of Medical and Health Sciences, University of Sargodha-Pakistan; ⁴Department of Chemistry, Faculty of Science and Technology, Government College University, Faisalabad-Pakistan

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The global demand for good quality food indicates that consumers are more concerned about a particular diet associated with good health and lower risk for certain ailments. Mushrooms are widely used as healthy nutritious food. In the present study, the nutritional composition of four different *Pleurotus* sp. was determined. Prior to extraction, all the selected mushrooms were subjected for proximate composition analysis. The protein, fat, ash, total carbohydrate, fiber and energy contents were in the range of (16.07-25.15%), (0.64-2.02%), (2.1-9.14%), (65.66-82.47%), (6.21-54.12%) and (342.20-394.30Kcal/100g), respectively. The spectrophotometric analysis showed that the concentration of protein was in the range of 45.78-33.47 mg/g in all *Pleurotus* sp. High performance liquid chromatographic analysis of sugars showed six different mono and disaccharides in all the selected mushrooms. The fatty acid profile by gas chromatography-mass spectrometry revealed that the main fatty acids in selected mushrooms were present in the order linoleic acid > oleic acid > palmitic acid. The results suggested that all the *Pleurotus* sp. could be considered as a rich source of nutrients.

To the Editor,

One of the world's biggest challenges is food insecurity, which is common in developing countries (1). The current food production systems are insufficient due to the continuing increase in world populations. Studies on the production of non-conventional protein as an alternative source of dietary supplement have increased with growing global demands for good quality food (2). Modern food technology can also extend a great deal for the production of certain kinds of food from mushrooms and related species (3). Among all mushrooms, the *Pleurotus* is the most cultivated in the world and

is commonly referred to as the Oyster mushroom (4). To date, almost 70 *Pleurotus* species have been reported, and its popularity has been increasing due to facility of cultivation, high yield potential and high nutritional and medicinal values. The shell-like cap with eccentric/lateral stipes along with cream, white, pink, yellow, dark gray or brown color are the specific features of the *Pleurotus* mushroom (5). They are considered to have great dietary importance, because they are low in calories and fats, and contain high amounts of protein, chitin, vitamins and minerals (Ca, P, Fe, K and Na). The nutritional composition of this mushroom ranges from 20-40%

Key words: *Pleurotus* sp., proximate composition, gas chromatography-mass spectrometry, high performance liquid chromatography, unsaturated fatty acids

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dry weight protein content, 7.7-14.2% fibers, and the fat constituent varies from 3-7%. They are also a major source of vitamins. Mushrooms contain 30-65% of soluble carbohydrates on dry weight basis, which includes monosaccharide, oligosaccharide and polymeric glycogen (6). *Pleurotus ostreatus*, called “Meat of the Forest”, is one of the edible mushrooms, and is reported to contain higher concentrations of amino acids, such as cystine, methionine and aspartic acid, compared to other edible mushrooms (7). These mushrooms could be grown commercially in the sub-tropical and in temperate zones of many countries of the world. In Pakistan wild oyster mushroom species grow on logs and stumps of trees in the forests of KPK and Azad Kashmir regions and other plantations in the plains of Punjab and Sindh during the rainy season of the monsoon (8).

In light of the above background, the current study is focused on the analysis of *Pleurotus* sp. to investigate their proximate composition along with sugar and fatty acid profile by HPLC and GCMS, respectively.

MATERIALS AND METHODS

Collection and pretreatment of Pleurotus sp.

The four different species of *Pleurotus* (Fries) Kummer mushroom including P1: *Pleurotus ostreatus* (white oyster mushroom) (Jacq. Ex. Fr.) Kumm., (imported from America), P3: *Pleurotus sajor-caju* (Phoenix oyster mushroom)(Fr.) (imported from Canada), P6: *Pleurotus sapidus* (Wild mushroom) (FW-133) and P8: *Pleurotus columbinus* (Blue oyster) (imported from UK) were collected from the mushroom culture bank of the Medicinal Mushroom Laboratory, Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan. All the selected mushrooms were self-harvested and dried. Taxonomic identification was performed with the assistance of the Medicinal Mushroom Laboratory, Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan. The specimen of each species was immediately ground in a domestic blender and reduced to fine dried powders and stored at 4°C before the extractions.

Proximate analysis of selected mushrooms

The dried powder of selected mushrooms was

examined for total carbohydrates, crude fiber, crude protein, crude fat and ash contents using the methods described hereunder.

Estimation of crude fat

The determination of crude fats of dried powder of selected mushroom was carried out by the method described by Barros et al. (9). Three grams of dried powder of selected mushroom were taken in a thimble and placed in an extraction tube of a Soxhlet apparatus; it was placed in a water bath and the temperature was adjusted so that continuous drops of water fell on the mushroom sample placed in the extraction tube. The process of extraction was carried out with petroleum ether (boiling point 40-60°C) for 16 h. Then the sample was removed from the extraction tube and the solvent was allowed to evaporate by placing the flask in a water bath at 100°C. The extract was placed in a hot air oven at 105°C for 30 min and dried completely, then allowed to cool in a desiccator and the weight of the dried extract was recorded. Crude fat percentage was calculated with the help of the following formula;

Crude fat (%) = Wt. of fat in sample (g) × 100/ Wt. of sample

Estimation of crude protein

Crude protein was determined by Macrokjeldahl apparatus previously described by Barros et al. (9). Protein percentage of mushrooms was determined by the following formula;

Crude protein (%) = 6.25 × Nitrogen (%)

The percentage crude protein in the original sample was calculated using the formula;

Crude protein (%) = (a-b) × 0.01 × 14.01 × c × 100 × 6.25 / d × e

(a = Titre value of the digested sample, b = Titre value of the blank sample, c = Volume to which the digested sample was made up with distilled water (100 mL), d = Volume of sodium hydroxide used for distillation (10 mL), e = Weight of dried fat-free sample)

Estimation of crude fiber

Crude fiber of the dried mushrooms was determined as previously described by Pushpa and Purushothama, (10). Percentage of the crude fiber was calculated by the formula;

Crude fiber (%) = 100 (crucible wt. before ash – crucible wt. after ash)/wt. of sample

Determination of ash contents

Ash contents of the dried mushroom sample were determined (11). A dried mushroom sample of 3 g was placed in a crucible and heated on an oxidizing flame until it stopped smoking. The crucible was placed in a muffle furnace for 6 h at 550°C until ash was obtained. This sample was allowed to cool in desiccators and then weighed. Ash contents were calculated with the help of following formula;

Ash (%) = wt. of ash in sample (g) $\times$ 100/ wt. of sample (g)

Determination of total carbohydrates

Total carbohydrates were calculated by difference as follows (11):

Total carbohydrates = 100 – (Total protein + Total fat + Total ash)

Determination of total energy

Total energy was calculated according to the equation below (12).

Energy (Kcal) = 4 $\times$ (g protein + g carbohydrate) + 9 $\times$ (g lipid)

Extraction of selected mushrooms

The selected mushrooms were extracted in water according to the method of Gangadevi et al. (13) with slight modification. Briefly, dried mushroom powder (20 g) was extracted with 200 mL of 80% methanol and ethanol using an orbital shaker (Gallenkamp, UK) for 8 h at room temperature. The extracts were filtered through Whatman No. 1 filter paper and evaporated through a rotary evaporator (EYELA, N-N Series, Rikakikai Co. Ltd. Japan) to yield the residue and stored at 4°C for subsequent analyses.

Quantification of proteins by Bradford assay

The sample of 10 μ L was taken in a sterilized Eppendorf tube and 1 mL of Bradford reagent was added to each tube. All the sample solutions were incubated at 37°C for 10-20 min along with the blank. Standard solutions of various concentrations were prepared for analysis to construct the standard curve and the absorbance of the standards and test samples was taken at 595 nm by spectrophotometer. Bovine serum albumin (BSA) was used as standard. The concentration of test samples was calculated through standard curve (14).

Determination of sugars by high performance liquid chromatography

The high performance liquid chromatography (HPLC) equipment (Shimadzu), an integrated system with a LC-10AT pump, a column Lichorspher (R)100 NH₂Purospher (R) STAR NH₂, 5 μ m, carbohydrates [mono, di and oligosaccharides) and a RID=RID-10A detector (Japan)] operating at RT. The mobile phase used was 70% CAN at a flow rate of 0.6 mL/min and the injection volume was 20 μ L. The compounds were identified by chromatographic comparisons with authentic standards. Heleno et al. (15) previously described the method for sample and standard preparation used with slight modification. The results expressed in g/100 g of dry weight, calculated by internal normalization of the chromatographic peak area. The linearity and sensitivity of the HPLC analysis was determined and the method validated by the instrumental precision, repeatability and accuracy, using *T. imbricatum* (15).

Fatty acid analysis of selected mushrooms by gas chromatography-mass spectrometry

Chromatographic separation of fatty acid methyl esters was accomplished with a gas chromatography-mass spectrometry (GCMS), Hewlett-Packard (Wilmington, DE), model 6890, equipped with electronic pneumatic control, a model 7683 with automatic liquid sampler. Sample (2 μ L) was introduced by split injection (50:1 ratio) onto a WCOT fused silica, chemically bonded capillary column (Chrompack CP-select CB for FAME, 100 m long, 0.25 mm inside diameter, 0.39 mm outside diameter, 0.25- μ m film thickness; Varian Walnut Creek, CA, USA). Helium 3 mL was used as carrier gas. The temperature gradient (70-250°C) consisted of the following steps: 70°C for 1 min; increased to 135°C at 90°C min⁻¹, hold for 1 min; increased to 160°C at 1.5°C min⁻¹; hold for 0.5 min, increased to 185°C at 1°C min⁻¹, hold for 0.5 min, increased to 195°C at 60°C min⁻¹, hold for 5.5 min, increased to 250°C at 90°C/min, hold for 3 min; total run time was 54.7 min. Injector temperature was 280°C; detector temperature was 300°C. Clapham et al. (16) previously described the method for sample and standard preparation used with slight modification. Fatty acids were identified according to their retention time using reference standard (GLC-63B, Nu-Check- Prep, Elysian, MN) and quantified using a Hewlett-Packard Chem station data system. Fatty acids quantified on dry matter basis

were lauric (C12:0), myristic (C14:0), palmitic (C16:0), palmetoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and α -linoleic (C18:3). Hepatodecanoic acid (C17:0, 0.4 mg/mL in *n*-hexane; Matreya, Pleasant Gap, PA, USA) was used as an internal standard (16).

Statistical analysis

Minitab software was used for computation and analysis of different parameters. Differences were analyzed by considering $p < 0.01$ statistically significant. The collected data was analyzed by means and standard deviation.

RESULTS

The results of proximate composition of selected mushrooms are given in Table I. The concentration of crude protein contents ranged between 16.07-25.15% on dry weight basis. In the present study we found that *Pleurotus* mushrooms P1, P3, P8 and P6 displayed the protein contents as 25.15% > 23.51% > 22.15% > 16.07%, respectively. The crude fat contents were present in the range of 0.64-2.02%. P8 expressed

fat contents of 2.02%. Other *Pleurotus* sp. P1, P3 and P6 showed fat contents 1.75% > 1.88% > 0.64%, respectively. Results of ash contents of mushroom were in the range of 2.1-9.14%. According to our research work, mushroom P1 exhibited maximum ash contents of 9.14% whereas P3, P6 and P8 showed 2.1% > 5.87% > 7.03%, respectively. In the present study, carbohydrate contents ranged between 65.66-82.47%. The mushroom P3 displayed the best carbohydrate contents (82.47%), whereas other *Pleurotus* sp. P1, P6 and P8 showed carbohydrate contents 66.54% > 72.52% > 65.66%, respectively. The mushroom P8 presented the greatest energy content of 394.30 Kcal/100g followed by P1, P3 and P6 in the range of 354.80 > 368.41 > 342.20 Kcal/100g, respectively. The dietary fiber contents were in the range of 6.21-54.12%; P3 showed the highest dietary fiber contents of 54.12% whilst other *Pleurotus* sp. P1, P6 and P8 exhibited 7.43% > 8.48% > 6.21%, respectively.

Determination of protein from selected *Pleurotus* sp.

The protein contents of mushrooms are not

Table I. Proximate composition of selected mushrooms.

Mushrooms	Crude protein (% DW)	Crude fat (%DW)	Ash (% DW)	Carbohydrates (% WD)	Energy Kcal/100g	Crude fiber (% WD)
<i>P. ostreatus</i> P1	25.15±.04 ^a	1.75±.0 ^c	9.14±.05 ^a	66.54±.04 ^c	354.80±.04 ^c	7.43±.04 ^b
<i>P. sajor-caju</i> P3	23.51±.0 ^b	1.88±.0 ^b	2.1±.03 ^d	82.47±.03 ^a	368.41±.04 ^b	54.12±.0 ^a
<i>P. sapidus</i> P6	16.07±.0 ^d	0.64±.0 ^d	5.87±.03 ^c	72.52±.04 ^b	342.20±.03 ^d	8.48±.04 ^b
<i>P. columbinus</i> P8	22.15±.0 ^c	2.02±.0 ^a	7.03±.05 ^b	65.66±.03 ^d	394.30±.04 ^a	6.21±.03 ^c

Values are expressed as mean±SD of triplicate experiments. Furthermore, mean carrying different superscripted letters vary significantly ($p < 0.05$) with 95% confidence

Table II. Quantification of soluble proteins from whole mushrooms powder by Bradford method.

Mushrooms	Proteins(mg/g)
<i>P. ostreatus</i>	45.78±0.8 ^a
<i>P. sajor-caju</i>	37.93±0.9 ^c
<i>P. sapidus</i>	33.47±0.5 ^d
<i>P. columbinus</i>	42.12±0.6 ^b

Values expressed as mean±S.D of triplicates of four *Pleurotus* spp. Furthermore, mean carrying different superscript letters vary significantly ($p < 0.05$) with 95% confidence.

only dependent on environmental factors and the stage of fruiting body, but also on species. All studied *Pleurotus* sp. showed high concentrations of protein. The concentrations of protein in *Pleurotus* sp. are given in Table II. Among the *Pleurotus* sp. *P. ostreatus* showed the maximum concentration of protein (45.7 ± 0.8 mg/g) followed by *P. columbinus* (42.12 ± 0.6 mg/g) > *P. sajor-caju* (37.93 ± 0.9 mg/g) and *P. sapidus* (33.47 ± 0.5 mg/g), respectively.

Detection of sugars from selected Pleurotus sp. by high performance liquid chromatography

Regardless of the various information associated with sugar, xylose, fructose, glucose, galactose, lactose and sucrose were the primary sugars examined

in the present mushrooms. The results are given in Table III. HPLC analysis of sugars determined six different mono and disaccharides. The results showed that fructose was present in all four *Pleurotus* sp. while other sugars may or may not be present in all mushrooms. The highest concentration of fructose was present in *P. sajor-caju* ($0.375 \pm 0.03\%$) followed by *P. sapidus* ($0.374 \pm 0.06\%$), *P. ostreatus* ($0.374 \pm 0.03\%$) and *P. columbinus* ($0.373 \pm 0.12\%$), respectively.

Fatty acid analysis of Pleurotus sp. by GCMS

This analysis identified over 25 different fatty acids (FAs) as shown in Table III. Linoleic ($18':2n6c$), oleic ($18':1n9c$) palmitic acids ($C16:0$), steric acid

Table III. Monosaccharide, disaccharide composition and fatty acid chemical finger print of selected mushrooms.

<i>Pleurotus</i> spps.	<i>P. ostreatus</i>	<i>P. sajor-caju</i>	<i>P. sapidus</i>	<i>P. columbinus</i>
Sugars(%age)				
Xylose	Nd	$0.486 \pm 0.12a$	$0.482 \pm 0.02b$	Nd
Fructose	$0.374 \pm 0.03a$	$0.375 \pm 0.03a$	$0.374 \pm 0.06a$	$0.373 \pm 0.12a$
Glucose	$0.39 \pm 0.05a$	$0.393 \pm 0.01a$	Nd	$0.391 \pm 0.05a$
Galactose	Nd	Nd	Nd	$0.723 \pm 0.01a$
Lactose	Nd	Nd	Nd	$1.715 \pm 0.07a$
Sucrose	$0.437 \pm 0.01b$	Nd	$0.455 \pm 0.01a$	Nd
Fatty acids (mg/g)				
16':0	2.671	3.809	1.246	4.229
18':0	0.413	0.979	0.258	1.193
18':1n9c	3.544	2.0103	1.170	6.075
18':2n6c	10.257	16.013	1.421	12.547
19':00	0.491	0.482	0.452	0.487
18':3n3	0.0202	0.036	0.031	0.196
TFA	17.40	23.33	4.58	19.73
SFA	24.65	20.77	48.7	30.33
MUFA	18.26	7.77	5.85	28.64
PUFA	54.06	52.51	30.5	47.31

Values are expressed as mean $\pm$ S.D of triplicates of four *Pleurotus* spp. Furthermore, mean carrying different superscript letters vary significantly ($p < 0.05$) with 95% confidence. Palmitic acids ($C16:0$), Steric acid ($C18:0$), Linoleic ($18':2n6c$), Oleic ($18':1n9c$), Linolenic ($18':3n3$) and Nonadecanoic acid ($C19:0$). Nd: not detected; C: carbon atoms; SFA: saturated fatty acids; MUFA: mono unsaturated fatty acids; PUFA: poly unsaturated fatty acids; TFA: total fatty acids

(C18:0), linolenic (18:3n3) and nonadecanoic acid (C19:0) were the main fatty acids found in the mushrooms. Linoleic acid was found in highest concentration in *P. columbinus* followed by *P. sajor-caju*, *P. ostreatus* and *P. sapidus*, respectively. Palmitic acid was present in all the studied mushrooms. The unsaturated fatty acids were more prevalent over the saturated fatty acids.

DISCUSSION

Traditionally, people prefer meat, vegetables and cereals to fulfill their dietary needs. There is a lesser supply of nutrients with the continuous increase in world population, which results in an increase in the cost of food. In this study, we found that in mushrooms the contents of crude proteins, fats, carbohydrates, dietary fibers, total ash and calorie values were in approximate ranges with each other and with the values as reported in previous studies (11) (17). Hung and Nhi, (17) reported protein contents for *P. ostreatus* (28.6%), which were non-significantly higher than our results. The results of raw fats concurred with the mushrooms present in the Delta state and Nigeria displayed fat contents between 0.9-2.58% (11). Our results for ash contents agree with data reported by Hung and Nhi (17) for *L. edodes*, *G. lucidum*, *P. ostreatus*, *V. volvacea* and *A. polytricha* in the range of (1.4% to 9% DW). These Authors also carried out a study on the carbohydrate contents *P. ostreatus* (61.3±0.2), and the dietary fiber in mushrooms present in the Delta state as well as Nigeria were in the range of 3.24-8.7% (11). Mosisa et al. (3) performed a comprehensive analysis of the nutritional quality of edible mushrooms (*Pleurotus sajor-caju*) grown in deproteinized whey medium and found the crude protein content of the single cell protein was 39.25%. Our results were slightly higher than these. Heleno et al. (15) studied sugars of five wild culinary-medicinal species of genus *Pleurotus* (Fr.) by high-performance liquid chromatography and reported three main sugars, sucrose, glucose and xylose, in various concentrations. The GC-MS analysis showed that the main fatty acids in selected mushrooms were linoleic acid > oleic acid > palmitic acid and our results are consistent with

previous studies. Total fatty acids were highest in *P. columbinus* > *P. ostreatus* > *P. sajor-caju* > *P. sapidus*. *P. columbines* and *P. ostreatus* had the greatest UFA concentration, which showed the high value HDL and low value of LDL cholesterol (18). These values may differ due to different environment, drying method, substrate and species of mushrooms.

The results of this study showed that *Pleurotus* sp. (*P. ostreatus*, *P. sajor-caju*, *P. sapidus* and *P. columbinus*) are very potent and active nutritious mushrooms. They have a high percentage of macromolecules, for which reason these mushrooms can be used as low cost healthy food. Moreover, they also showed the potential to be developed into other products such as medicines. It would be an excellent alternative for many health conscious people and could also add to the value of the food products. Mushrooms can be recommended for the countries suffering from insufficient nutrition; especially developing or third world countries.

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LETTER TO THE EDITOR

A VERSATILE METHOD FOR GENE DOSAGE QUANTIFICATION: MULTIPLEX PCR AND SINGLE BASE EXTENSION FOR COPY NUMBER AND GENE-CONVERSION IDENTIFICATION OF *SMN* GENES

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A comparison of the individual genomes within a species demonstrates that structural variation, including copy number variation (CNV), is a major contributor to phenotypic diversity and evolutionary adaptation. CNVs lead to the under/over-expression of a gene, according to the changes in the gene dosage, which account for the development of a number of genomic disorders. Thus, the development of efficient, rapid and accurate CNV screening is of fundamental importance. We report a method that enables the simultaneous determination of the copy numbers of several different targets as well as the discrimination among highly similar/almost identical targets that differ by only one single nucleotide variant, which establishes their copy numbers. The PCR co-amplification and single-base extension technologies are used to identify the copy number of a target sequence relative to a reference sequence of known genomic copy number in a given sample. This efficient and accurate quantification platform was successfully used to quantify the copy numbers of the primary spinal muscular atrophy-determining gene, *SMN1*, and the disease modifier gene, *SMN2*. The reliability, low-cost and potential for high-throughput make our method suitable for screening large populations as well as for use as a tool in clinical settings for genetic diagnosis/prognosis.

To the Editor,

Copy number variations (CNVs) are a form of genomic diversity involving DNA sequences >50 bp that are present in the genome in a variable number of copies (1). In humans, CNVs are a primary source of genetic variation (2), with up to 20% of the genome exposed to CNV (3). Many human disease conditions have been identified that are either caused by CNVs or that have a relative risk that is increased

by CNVs (4-6). A number of different tools have been developed to assess CNV. Approaches, including quantitative real-time PCR (7) and multiplex ligation-dependent probe amplification (MLPA) (8) could manifest significant false-positive rates and require an increased handling time and cost, while the recently introduced next generation sequencing (NGS) approach presents substantial computational and bioinformatics challenges (1). Here, we describe

Key words: hereditary diseases, copy number variation, gene dosage, SNPs identification, multiplex-PCR, single base extension

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a method for determining the CNV within a genome. This method is based on a specific multiplex PCR protocol that involves the amplification of the target locus/loci and reference locus/loci in a single reaction followed by SBE genotyping and capillary gel electrophoresis. The assay we developed provide an efficient and reliable method to analyse gene deletions and duplications. A comparison of the target signal over the reference signal for the test and control sample(s) results in a dosage quotient, indicating the copy number of the target region in the test sample. We introduce this method and verify its robustness and versatility by applying it as a diagnostic protocol for detecting *SMN1* deletion/conversion and for further determining the copy numbers of the *SMN1* and *SMN2* genes that are involved in spinal muscular atrophy (SMA). SMA is a severe neuromuscular disease which results in progressive muscle weakness and paralysis (9). The *SMN1* gene is located on chromosome 5q13 and in 95% of SMA cases is deleted. A nearly identical gene, *SMN2*, codes for the same protein product as *SMN1*, although at much lower rate, and decreases the severity of the SMA phenotype in a copy number-dependent manner (10). We selected the SMA genetic locus to validate this method because it is a well characterised disease-causing locus for

which SNP identification and CNV quantification have relevant diagnostic and/or prognostic roles.

MATERIALS AND METHODS

Subjects

We genotyped the DNA samples of 160 subjects. All the DNAs of the subjects were previously characterized for *SMN1* and *SMN2* copy numbers by multiplex real-time PCR (11) and by MLPA (12). Informed consent was obtained from all subjects.

Nucleic acid extraction

The genomic DNA was isolated from the peripheral blood leukocytes by a PuregeneTM DNA Purification Kit (Gentra Systems, Milan, Italy), according to the manufacturer's protocol. The DNA concentration was determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Milano, Italy).

Primer design

The PCR primers that flanked the marker polymorphisms in exon 7 and in exon 8 of the target *SMN* genes as well as the primers that were used to amplify reference genes Albumin (*ALB*) on chromosome 4 and Coagulation Factor VIII (*F8*) on chromosomes X, were designed by using Primer3 (<http://www-genome.wi.mit>).

Table I. PCR (for and rev) and SBE (sbe) primer sequences.

Primer name	5'-3' sequence
SMN_exon7_for	TGTCTTGTGAAACAAAATGCTT
SMN_exon7_rev	CACTTTCATAATGCTGGCAGA
SMN_exon8_for	CCACTAAAGAAACGATCAGACAGA
SMN_exon8_rev	TTTTCTCAACTGCCTCACCA
ALB_for	CCGTGGTCCTGAACCAGTTA
ALB_rev	CTCCTTCTCAGAAAGTGTGCAT
F8_for	TGGCATGGAAGCTTATGTCA
F8_rev	AGGGAGCATAGTCCCAGTCC
SMN_exon7_sbe	AATGTGAGCACCTTCCTTCTTTTGTATTGTTGTCT
SMN_exon8_sbe	GTGCTGGCCTCCCACCCCCACC
ALB_sbe	CTGCACAGAATCCTTGGTGAACAGGCGACC
F8_sbe	AGCTTATGTCAAAGTAGACAGCTGTCCAGAGGAACC CCAAC

edu/cgi-bin/primer/primer_3www.cgi). The primers were designed to have similar melting temperatures and different PCR product lengths, as is optimal for multiplex PCR. The SBE primers were designed to terminate amplification one nucleotide before the nucleotide of interest and with different lengths to avoid peak overlaps and interferences. Both of the SBE oligonucleotides that were used to detect nucleotide substitutions between the *SMN1* and *SMN2* genes were reverse primers designed on the (-)-strand. Thus, the oligonucleotide that was used to detect the C and T residues in exon 7 incorporated G for *SMN1* and A for *SMN2*, while the oligonucleotide that was used to detect the G and A residues in exon 8 incorporated C for *SMN1* and T for *SMN2*. All of the PCR and SBE primer sequences are included in Table I.

Multiplex PCR and SBE

The multiplex PCRs were performed in a final volume of 25 μ l. We combined the template (30 ng of genomic DNA) with the KAPA2GFastHSReadyMixPCR Kit Premix (Kapa Biosystems, Wilmington, MA, USA) and locus-specific primers (10 μ M each; Sigma Aldrich), as recommended by the KapaTaq protocol. The thermocycling conditions consisted of an initial denaturation step of 95°C for 2 min, followed by 26 cycles of 95°C for 15 s, 56°C for 15 s and 72°C for 7 s, with a final extension step at 72°C for 30 s. According to the manufacturer's instructions, 3 μ l of PCR products were incubated with Exo-SAP IT (Amersham Biosciences) prior to the primer extension reaction. Primer extension was carried out with the SNaPshot Multiplex Ready Reaction Mix (Applied Biosystem). The reaction was performed in a total volume of 10 μ l, containing 3 μ l of cleaned PCR products, 1 μ l of SNaPshot premix, 1 μ l each of 0.2 μ M SBE primer and 2 μ l of nuclease-free H₂O. The primer extension thermocycling conditions consisted of 26 cycles of 96°C for 10 s, 50°C for 15 s and 60°C for 30 s. Following the primer extension, the reactions products were purified by SAP (Amersham Biosciences), according to the manufacturer's instructions. The cleaned products were combined with 0.2 μ l of GeneScan-120 LIZ Size Standard Mix and 9.8 μ l of formamide and run on an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems). The peaks of dye intensities corresponding to extensions of the SBE primers were determined by inspecting the output from the ABI 3730 DNA Analyzer.

Statistical analysis

The quantification of the *SMN1* and *SMN2* copy numbers was performed independently for exon 7 and exon 8 by dividing the height of each gene-specific peak by the sum of the 2 endogenous control peaks (*F8* and *ALB*). The *ALB* gene on chromosome 4 is present in 2 copies per diploid genome. *F8* on chromosome X is present as a single copy gene per male diploid genome. The ratio was then compared, separately for males and females, to the ratios obtained from the three control samples with known *SMN1* and *SMN2* copy numbers to obtain a dosage quotient that indicated the copy number of each *SMN* gene.

RESULTS

We used the PCR co-amplification and SBE technologies to determine the copy number of a target sequence of an unknown genomic copy number relative to a reference sequence of a known genomic copy number. Briefly, upon target and reference sequence co-amplification, the excess dNTPs and primers were removed by shrimp alkaline phosphatase (SAP) and exonuclease treatment. The SBE of a primer that was specific for the target sequence as well as of a primer that was specific for the reference sequence was carried out in the presence of fluorescently labelled ddNTPs. The unincorporated fluorescent ddNTPs were then removed by SAP, and the extension products were readily identified using a DNA sequence detector. The quantification of the target sequence is achieved by the comparison of the target fluorescent signal *versus* the reference signal, exploiting the fundamental concept that the accumulation of fluorescence is proportional to the amplification of genomic regions. We utilized qualitative and quantitative capacities of this method by applying it as a diagnostic protocol for the identification of SMA carriers and for the characterization of affected individuals, concurrently establishing the copy number of the primary SMA-determining gene *SMN1* and the potential disease modulator gene *SMN2*. The assay was applied to a cohort of 160 individuals with different *SMN1* and *SMN2* copy numbers, including 88 apparently healthy individuals from the general population, 14 SMA-affected patients and 58 SMA carriers (Table II).

Base pair exchanges, C-to-T at position +6 in exon

7 (c.840C>T) and G-to-A in the untranslated region of exon 8 were used to distinguish between the two *SMN* genes (Fig. 1A), while the regions of the *ALB* and *F8* genes were used as internal references (Fig. 1A and Materials and Methods). We unambiguously identified the *SMN1* and *SMN2* peaks as well as the reference peaks from the genotyping outputs (Fig. 1B).

Two independent references were used simultaneously to avoid biases due to possible individual variations in the copy number of one

reference sequence. Because *ALB* on chromosome 4 is present in two copies per diploid genome and *F8* on chromosome X is present in two copies in females and in a single copy in males, we examined the reliability of our system by looking at the relative ratios of the two reference signals across males and females. The *ALB*/*F8* ratios found in males (1.85 ± 0.10) were consistently doubled with respect to those found in females (0.98 ± 0.12), underlining the reliability and the quantitative nature

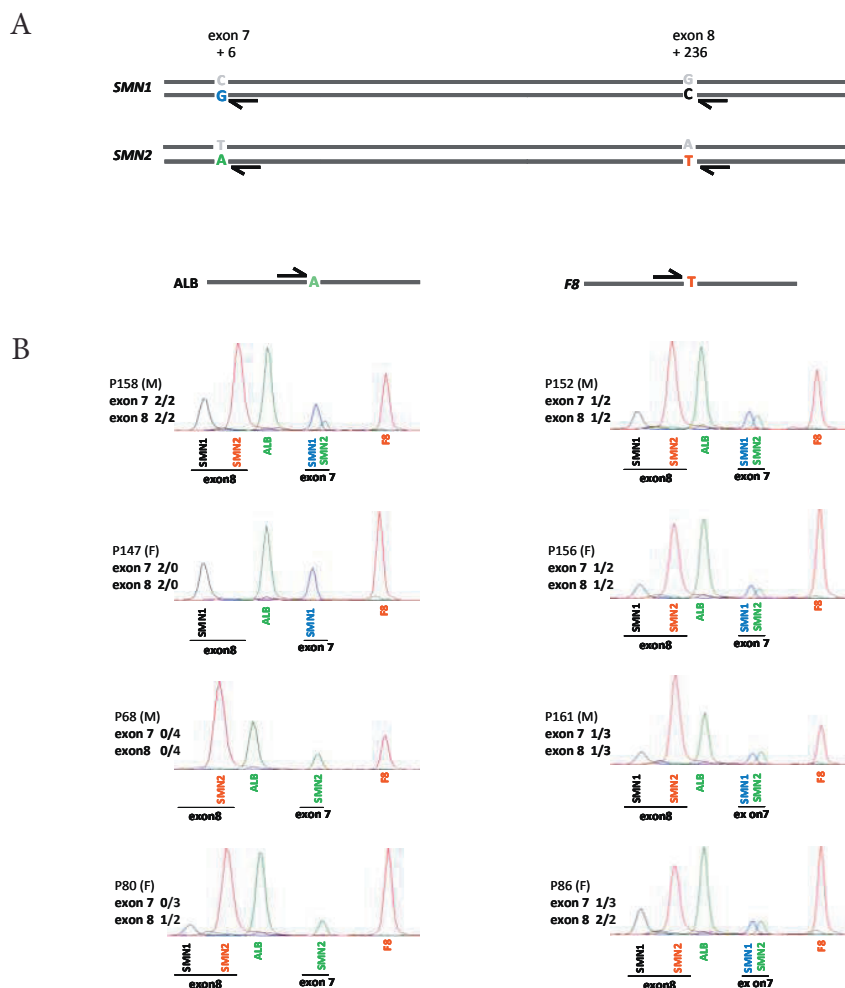


Fig. 1. SBE genotyping performed for target *SMN* genes and the reference Albumin and Factor 8 genes. **A)** For target *SMN* genes genotyped residues were C/T at position +6 in exon 7 and G/A at position +236 in exon 8. For reference albumin (*ALB*) and factor8 (*F8*) genes, genotyped residues were A at position +102 in exon 12 and T at position +52 in exon 8, respectively. Arrows represent SBE primers. **B)** SBE genotyping of multiplex PCR analysis from healthy controls, SMA patients and carriers with different *SMN1*/*SMN2* ratios. *SMN1* and *SMN2* in exons 7 and 8 as well as *ALB* and *F8* are indicated in colour code under respective peaks in sequencing outputs. Sample name, gender and *SMN1*/*SMN2* copy numbers, separately reported for exon 7 and exon 8, are indicated on the leftside of each output. F = female; M = male; Individuals P80 (affected subject) and P86 (healty carrier) experienced gene conversion of *SMN1* to *SMN2* in exon 7. Males have only one copy of the *F8* gene since it is on chromosome X.

of the assay. In addition, the fixed relative ratios between the two reference signals across individuals indicated stability in their copy numbers, confirming them as suitable references in all of the samples. It is important to note that the reference sequence can be any genomic region with stable copy numbers. Control samples with known *SMN1*/*SMN2* copy numbers (1/1, 1/2, 1/3, 2/2, 2/1 and 3/1) were run alongside the test samples. First, the controls were used to determine the proportionality between *SMN1* and *SMN2* relative signal intensities, separately for exon 7 and exon 8, showing the linearity of the assay in response to the varying copy numbers of the two *SMN* genes (exon7 $R^2=0.9915$ and exon8 $R^2=0.9653$) and further confirming the quantitative nature of the assay. Second, the target-to-reference ratios in the control subjects allowed for the assessment of the *SMN1* and *SMN2* copy numbers in the test samples with an unknown *SMN* dosage. The copy numbers of the *SMN1* and *SMN2* genes in the test samples (T) compared to those of the control samples (C) were calculated separately for males and females by the following equations:

$$\frac{\text{Peak heights SMN1(T)}/[\text{ALB}\pm\text{F8(T)}]}{\text{Peak heights SMN1(C)}/[\text{ALB}\pm\text{F8(C)}]}$$

and

$$\frac{\text{Peak heights SMN2(T)}/[\text{ALB}\pm\text{F8(T)}]}{\text{Peak heights SMN2(C)}/[\text{ALB}\pm\text{F8(C)}]}$$

This comparison resulted in a dosage quotient that indicated the copy number of each *SMN* gene in the test sample.

For each individual, we quantified the *SMN1* and *SMN2* dosage independently for exon7 and exon 8, enabling the identification of the gene-conversion events that result in the creation of a hybrid *SMN* gene. The gene conversion of *SMN1* to *SMN2* in exon 7 is one cause of SMA. The +6 C-to-T substitution in *SMN2* exon 7 decreases the activity of an exonic splice enhancer and alters the splicing pattern so that the *SMN2* mRNA excludes the exon 7 sequence. Consequently, *SMN2* produces insufficient amounts of the full-length *SMN* transcript and protein to rescue the SMA

Table II. A cohort of 160 individuals with different *SMN1* and *SMN2* copy numbers.

Number of subjects	Genotype (<i>SMN1</i> : <i>SMN2</i>)	Expected total copy number (<i>SMN1</i> + <i>SMN2</i>)	Measured total copy number (mean $\pm$ SD)	exon7 measured <i>SMN1</i> / <i>SMN2</i> ratio (mean $\pm$ SD)	exon8 measured <i>SMN1</i> / <i>SMN2</i> ratio (mean $\pm$ SD)	Expected <i>SMN1</i> / <i>SMN2</i> ratio
5	2:0	2	2.28 $\pm$ 0.22			
4	1:1	2	2.11 $\pm$ 0.17	1.07 $\pm$ 0.03	1.10 $\pm$ 0.02	1.00
20	1:2	3	3.08 $\pm$ 0.29	0.52 $\pm$ 0.03	0.54 $\pm$ 0.06	0.50
40	2:1	3	3.02 $\pm$ 0.32	2.05 $\pm$ 0.06	2.00 $\pm$ 0.02	2.00
32	1:3	4	3.87 $\pm$ 0.34	0.34 $\pm$ 0.03	0.35 $\pm$ 0.02	0.33
36	2:2	4	3.97 $\pm$ 0.31	1.09 $\pm$ 0.04	1.11 $\pm$ 0.03	1.00
2	3:1	4	4.15 $\pm$ 0.21	3.02 $\pm$ 0.10	2.95 $\pm$ 0.05	3.00
5	2:3	5	5.03 $\pm$ 0.21	0.62 $\pm$ 0.03	0.67 $\pm$ 0.06	0.66
2	1:4	5	4.85 $\pm$ 0.39	0.26 $\pm$ 0.01	0.22 $\pm$ 0.02	0.25
9	0:3	3	3.12 $\pm$ 0.14			
4	0:4	4	4.45 $\pm$ 0.22			
1	0:5	5	5.22 $\pm$ 0.20			

phenotype. However, the conversion of *SMN2* to *SMN1* in exon 7 could be used as a therapeutic approach for SMA (13). By identifying individuals with unequal *SMN1/SMN2* ratios in the two exons, we were able to distinguish between the hybrid genes derived from conversion events. Individual P86 had an *SMN1/SMN2* ratio of 1:3 for exon 7 and 2:2 for exon 8, while individual P80 had a ratio of 0:3 in exon 7 and 1:2 in exon 8 (Figure 1B), indicating a conversion from *SMN1* to *SMN2* in exon 7. Due to this conversion, individuals P86 and P80 became an SMA carrier and an SMA-affected individual, respectively.

We analysed each sample at least three times obtaining analogous results, demonstrating the reproducibility of our detection system for gene dosage determination (Table II). The measured copy numbers were in accordance with the genotypes that were previously determined by real-time PCR (11) and MLPA (12), proving that we can successfully assign all genotypes with different *SMN1/SMN2* gene copy numbers, including *SMN1/SMN2* ratios equal and not equal to one. We initially designed SBE oligonucleotides on the (+)- and (-)-strands for to detect nucleotide substitutions in exon 7 and exon 8 of the *SMN* genes as well as several oligonucleotides to genotype different *ALB* and *F8* residues. These oligonucleotides were used in various combinations to obtain the optimal output pattern, *i.e.*, comparable peak heights and clear peak separation to avoid interference due to overlapping signals (data not shown). All of the tested combinations gave distinct, well-separated peaks; however, for some combinations, there was a large discrepancy in the signal intensities favouring one peak (up to 20-fold). We were always able to correctly determine the copy number of both of the *SMN* genes. Good peak separation was obtained using SBE oligonucleotides that differed in length by at least 3 nucleotides.

DISCUSSION

Here, we report a molecular tool for the targeted detection and characterisation of CNVs. The PCR co-amplification and SBE technologies were used

to determine the copy number of a target sequence of unknown genomic copy number relative to a reference sequence of known genomic copy number. This is a fluorescence-based method that can be used to simultaneously determine the copy numbers of several different targets as well as to discriminate among highly similar/almost identical targets that differ by only one SNP, establishing their copy numbers. To fully exploit the potential of this method, we validated it by genotyping the human SMA locus, evaluating the *SMN1* and *SMN2* genes copy number at the same time. A previous method based on the principle of primer extension has been developed for genotype determination at the *SMN* locus (14). However, this method is more laborious and time-consuming because it is based on two independent multiplex and complementary PCRs before the primer extension reaction, including a competitive PCR requiring a known amount of pre-constructed internal controls. Furthermore, this method is not able to detect the conversion of *SMN1* to *SMN2* because it relies only on exon 7 of both genes. The method we report allows for the complete genotyping of the *SMN* locus, including the gene conversions, by the concurrent identification of SNPs in exon 7 and exon 8 of the two *SMN* target genes and of arbitrary residues in *ALB* and *F8* reference genes. We were able to accurately establish the copy number of the primary SMA-determining gene *SMN1* and the potential disease modulator gene *SMN2* in a cohort of 160 individuals previously genotyped in our laboratory (11-12). We successfully identified all of the carriers and correctly determined the copy numbers of the *SMN2* gene in the SMA patients. The precise assessment of the *SMN2* copy number is particularly important, as it could have a prognostic relevance for affected subjects (10) and as *SMN2* is a common target in gene therapy (13). The assay is based on the recognition of a specific fluorescent signal, circumventing sequence/probe cross-hybridisation that could contaminate the final analytical results, as in MLPA-based and real-time PCR-based methods. In conclusion, we report a rapid and simple quantitative assay that allows for the identification and allele-specific characterisation of CNVs.

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LETTER TO THE EDITOR

TRANSVAGINAL COLOR DOPPLER SONOGRAPHY COMBINED WITH COLPOSCOPY FOR DIAGNOSIS OF EARLY STAGE CERVICAL CANCER AND PRECANCEROUS LESIONSHR. WANG¹, Y. LIN², XY. ZHANG³ and XT. MA²

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This study aims to assess the value of transvaginal color Doppler sonography (TVCD) combined with colposcopy for diagnosis of early stage cervical cancer and precancerous lesions. From January 2015 to September 2016, one hundred patients, diagnosed as having early stage cervical cancer and precancerous lesions by histopathological study, were selected as the observation subjects. All patients were examined by TVCD and colposcopy, and the results were compared with histopathological findings. The diagnostic accuracy, sensitivity and specificity of TVCD alone, colposcopy alone, and TVCD combined with colposcopy in early stage of cervical cancer and precancerous lesion were compared. The sensitivity, specificity and accuracy of TVCD combined with colposcopy were significantly higher than that of TVCD ($P < 0.05$), and the sensitivity was significantly higher than that of colposcopy ($P < 0.05$). The high sensitivity, specificity and accuracy of TVCD combined with colposcopy on the diagnosis of invasive cervical carcinoma (ICC) and cervical intraepithelial neoplasia (CIN) can provide a scientific basis for its use in clinical diagnosis.

To the Editor,

Cervical cancer is a type of malignant tumor, but it takes a long time to develop from precancerous lesions to early cancers, which provides time and opportunity for early diagnosis (1). Colposcopy and biopsy are the current methods for diagnosis of cervical intraepithelial neoplasia (CIN) and early stages of cervical cancer. It has been demonstrated that Doppler is a well-recognized method for detection of neo-vascularization and blood flow (2). Furthermore, cancer cervix is well-known to be associated with neo-vascularization. Transvaginal color Doppler sonography (TVCD) allows *in vivo* assessment of tumor angiogenesis and vascularization (3, 4). Previous studies have disclosed its role in

some gynecological malignancies, such as ovarian cancer and endometrial cancer (5, 6). This study was performed to evaluate the application value of TVCD combined with colposcopy in the diagnosis of early cancer and precancerous lesions.

MATERIALS AND METHODS*Clinical data*

This observational study was conducted from January 2015 to September 2016, and included 100 cases with suspected early Ca cervix or CIN as observation subjects. The mean age of the patients was 42.6 ± 1.3 years (range: 22-72 years). Patients were included in the study if they were suspected with early cervical cancer and

Key words: *transvaginal color Doppler sonography, colposcopy, early stage cervical cancer, precancerous lesions*

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precancerous lesions. Exclusion criteria were patients with cervical cancer or combined with malignant tumor. There was no significant difference in the general data between patients. All patients gave informed consent to participate in the study.

Methods

All patients were examined by TVCD and colposcopy, and compared with histopathological findings. The diagnostic accuracy, sensitivity and specificity of TVCD alone, colposcopy alone, and TVCD combined with colposcopy in early cervical cancer and precancerous lesions were compared.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) was used for data processing. Measurement data were presented as (mean $\pm$ standard difference). Student's *t*-Test was used for comparison between groups. The categorical data were described as the rate (%). The χ^2 test or Fisher's exact test was used to assess differences in categorical data between the two groups. All results were considered significant at the 0.05 level.

RESULTS

Sensitivity, specificity and accuracy of the three inspection methods

Histopathological examination found that there were 21 patients with early cancer, 36 cases with precancerous lesions and 43 cases with cervicitis among the 100 cases. The sensitivity, specificity and accuracy of TVCD combined with colposcopy were significantly higher than those of TVCD alone ($P < 0.05$), and the sensitivity of TVCD combined with colposcopy was significantly higher than that of colposcopy alone ($P < 0.05$) (Table I).

TVCD manifestations in different patients

By observing the color Doppler ultrasound images of patients, we found that the percentage and mean value of cervical enlargement and the peak systolic velocity (PSV) were all significantly higher than those of patients with cervicitis ($P < 0.05$), but the resistance index was significantly lower than that of patients with cervicitis ($P < 0.05$), as shown in Table II.

DISCUSSION

TVCD, as a noninvasive technique, is a new type of examination developed by ultrasound in recent years. It has the advantage of wide viewing angle, clear images, no need to maintain a full bladder state, and it is not affected by intestinal gas and fat (7). Color Doppler allows easy identification of blood vessels within the tumoral tissue and gives a tumor blood flow mapping. However, studies regarding color Doppler ultrasound for the assessment of intratumoral blood flow in cervical cancer are still limited. Previous study has demonstrated that intratumoral blood flow assessed by TVCD was correlated with some tumor features, such as cell proliferation index and pelvic lymph node metastases in patients with ICC, but the blood flow detection rate was low, only 46.2% tumors showed an intratumoral color-coded signal (8). Later, by using the power Doppler ultrasound, Wu et al. found a 100% blood flow detection rate (9). In addition, Alcazar et al. found that there was a more frequent abundant flow in squamous carcinomas than in adenocarcinomas, and also showed that intratumoral blood flow correlates with some tumoral features, such as histologic grade, tumor volume and tumor stage (10).

Colposcopy is one of the commonly used methods for the examination of cervical lesions, through the direct observation of cervical changes, including color, surrounding mucosa and other aspects (11). Meanwhile, iodine solution can also be used for further examination. Therefore, it has a good application value in the diagnosis of early cancer and precancerous lesions.

In this study, the sensitivity, specificity and accuracy of TVCD in the diagnosis of early cancer and precancerous lesions were 72.54%, 86.04% and 78.72%, respectively; the sensitivity, specificity and accuracy for colposcopy were 90.2%, 100% and 94.68%, respectively; while, the sensitivity, specificity and accuracy of TVCD combined colposcopy were 98.04%, 100% and 98.93%, respectively. The sensitivity, specificity and accuracy of TVCD combined with colposcopy were higher than those of TVCD alone, and the sensitivity of

Table I. Sensitivity, specificity and accuracy of the three inspection methods.

Inspection method	Diagnostic results	Pathological diagnosis results		Sensitivity	Specificity	Accuracy
		Precancerous lesions and early cancer	Cervicitis			
TVCD	Precancerous lesions and early cancer	37	6	72.54	86.04	78.72
	Cervicitis	14	37			
Colposcopy	Precancerous lesions and early cancer	46	0	90.20	100.00	94.68
	Cervicitis	5	43			
TVCD combined with colposcopy	Precancerous lesions and early cancer	50	0	98.04 ^{ab}	100.00 ^a	98.93 ^a
	Cervicitis	1	43			

(Note: compared with TVCD, ^a $P < 0.05$; compared with colposcopy, ^b $P < 0.05$)

Table II. TVCD manifestations in different patients.

Classification of disease	Percentage of cervical enlargement (%)	Average value of cervical enlargement (mm)	Peak systolic velocity (cm/s)	Resistance index
Early cancer (n=21)	85.71 (18/21) ^a	42.58±0.35 ^a	11.56±2.56 ^a	0.40±0.06 ^a
Precancerous lesions (n=36)	94.44 (34/36) ^a	42.54±0.32 ^a	9.56±2.15 ^a	0.51±0.05 ^a
Cervicitis (n=43)	18.60 (8/43)	27.56±0.21	8.54±2.36	0.60±0.16

(Note: compared with cervicitis, ^a $P < 0.05$)

TVCD combined with colposcopy was higher than that of colposcopy alone, suggesting that there were high sensitivity, specificity and accuracy of TVCD combined with colposcopy in the diagnosis of early cancer and precancerous lesions. By observing the color Doppler ultrasonography of patients, the results found that the percentage and mean value of cervical enlargement and PSV were all higher than those of patients with cervicitis, but the resistance index was lower than that of patients with cervicitis, suggesting that color Doppler images could observe the changes of cervical morphological and blood flow, providing a scientific basis for clinical diagnosis of early stage cervical cancer and precancerous lesions by a noninvasive technique.

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LETTER TO THE EDITOR

ANALYSIS OF QUALITY NURSING OF POSTOPERATIVE INCISION INFECTION IN UROLOGICAL PATIENTS

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To study the effect of quality nursing on postoperative incision infection in urological patients, 200 subjects admitted to our hospital between June 2016 and June 2017 were included in this study and divided into a quality nursing group (group A) and a general nursing group (group B), 100 in each group. Blood loss, blood transfusion, hospital stay, incision healing, incision infection, and self-rating depression scale (SDS) scores in both groups were compared. It was found that the bleeding volume in group A was significantly less than that in group B, and there was significant difference between the two groups ($P < 0.05$) while the difference in blood transfusion rate and hospital stay between the two groups was not significant ($P > 0.05$); the number of patients of level 1 healing in group A was larger than that of group B while the number of patients of level 2 and level 3 healing was smaller than that of group B, with significant differences ($P < 0.05$); the number of infection cases in group A was significantly lower than that in group B, and the difference was significant ($P < 0.05$); the SDS score of group A was lower than that of group B, with significant differences ($P < 0.05$). Therefore, quality nursing had a certain effect on the infection of postoperative incision of urological patients, which had positive significance for incision healing. Reducing the effect of bacterial infections in operated patients is important for rapid healing and patient health. Using a long-lasting antibacterial can protect the patient and reduce the incidence of other infections.

To the Editor,

Currently, the urology department, covering urinary tract diseases such as urinary stones, kidney stones, prostatic hyperplasia and testicular inflammation, is one of the departments with a high incidence of infection (1). For urological patients, surgical treatment is the most common and most effective treatment (2). However, surgery can cause damage to a patient's physical functions and lead to complications (3, 4), the latter of which are commonly caused by wound infection and can influence recovery. Marković-Denić et al. (5) studied the incidence of urological infection and found that

the total incidence rate was 5.9%. Niu et al. (6) divided infected patients into an experimental group and an observation group to compare site of infection with the uninfected patients. The pathogen test results and the incidence of nosocomial infections and prognosis, regarding the clinical characteristics of the two groups found that the mortality of the infected group was significantly higher than that of the observation group. Therefore, they held that operations should be reduced, as well as hospital stay and time of indwelling catheter. In this paper, the effect of quality nursing on incision infection of patients after urinary surgery was studied to provide

Key words: urology, infection, quality nursing

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theoretical and technical support for postoperative incision healing measures.

MATERIALS AND METHODS

Study subjects

Two hundred urological patients admitted to our hospital between June 2016 and June 2017 were included in this study. The subjects were divided into a quality nursing group (group A) and a general nursing group (group B), 100 in each group. The basic information of the subjects is shown in Table I. The ratio of male to female, age, weight, type of disease, and endoscopic surgery were not significantly different between the two groups. All the subjects were informed about the experimental procedures and signed informed consent.

General nursing method

After incision stitching was performed on the patients in group B, the incisions were covered with dry dressings, which were changed every three days. During the hospital stay, the patients were adjusted to a supine position whilst asleep with few turn overs. In addition, the rooms were given disinfection every three days to avoid cross-infection phenomenon. Surgical aseptic operation was strictly implemented. Patients with urinary tract irritation

symptoms underwent a second urine test and were treated with antibacterial agents and asked to drink water. Then, their body temperature, urine and urethral secretions were observed and antibiotics were applied to control infection. After extubation, the urinating condition of the patients was controlled and doctors were called when a patient had difficulty in urination. After surgery, the patients were allowed to have rice soup and provided with psychological guidance.

Quality nursing method

Before surgery, the patients in group A were adjusted to a comfortable position with the help of the nursing staff and told about the brief introduction of the surgery to reduce their sense of fear. The specific information was as follows:

Patients who were not awake after surgery took the position of lying on one side to ensure smooth breathing and they were not allowed to change the lying position until their blood pressure recovered to normal level. If bleeding was seen from the incision site, timely treatment was performed.

Patients with abnormal urination were comforted to alleviate their mental pressure. If they felt urinary tract irritation, they were suggested to rest and drink plenty of water in order to increase urine output; if the situation

Table I. Basic information of patients.

Group	Male: female	Age (years old)	Weight (kg)	Disease type	Endoscopic surgery
quality nursing group (group A)	75:25	29~79	59~74	Kidney stones, bladder stones, ureteral calculi and urethral stones	Urethral bladder balloon lithotripsy surgery, ureteroscopic pneumatic lithotripsy surgery, skin nephrolithiasis pneumatic lithotripsy surgery, urethral prostate gasification electrotomy surgery
general nursing group (group B)	78:22	30~82	56~75	Kidney stones, ureteral calculi, urethral calculi and bladder stones	Urethral bladder balloon lithotripsy surgery, ureteroscopic pneumatic lithotripsy surgery, skin nephrolithiasis pneumatic lithotripsy surgery, rethral prostate gasification electrotomy surgery

was serious, they were given Harnal. If there was urine retention, their position was changed and hot compress was applied to their lower abdomen. The bedding was immediately changed if contaminated due to urinary incontinence.

The drainage tube was fixed and was changed every week. The urethra was disinfected twice a day to prevent contamination. Timely diagnosis and treatment were performed on the patients if an abnormal urine state was found. Routine examination and bacterial culture of urine were performed.

Six hours after surgery, the patients could eat rice soup and other liquid foods if there were no gastrointestinal adverse reactions. They took several small meals made up of low-fat digestible foods, accompanied with fresh vegetables and fruits. In addition, they drank a lot of water rather than strong tea to ensure a smooth bowel movement.

Communication with the patients was performed to help them relieve mood. The temperature and incision conditions of the patients were recorded daily. The confusions of the patients were explained to make them relaxed.

Before the patients were discharged from hospital, their family members were told that the patients had to avoid doing physical labor or waist and limb movement in the early days and that they should undergo regular follow-up examinations.

The incision healing conditions of the patients were evaluated based on three levels: level 1, level 2 and level 3, with their standards as follows:

Level 1: good healing results, without discomfort reactions.

Level 2: poor healing results, with redness, hydrops and induration on the incision.

Level 3: suppuration of the incision, which needed drainage.

In this study, levels 2 and 3 were regarded as infection situations; the calculation formula for the infection rate was $Z = \frac{A+B}{C}$, where A refers to the number of level 2 patients, B refers to the number of level 3 patients and C refers to the total number of patients (7).

Observation indicators

The surgical conditions, and infection indicators of the patients were observed, with SDS scoring made

on the patients and the results displayed. SDS is a self-rating depression scale which is recommended by the U.S. Department of Education and Health for psychopharmacology research, mainly including the following 20 items: blue mood; big mood fluctuations in the day; tendency to cry; difficulty in falling asleep; poor appetite; diminished sexual interest; loss of weight; defecation difficulty; palpitation; sleepiness; slowness in thinking; decreased ability; uneasiness; feeling hopeless; easily irritated; hesitation in making decisions; feeling of being useless; feeling empty; feeling no sense of value; loss of interests for anything.

All the above items were scored from 1 to 4; 1 indicated few or no such situation; 2 indicated it happened sometimes; 3 indicated it happened most of the time; 4 indicated it happened almost all the time. And the depression degree was calculated according to the following equation:

Depression degree = cumulative score of all the 20 items / 80.

The greater the result, the deeper the depression degree (8).

Statistical analysis

The data in this study was processed by SPSS 19.0, with the counting data expressed with frequency method and group comparison tested with χ^2 . $p < 0.05$ indicated that the difference had statistical significance.

RESULTS

Surgical conditions

The intraoperative blood loss of group A was obviously less than that of group B, with significant differences ($p < 0.05$) (Table II). Furthermore, there were no significant differences in incidence of transfusion and hospital stay between the two groups ($p > 0.05$), suggesting that patients in group A who received quality nursing had less bleeding during surgery, which reflects the role of relieving the nervous tension before the operation.

Comparison of healing and infection rates

As shown in Fig. 1, the number of level 1 patients in group A was 86, much higher than 63 in group B, while the number of level 2 and

level 3 patients was obviously lower than that in group B. There were statistical significances in differences in the number of patients of the three levels between the two groups ($p<0.05$).

As shown in Table III, the infection rate of group B was significantly higher than that in group A, with statistical significance ($p<0.05$), and the number of patients with incision shallow infection, incision deep infection and organ and luminal infection was smaller in group A than in group B, suggesting that quality nursing could improve incision healing results and reduce infection. By comparing the incision infection rate and healing condition of the two groups, it was found that the incision infection and recovery results of group

A were better than group B, and incision healing conditions reflected the role of quality nursing.

SDS scoring

The SDS score of group A was 41.11 ± 5.67 , obviously lower than 53.11 ± 8.26 in group B, with statistical significance ($p<0.05$), suggesting that the mental status of patients in group A was better than that of patients in group B (Table IV).

DISCUSSION

In recent years, with the development of medical technologies, the emphasis on urological departments

Table II. *Surgical conditions of the patients.*

Group	Number of cases	Intraoperative blood loss (mL)	Incidence of transfusion (%)	Hospital stay (day)
quality nursing group (group A)	100	$89\pm37.1^*$	12 (11.1)	4.6 ± 1.1
general nursing group (group B)	100	104 ± 49.7	14 (13.2)	5.4 ± 2.1

Table III. *Infection rate of the two groups.*

Group	Number of cases	Infection rate (%)	Incision shallow infection	Incision deep infection	Organ and luminal infection
quality nursing group (group A)	100	14%*	9	5	0
general nursing group (group B)	100	37%	21	15	1

* indicates that compared with group B, $P<0.05$

Table IV. *SDS scoring of the two groups.*

Group	Number of cases	SDS score
quality nursing group (group A)	100	$41.11\pm5.67^*$
general nursing group (group B)	100	53.11 ± 8.26

* indicates that compared with group B, $P<0.05$

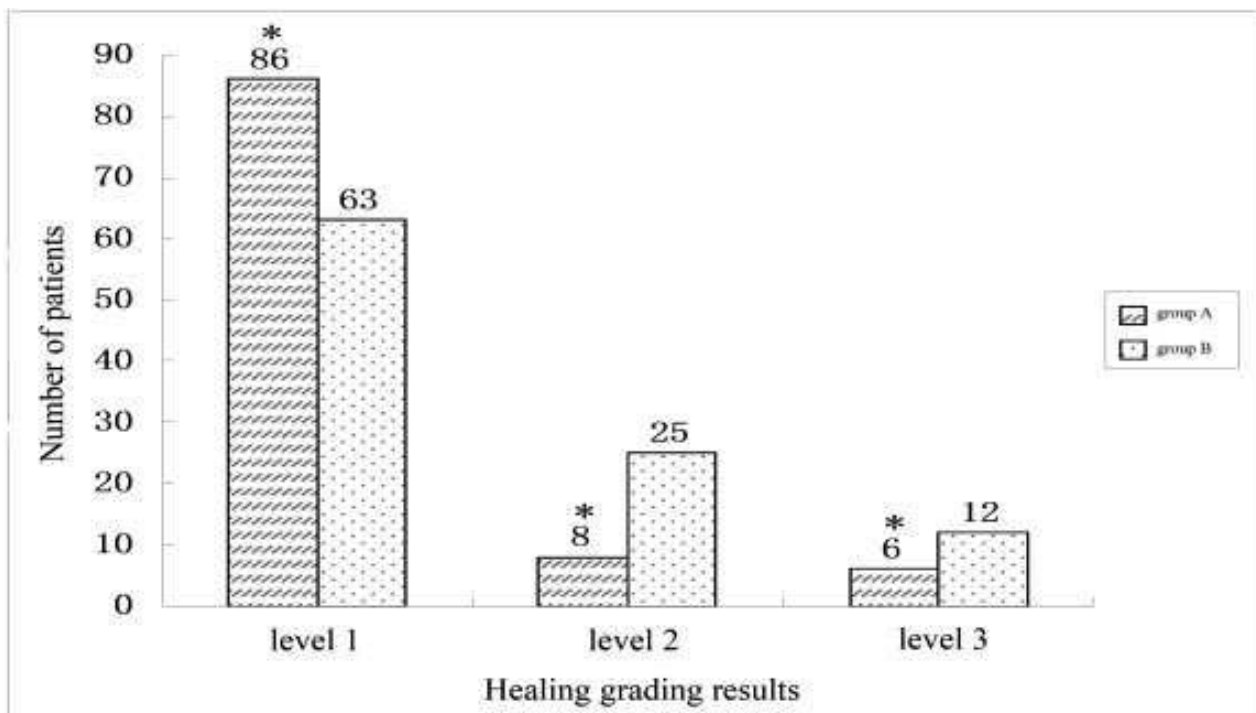


Fig. 1. Healing grading results of the incision of the patients in the two groups.

* indicates that compared with group B, $P < 0.05$

has grown. Among current surgical treatment, endoscopic surgery is a commonly used method (9). In addition to incision infection, urological infections also includes respiratory, blood and urinary tract infections (10). In the course of treatment, because of the need for an indwelling catheter, a little carelessness will cause bacteria, leading to urinary tract infection. In addition, the irrational use of antibiotics also increases the risk of infection (11, 12). Therefore, quality care is one of the most effective ways to prevent infection. Nursing, both before and after surgery, is a response to diagnosis or treatment of existing or potential health problems. As most patients feel anxious and nervous before surgery, it is necessary to give them guidance and comfort. In the present study, the bleeding volume of group B was significantly higher than that of group A, which suggested that the posture adjustment was beneficial to the improvement of the recovery of the patient. Similarly, quality nursing also helped to reduce the infection rate and improve the healing grade. The

SDS scoring results showed that the depression degree of patients in group A was lower than that in group B, suggesting that postoperative care could not only reduce the infection of the incision, but also ease the psychological tension. Therefore, communication with patients before surgery could reduce the amount of intraoperative blood loss, and nursing for abnormal micturition could reduce the infection rate of incision as well as improve the healing level of patients. It is an effective nursing method and is worth being promoted for wide application.

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LETTER TO THE EDITOR

EFFECT OF POLYDATIN ON EXPRESSION OF p53 AND NOTCH1 IN BRAIN TISSUE OF ISCHEMIC CEREBROVASCULAR DISEASEFY. CHEN¹, XY. FANG² and H. ZHANG³

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Secondary injury of brain tissue following cerebral infarction exhibits a complicated pathogenesis that is attributed to the induction of apoptosis and inflammatory response. The present study aimed to investigate the polydatin neuroprotective effects and their mode of action in cerebral ischemic injury. A total of 80 healthy adult male Sprague-Dawley rats were randomly divided into a Sham operated group, a permanent middle cerebral artery occlusion (MCAO) group, a Polydatin low dose group and a Polydatin high dose group. A total of 20 rats were used in each group. The right MCAO model of rats was established using the modified Longa suture method. The animals in the intervention group were injected intraperitoneally with 12.5mg/kg and/or 50mg/kg of polydatin following infarction. The expression levels of p53 and Notch 1 were measured by immunohistochemical techniques. The degree of left limb hemiplegia in the MCAO group was different compared with that of the low and high dose group of polydatin. The score for the neurological function in the high-dose group of polydatin was lower than that in the MCAO group (High vs MCAO: 2.4 ± 0.31 vs 3.9 ± 0.23 , $P < 0.05$). The results indicated that high dose polydatin could reduce brain edema following cerebral infarction and improve the behavioral score in rats.

To the Editor,

Cerebrovascular disease is one of the three major diseases that lead to human death. This disease is caused by multiple etiologies such as atherosclerosis and is associated with various diseases, namely diabetes mellitus, hypertension, dyslipidaemia and so on (1). The mechanisms that are associated with tissue injury following cerebral ischemia are complex and include apoptosis, inflammatory response, oxidative free radical damage, excitotoxic amino acid effects, mitochondrial damage, energy depletion, and calcium overload (2).

The p53 gene is a tumor suppressor gene that

is located in the human chromosome 17p13.1 and encodes a 393 amino acid 53kD nuclear phosphorylation protein. The p53 protein plays an important role in the process of apoptosis following cerebral ischemia. It induces apoptosis via the transcriptional-dependent pathway and the non-transcriptional-dependent pathway (3). The Notch 1 signaling pathway exhibits proinflammatory effects and is involved in the inflammatory response following cerebral ischemia (4). Therefore, the use of drugs that inhibit the activation of p53 and Notch 1 signaling pathways induced by cerebral ischemia provides two important approaches for the treatment

Key words: polydatin, brain protection, cerebral infarction, p53, Notch 1

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of ischemic cerebrovascular diseases.

Polydatin (resveratrol-3-O- β -mono- D-glucoside, the glycoside form of resveratrol) is the natural precursor of resveratrol that is widely found in natural plants and/or fruits, such as grapes, peanuts, cassia seed, and reed and *Polygonum cuspidatum*. It possesses antioxidant, radical scavenging and anti-apoptotic effects, while it further protects the blood vessel endothelium (5).

The aim of the present study was to evaluate the effect of polydatin on the expression of p53 and Notch 1 proteins in a cerebral ischemia rat model.

MATERIALS AND METHODS

Reagent and drugs

The experimental drugs included Polydatin (Xi'an Huacui Biotechnology Co., Ltd. China) and Chloral hydrate (Tianjin Chemical Reagent Three Factory, China). The main chemical reagents of the immunohistochemical experiments included rabbit anti-rat p53 polyclonal antibody (Bioworld, USA), rabbit anti-rat Notch1 polyclonal antibody (Abcam, USA), ABC (Avidin-Biotin Complex) reagent kit (Hebei Bohai Bioengineering Co., Ltd.), DAB (3,3'-Diaminobenzidine) color reagent kit (Beijing Zhongshan Biological Engineering Co., Ltd.), sodium chloride (Tianjin Chemical Reagent Three Factory), sodium hydrogen phosphate (Tianjin Chemical Reagent Three Factory), citric acid (Tianjin Chemical Reagent Three Factory), xylene (Tianjin Chemical Reagent Three Factory) and paraformaldehyde (analytical purity) (Tianjin Chemical Reagent Three Factory). The primary solutions included Polydatin with $\geq 98\%$ purity, made in 0.9% sodium chloride solution. The antigen repair solution A consisted of 101 g citric acid and 100 ml double distilled water. The Antigen repair solution B consisted of 14.705 g citric acid and 500 ml double distilled water.

Animals

A total of 80 healthy adult male Sprague-Dawley rats (weight: 265.34 ± 2.48 g; age 9.84 ± 4.31 weeks) were supplied by the animal experimental center of the Basic Medical College of Hebei Medical University. The rats were fed with standard diet and purified water. The feeding environment was a multi-layer flow framework from the animal experimental center in the Second Affiliated hospital of the School of Medicine of Zhejiang University.

Prior to the study, the animals were acclimatized for 2 weeks under laboratory conditions. The animals were kept under standard conditions, namely 19-23°C, humidity range of 35-55% and 12-hour light/dark cycle. The study was approved by the Ethics Committee of the Second Affiliated hospital of the Zhejiang University School of Medicine.

The rats were randomly divided into four groups as follows: Sham operated group, MCAO group, Polydatin low dose group (MCAO + Polydatin 12.5 mg/kg) and Polydatin high dose group (MCAO + Polydatin 50 mg/kg). A total of 20 animals were used per group. The polydatin treatment group received 50 mg/kg of polydatin solution that was injected intraperitoneally immediately after cerebral infarction, whereas the low dose of polydatin treatment group received 12.5 mg/kg polydatin solution that was injected intraperitoneally immediately after cerebral infarction. The MCAO and the Sham groups received the same volume of normal saline solution injected intraperitoneally immediately after cerebral infarction.

Establishment of MCAO animal model

The MCAO rat model was established using the suture method. A nylon line with a length of approximately 3 cm was selected, and its head was rounded off on an alcohol lamp. Specific marks were used at 18 mm and 20 mm that corresponded to the head and the distance from the head end, respectively.

The right middle cerebral artery occlusion (MCAO) models were made by the Longa suture method. The rats were weighed and anesthetized with intraperitoneal injection of 10% chloral hydrate. Following the successful induction of anesthesia, the rats were held in the supine position on the operating table. The conventional skin and disinfection processes were carried concomitantly and a median neck incision of approximately 2 cm was selected. The glandular tissue of the neck and fascia were carefully separated, exposing the right neck triangle. The right common carotid artery (CCA), the external carotid artery (ECA) and the internal carotid artery (ICA) were also carefully separated. The CCA was clipped with a small artery clamp. The trunk and branches of ECA were dissociated, and their branches were coagulated by bipolar electrocoagulation pen. The ECA was ligated and separated at approximately 3 to 4 mm from the bifurcation

of CCA. The ICA was isolated in the skull base direction and its trunk was exposed. Following a small cut on the stump of ECA, the prepared thread plug was inserted into the ICA intracranial segment. The length of the incoming bolt was approximately 18.5 ± 0.5 mm from the CCA bifurcation. During that process, a slight resistance was applied, and the plug was inserted into the intracranial anterior cerebral artery in order to block the opening of the middle cerebral artery. The remaining end of the thread plug was ligated and cut off, and the CCA micro artery clip was removed. The subcutaneous tissue and skin were sutured layer by layer following disinfection.

Rat model of sham group

The preparation procedure of the sham group was the same as that of the MCAO rat model, although the blood vessels were not inserted after the plug.

Behavioral assessment

Following the establishment of the animal model, the rats were evaluated by the modified Longa classification method at 24 h post-operatively. The following scoring system was used: 0 score: no neurologic impairment; 1 score: the rat could not stretch the forelimb (left) of the paralyzed side; 2 score: the rat could stretch the forelimb (left) of the paralyzed side; 3 score: the rats could form a large circle of the paralyzed side (left side) during walking; 4 score: the rat could form a small circle of the paralyzed side (left side) during walking; 5 score: the rat was leaning towards the paralyzed side (left side) while walking.

Determination of water content in brain tissue

The water content of the brain tissue was measured by the wet and dry weight method. The brain tissue exhibited approximately 2 mm thickness and was used for the measurement of the cerebral water content. The brain tissue was divided into two sides: the lesion side and lesion contralateral.

The brain tissue was placed in the pre-weighing (A) foil and immediately weighed (B). B-A is wet weight. The brain tissue was wrapped with aluminum foil, and placed in the oven and baked for 24 h at 100°C . The brain tissue was then removed under room temperature and weighed (C). C-A denoted dry weight. The water content of both sides of the brain was calculated by using the following

formula: $(\text{Brain tissue wet weight} - \text{Brain tissue dry weight}) / \text{wet weight} * 100\%$, that is $(B - C) / (B - A) * 100\%$.

Determination of p53 and Notch1 expression levels by immunohistochemical methods

The rats in each experimental group were decapitated following the second anesthesia. The brain tissues of each group were stored in 4% paraformaldehyde solution at 4°C . The preservation process was fixed for more than 24 h but not more than 7 days. The dehydration process was conducted using conventional gradient dehydration, while xylene was used for transparency. The samples were embedded with paraffin and continuous slices with a thickness of 5 mm were made. Immunohistochemical staining was conducted using ABC (Avidin-Biotin Complex) staining. Following the development of color by DAB (3,3'-Diaminobenzidine), hematoxylin solution was used for the formation of the dye, and the process was as follows. The tissue sections were placed in xylene for 10 min. Following the replacement of xylene, the samples were soaked for an additional 10 min period in absolute ethanol. Following the replacement of the anhydrous ethanol, the samples were soaked for 10 min. Subsequently, they were soaked in 95% ethanol solution for 8 min and in 80% ethanol solution for an additional 5 min. The samples were rinsed three times with 0.01M PBS (Phosphate Buffered Saline) for 5 min each time. The slices were placed in the antigen repair fluid, and the antigen was repaired by heating in a microwave for 20 min. The antigen repair solution was naturally cooled at room temperature. Following incubation with DAB for 5-10 min, hematoxylin staining was used for 30 s, and hydrochloric acid alcohol was added to the samples for 15 s. A solution containing 1% ammonia was used to recover the color to blue for 2 s. The dehydration and mounting was conducted as follows. The tissue sections were placed in 80% ethanol solution for 30 s and subsequently soaked in 90% ethanol solution for 30 s, in 95% ethanol solution for 1 min and in absolute ethanol for 2 min. Following the replacement of the anhydrous ethanol, the samples were further soaked for 2 min. Finally neutral gum was used for mounting.

The sections were observed under an optical microscope, and dyed brown in a positive cell. The lesions were observed on the lateral cortex, and 3 slices were obtained from each specimen. Each slice was observed

in a 400-fold field of view, and five non-repetitive fields around the infarct were counted in order to obtain the average of each field of view.

Statistical processing

SPSS 13 statistical software was used for statistical analysis. The neurological function score was assessed between the different groups by One-way ANOVA with Tukey's test. The one-way ANOVA and SNK-q detection were used to analyze the difference between the water content and the immunohistochemical results of the brain tissue. The significance level was set at $\alpha=0.05$.

RESULTS

Effect of polydatin on the expression of p53 and Notch1 proteins

The number of p53 and Notch 1 immunoreactive cells in the high dose of polydatin was significantly lower than that in the MCAO group at 24 h following cerebral infarction ($P>0.05$). In the low dose group

of polydatin, the positive cells in the MCAO group decreased, although the difference was not statistically significant ($P > 0.05$). The details are shown in Fig. 1.

Neurological function score

Different degrees of left limb hemiplegia were noted in the MCAO group compared with the high dose and low dose groups of polydatin. No apparent neurological deficit was noted in the rats of the Sham group. The neurological score of the high dose group of polydatin was higher than that of the MCAO group (High vs MCAO: 2.40 ± 0.31 vs 3.90 ± 0.23 , $P < 0.05$). No significant change was noted in the neurological function score between the low dose group and the MCAO group (Low vs MCAO: 3.30 ± 0.21 vs 3.90 ± 0.23 , $P > 0.05$) (Table I).

Determination of brain tissue water content

No significant difference was noted in the water content of the contralateral brain tissue between

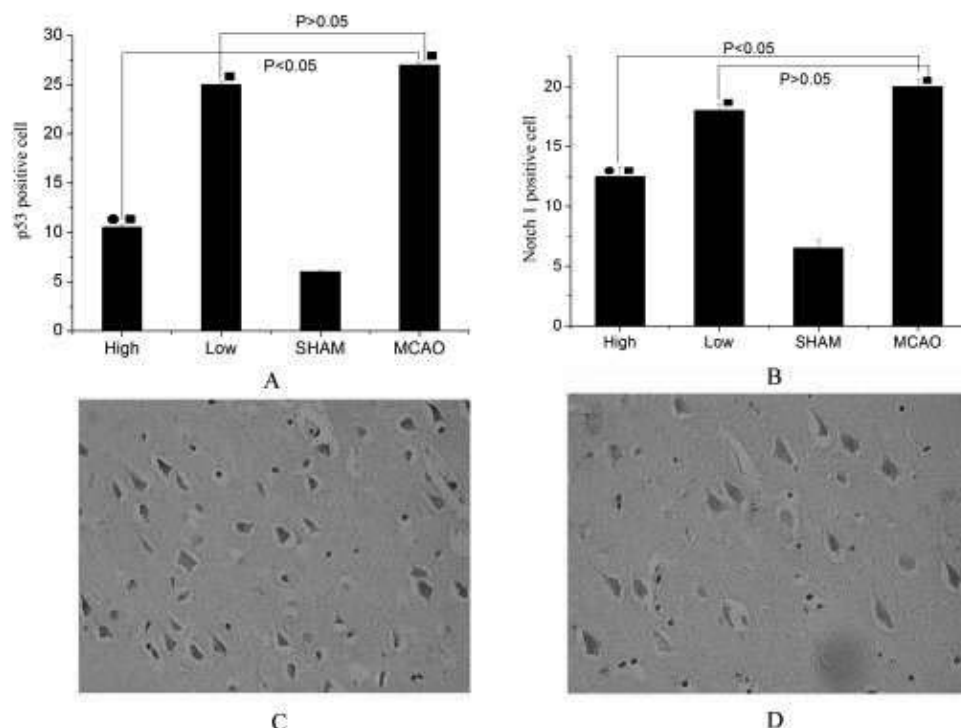


Fig. 1. Effect of polydatin on the expression levels of p53 and Notch1. A) Polydatin decreased the p 53 positive cells following MCAO. ■ $P < 0.05$ vs Sham group; • $P < 0.05$ vs MCAO group. B) Polydatin decreased the Notch 1 positive cells following MCAO. ■ $P < 0.05$ vs Sham group; • $P < 0.05$ vs MCAO group. C) p53 immuno-positive cells at 24 h following MCAO operation in the MCAO group X400; D) Notch 1 immuno-positive cells at 24 h following MCAO operation in the MCAO group x400.

the Sham and the MCAO groups with regard to the administration of the large dose of polydatin and the low dose of polydatin at the 24 h time period following surgery ($P > 0.05$). The water content of the brain tissue in the MCAO group was significantly higher than that noted in the Sham group (MCAO vs Sham: $84.46 \pm 0.37\%$ vs $77.78 \pm 0.31\%$, $P < 0.05$). A slight decrease ($83.4 \pm 0.52\%$) was observed in the water content of the brain tissue in the small dose group of polydatin, although no significant difference was evident compared with the MCAO group ($P > 0.05$). The water content of the lateral brain tissue in the high dose group of polydatin decreased to $81.15 \pm 0.41\%$, which was significantly

lower compared with that noted for the MCAO group ($P < 0.05$) (Fig. 2).

DISCUSSION

The Notch ligand in *Drosophila* is denoted Delta Serrate, whereas the Notch ligand in the nematode is denoted Lag 2 (6). Therefore, Notch ligands are also known as DSL proteins (7). A total of 5 types of Notch ligands have been identified in mammals: Delta-like 1, 3, 4 and Jagged 1, 2 (8). Notch ligands are transmembrane proteins that are expressed on the surface of cells (9). The extracellular fraction contains several tandem epidermal growth factor-

Table I. Neurological deficit scores.

Group	Score						Average score
	0	1	2	3	4	5	
Sham+Vehicle	10	—	—	—	—	—	—
MCAO+Vehicle	—	—	—	3	5	2	$3.90 \pm 0.23 \blacktriangle$
12.5mg/kg polydatin	—	—	1	5	4	—	$3.30 \pm 0.21 \blacktriangle$
50mg/kg polydatin	—	2	3	4	1	—	$2.40 \pm 0.31 \blacktriangle \star$

Note: One-way ANOVA-Tukey's multiple comparison test was used (20 animals in each group). $\blacktriangle P < 0.05$ vs Sham group; $\star P < 0.05$ vs MCAO group

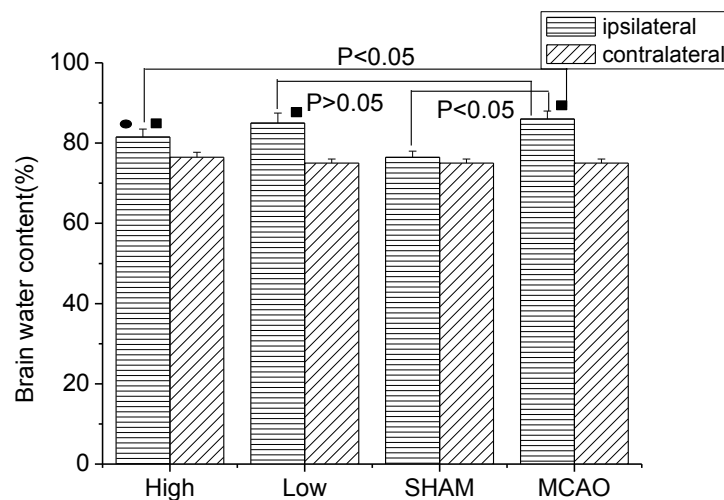


Fig. 2. Effects of polydatin on brain water content following MCAO. $\blacksquare P < 0.05$ vs Sham group. $\bullet P < 0.05$ vs MCAO group

like repeats and the conserved DSL domains that are necessary to bind and activate the Notch receptor (10). The intracellular portion of the ligand contains various short and variable sequences, although its function remains unclear (11). The Notch signal is transmitted between adjacent cells through the interaction of the Notch receptor with the ligand (12). Additional studies have indicated that Notch 1 regulates the expression of p53 in gliomas. Thus, the Notch 1-mediated inflammatory pathway and the p53-mediated apoptotic pathway may be closely related. Furthermore, Notch1 plays an important role in the tissue damage following cerebral ischemia, although the specific mechanism remains to be elucidated (13-14).

In the present study, the expression levels of p53 and Notch 1 in the brain tissue increased significantly following 24 h of cerebral infarction. Polydatin can relieve cerebral edema following cerebral infarction, and exerts a neuroprotective effect on focal cerebral infarction. Polydatin can inhibit the elevation of p53 and Notch 1 expression in the cerebral cortex following cerebral infarction. With regard to the interaction of the p53-mediated apoptotic pathway and the Notch 1-mediated inflammatory response, the inhibitory effect of polydatin on this pathway may be one of the mechanisms responsible for its neuroprotective effect that is similar to that noted by other natural polyphenols. In conclusion, high dose polydatin could reduce brain edema following cerebral infarction and improve the behavioral score in rats.

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LETTER TO THE EDITOR

EFFECT OF DEXMEDETOMIDINE ON STRESS REACTIONS AND CELLULAR IMMUNE FUNCTION OF PATIENTS IN PERIOPERATIVE PERIOD FOLLOWING RADIAL RESECTION FOR RECTAL CARCINOMA

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The study aimed to assess the effects and the further mechanism of action of dexmedetomidine with regard to stress reactions and cellular immune function of patients during the perioperative period following radical resection for rectal carcinoma. A total of 36 patients with rectal carcinoma were selected for radical resection under general anesthesia. The patients were divided into two groups, namely an experimental and a control group. In the experimental group (dexmedetomidine group) 1 µg/kg/bw dexmedetomidine was injected intravenously 10 min prior to the induction of general anesthesia, and then infusion was carried out at a rate of 0.2 µg·kg⁻¹·h⁻¹ for 30 min prior to the end of surgery. With regard to the control group, the same amount of normal saline (NS) was infused with the same method as the experimental group. Controlled intravenous analgesia was conducted following surgery to all of the patients. Regarding the effect of dexmedetomidine on the reaction of stress, a decrease of VAS scores was noted in the experimental group following extubation compared with the control group ($P<0.05$). Furthermore, a significant decrease in the consumption of morphine in the first 24 h was observed that was accompanied by a decrease of plasma cortisol levels at 6 and 24 h following surgery compared with the control group. The levels of IFN-γ/IL-10 in the experimental group were lower than those of the control group ($P<0.05$). The percentages of CD8⁺ and CD4⁺/CD8⁺ cells in the experimental group were increased compared with those of the control group ($P<0.05$). By infusing dexmedetomidine continuously, stress reactions during the perioperative period were significantly decreased, whereas the analgesic effects of opioid were increased.

To the Editor,

Stress reactions during the perioperative period are mainly caused by surgery simulation, pain, hemorrhage and oxygen deficit (1). Stress reactions determine an increase in tumor growth rate and tumor metastatic rate (2). These effects can be stimulated by surgery, the method of anesthesia and the anesthetics used.

Currently, opioids are the first choice for

controlling traumatic stress during surgery, since they increase the pain threshold (3, 4). However, certain studies have demonstrated that postoperative hyperpathia, postoperative chronic pain syndrome and postoperative immunosuppression were partially related to the opioid drugs used during the perioperative period. Therefore, in an effort to minimize the use of opioid drugs, various studies have proposed a multimodal anesthesia concept, which suggests the

Key words: dexmedetomidine, rectal carcinoma, stress response, immune function

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inhibition of the interaction between sympathy and noxious stimulation of the somatic nerve via the application of multiple drugs acting by a different mechanism (such as ketamine, steroid and α 2-AR agonist) that in turn reduces stress reaction during the perioperative period (5). Dexmedetomidine (DM) is a new-style α 2-AR agonist, which can be used to achieve pharmacologically a “sympathectomy” effect for sedation, hypnosis and antinociception (6, 7). The present study aimed to assess the effect of dexmedetomidine on stress reaction and immunological function of the patients during the perioperative period following radical resection for rectal carcinoma.

MATERIALS AND METHODS

Patients

Thirty-six patients diagnosed with middle and high rectal cancer, namely adenocarcinoma and/or adenosquamous carcinoma (distance between cancer center and anus >5cm), in the Affiliated HongQi Hospital of the MuDanJiang Medical University from November 2015 to January 2017 were selected. All the patients selected were of grade A and B according to the improved Dukes grade (0-II stages according to TNM stages of UICC) and were scheduled to have Dixon surgery. The present study was randomized and included a placebo control. During the preoperative assessment, patients of grades I-II according to the American Society of Anesthesiologist (ASA) were recruited with an age range of 30-70 years and a weight range of 50-80 kg. The selected patients were randomly divided into two groups of 18 subjects: a control group and an experimental group (dexmedetomidine group). All patients signed informed consent prior to surgery which was carried out by a surgeon of the same group and the same inpatient area. The medical Ethics Committee of the Affiliated HongQi Hospital of the MuDanJiang Medical University approved the study protocol. Prior to the surgery, all the patients exhibited normal hepatorenal function and were not treated with chemotherapy and/or radiotherapy. The patients were also free of immunological disease, endocrine disease, metabolic disease and hormone therapy and/or hormone application history. Prior to surgery, the patients did not consume opioid drugs and/or non-steroid anti-inflammatory drugs. The patients with psychosis,

alcohol addiction and congenital heart disease were excluded. In addition, the patients with blood transfusion during the perioperative period and infection and/or serious complications following surgery were excluded. Prior to recruitment of the participants, informed consents were signed by all patients and/or their relatives and were retained along with their remaining clinical history.

Experimental apparatus and materials

Anesthetics: Dexmedetomidine hydrochloride was purchased from Jinan Hongfangde Pharmatech Co., Ltd., whereas Remifentanyl Hydrochloride for Injection from Yichang Humanwell Pharmaceutical Co., Ltd. Propofol Injection was purchased from Xi'an Libang Pharmacy Co., Ltd. Fentanyl was from Yichang Humanwell Pharmaceutical Co., Ltd. and midazolam from Shanghai Hanxiang Biotechnology Co., Ltd. Rocuronium Bromide Injection was obtained from Hebei Kaisheng Medical Technology Co., Ltd. and Tropisetron hydrochloride from inan Lyxin Chemical Technology Co., Ltd. Morphine hydrochloride was obtained from Shenyang Guangda Pharmacy Co., Ltd. and Sevoflurane for Inhalation from Jiangsu Hengrui Pharmaceutical Co., Ltd. Ephedrine hydrochloride was purchased from ChifengArker Pharma Co., Ltd. and Heparin sodium from Wuhan Desheng Biochemical Technology Co., Ltd.

The cortisol ELISA KIT was obtained from Shanghai Fusheng Bio-technology Co., Ltd., whereas the IFN- γ kit from Wuhan MSKBIO Technology Co., Ltd. and the IL-10 kit from Wuhan MSKBIO Technology Co., Ltd.

The experimental apparatus comprised 100 μ l single channel Pipettes (American RAININ) and 50 μ l 8 channels Pipettes (American RAININ). The Ease China electronic DG5033A ELIASA was from Nanjing Ease China Electronic Group Medical Equipment Co., Ltd. and the anesthesia apparatus from Yian Medical treatment. The electronic epidural analgesic tube was from ZhangjiagangLongyi Medical Apparatus and Instruments Co., Ltd. and the flow cytometer (FCM) from Becton Dickinson Co.

Anesthesia

Prior to anesthesia, the experimental drug was administered using an injection volume of 50 ml. The control group was administered 50 ml of normal saline (NS), whereas the experimental group was administered

48 ml NS + 2 ml dexmedetomidine (1mg/2ml). The anesthetist of the experimental group was not aware of the nature and type of medicine used. A total of 1 μ g/kg of experimental medicine was intravenously infused for all patients 10 min prior to the induction of anesthesia. Subsequently, the drug was administered at a rate of 0.2 μ g·kg⁻¹·h⁻¹ for 30 min prior to the end of the surgery. Following surgery, the endotracheal tube was removed and the patients were transferred to the Post-anesthesia care unit (PACU).

The postoperative analgesia was conducted as follows: Following extubation, the patients were connected to an electronic epidural analgesic tube (BOT-6) and controlled intravenous analgesia was carried out. The recipe of analgesic included 0.5 mg/ml of morphine hydrochloride and 5 mg of Tropisetron hydrochloride in 0.9% normal saline that was diluted at a final volume of 150 ml. The initial capacity was set at 2 ml and the background infusion dosage was set at 3 ml/h. Patient control analgesia (PCA) was set at a rate of 1 ml/event and the locking time was 10 min. The infusion was maintained for 24 h following surgery and the therapeutic effect was evaluated by an anesthetist.

Observation index

The hemodynamic parameters of the patients IBP and HR, the incidence rate of the adverse cardiac events and the frequency of use of the vasoactive agent were observed continuously during surgery.

The clinical medication and manifestation was recorded as follows: The dosage of propofol and remifentanyl used during surgery was estimated and the total morphine dosage for postoperative analgesia was recorded. The recovery time and the extubation time of the patients were also recorded.

The pain score was assessed using the VAS scoring. The results were conducted at 2, 6, 12 and 24 h following surgery by a medical professional who was blinded to the groups of the patients.

The stress index was assessed using peripheral venous blood prior to anesthesia (T0), following trachea cannula (T1) and following the removal of the tube (T3). The plasma cortisol concentration was measured by chemiluminescence.

The immune index was assessed using peripheral venous blood by an ELISA method specific for the

detection of IFN- γ and IL-10 in blood. Subsequently, the value of IFN- γ /IL-10 was calculated. FCM was used to test the levels of CD3+%, CD4+%, CD8+% and CD4+/CD8+ of T-lymphocytes.

Statistical analysis

SPSS19.0 was used to process measurement data. The data were expressed as mean value $\pm$ standard deviation ($\bar{x} \pm s$). The comparisons among plasma cortisol concentration, IFN- γ , IL-10, IFN- γ /IL-10, CD3+% and CD4+/CD8+ were performed by univariate analysis. The SNK method was used for the comparison between two groups of variables. Student's *t*-test was used for the evaluation of the differences of the parameters age, BMI, operation time and extubation time. Ranked data (VAS score) was subject to the rank sum test. Differences with regard to ASA class and vasoactive agent usage rate were evaluated by the *chi*-squared test. A value of $\alpha=0.05$ was set as the standard for the statistical comparison and differences were significant when $P<0.05$.

RESULTS

General clinical data

No significant differences were noted for age, gender, BMI, ASA class, operation time, vasoactive agent dosage and extubation time of the patients in the two groups ($P>0.05$).

Comparison of stress reaction, opioid use and analgesic effect during perioperative period

The plasma cortisol concentration of the control group was higher than that of the experimental group at 6 h and 24 h following the surgery ($P<0.01$) (Fig. 1).

The consumption of morphine by the experimental group at 24 h following surgery was lower than that of the control group ($P<0.05$). During the perioperative period, the VAS score of the control group was higher than that of the experimental group ($P<0.05$) (Table II).

The propofol dose used for patients in the experimental group was lower than that used for patients in the control group, although the difference was not significant ($P>0.05$). However, the use of remifentanyl in patients of the experimental group was apparently lower than that of the patients in the control group ($P<0.05$) (Table II).

Table I. General clinical data of the patients.

Index	Control group	Experimental group	P value
Age (years)	58.26±6.31	69.84±6.22	0.76
Gender (female/male)	6/12	5/13	0.51
BMI(Kg/m ²)	22.33±1.21	22.57±1.08	0.38
ASA class(I/II)	7/11	8/10	0.69
Operation time (min)	212.35±27.28	226.63±20.12	0.65
Ephedrine usage rate	5/18	7/18	0.51
Atropine usage rate	4/18	6/18	0.65
Extubation time (Min)	21.31±4.98	21.08±5.27	0.73

Note: The parameters age, BMI, operation time, extubation time were compared by Student's *t*-test, whereas the variables gender, ASA, ephedrine usage rate, atropine usage rate were compared with chi-squared test.

Table II. The doses of propofol, remifentanyl and morphine used, the extubation time and the VAS score in the groups following the surgery.

Group	Control group	Experimental group
Cases (n)	18	18
24-h morphine consumption (mg)	96.01±9.39	102.68±9.34 [#]
Propofol(mg)	1498.28±536.26	1221.38±457.98
Remifentanyl (ug)	4403.71±801.92	3500.91±492.93 [#]
Extubation time (min)	22.17±5.03	21.82±5.36
2 h during surgery	4.41±0.69	3.06±0.44 ^a
6 h after surgery	3.20±0.51	2.19±0.41 ^a
12 h after surgery	1.58±0.48	1.24±0.50 ^b
24 h after surgery	0.98±0.31	0.66±0.34 ^b

Note: Compared with the control group, [#]*P*<0.05; Rank sum test was carried out and compared with the control group, ^a denotes *P*<0.01 and ^b denotes *P*<0.05.

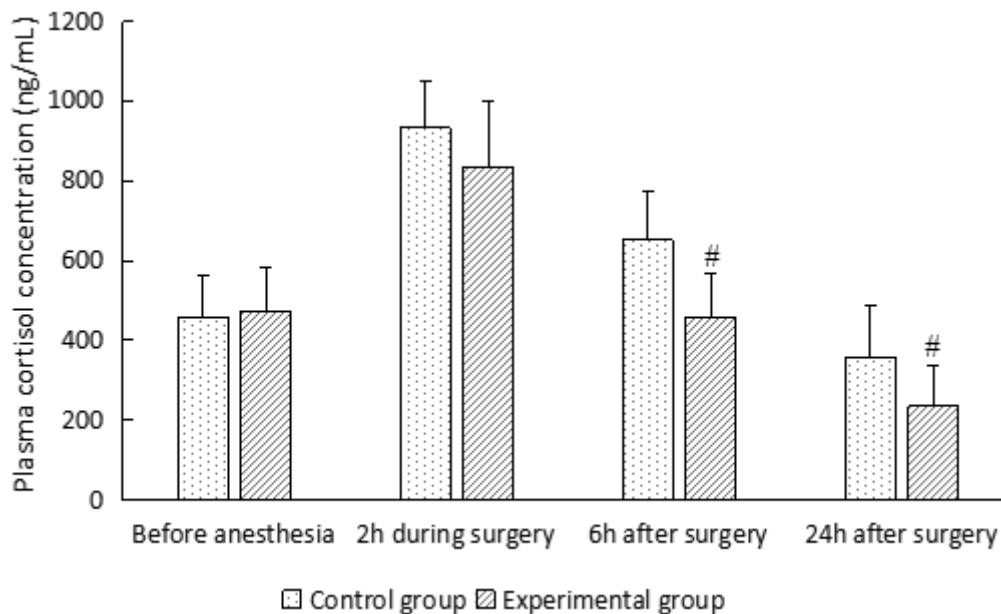


Fig. 1. Comparison of plasma cortisol concentration with the control group, [#] $P < 0.01$.

Immunological balance and Change of T-lymphocytes subsets

As shown in Fig. 2A, the IFN- γ concentration levels of both groups at time-points T1, T2 and T3 were lower than those at T0. The change in the concentration of IL-10 in the control group exhibited no significant difference. With regard to the experimental group, the concentration levels of IL-10 at the T1, T2 and T3 time points were higher than those noted at T0. ($P < 0.05$). In addition, the ratios of IFN- γ /IL-10 in the control and experimental groups following surgery were higher than those noted prior to the surgery (T0) ($P < 0.05$). The ratio of IFN- γ /IL-10 in the experimental group at the T1, T2 and T3 time points was significantly lower than that noted in the control group ($P < 0.01$).

As shown in Fig. 2B, the percentages of CD3⁺ and CD4⁺ cells at the T1 and T2 time points were greatly decreased compared with those at T0 prior to surgery ($P < 0.05$). The comparison between groups indicated that the percentage of CD8⁺ cells in the experimental group was significantly higher than that of the control group, while the ratio of CD4⁺/CD8⁺ of the experimental group was significantly lower than that of the control group ($P < 0.01$).

DISCUSSION

In the present study, the remifentanyl dosage of the experimental group was evidently lower than that of the control group, which agrees with the aforementioned hypothesis. Following infusion with dexmedetomidine, the VAS scores of the patients after surgery were significantly decreased. This indicated that the α_2 receptor agonist exhibited a beneficial effect on analgesia. The data indicated that dexmedetomidine could inhibit the central sympathetic system and decrease the plasma concentration of cortisol. Similarly, since the analgesic effect was obvious, the total morphine dosage used at 24 h following surgery in the experimental group was lower than that used in the control group.

The dynamic equilibrium of the T cell subset is an important part of immune regulation, and the balance adjustment of the Th1/Th2 ratio is a significant regulatory mechanism (8, 9). It has been shown that the ratio of IFN- γ /IL-10 is used to reflect the deviation of Th1/Th2 indirectly (10, 11). Dexmedetomidine can be used as an anti-sympathetic stimulant. Thus, the immunological balance is indirectly influenced

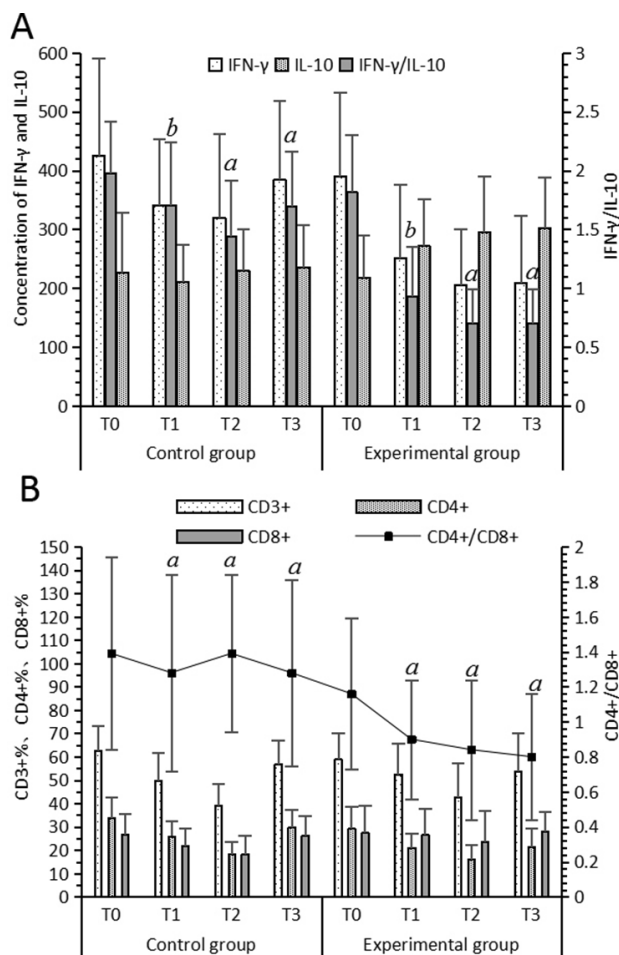


Fig. 2. Immunological balance and change in the T-lymphocyte subsets. **A)** Concentration change of IFN- γ and IL-10. **B)** Change in the T-lymphocyte subsets; Rank sum test was conducted for the comparison with the control group; *a* denotes $P < 0.01$; and *b* denotes $P < 0.05$.

(12). In the current study, the CD3+, CD4+ and CD8+ cells of the experimental subjects in the control group that were detected at 2 h during the surgery and 6 and 24 h following the surgery were significantly lower than those noted prior to surgery, suggesting that the immune protection mechanism was further weakened under general anesthesia. Since no significant difference between the operative wound and the anesthetics was noted (normal saline was used for comparison), the IFN- γ /IL-10 and CD4+/CD8+ ratios of the patients in the experimental group at 6 and 24 h following surgery were clearly

lower than those of the patients in the control group. In addition, the percentage of CD8+ cells in the experimental group at 6 and 24 h following surgery was apparently higher than that noted in the patients of the control group, which indicated that the immunological function was influenced when the intervention factor (dexmedetomidine) was added. The total number of T-lymphocytes was decreased and the ratio of CD4+T cell to CD8+T cell was reversed. Concomitantly, the Th1 and Th2 cells deviated from the baseline values, which highlighted that the cellular immunity was further inhibited.

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LETTER TO THE EDITOR

METHYLMERCURY-L-CYSTEINE TARGETING L-TYPE AMINO ACID TRANSPORTER CONJUGATE CYTOTOXICITY ON C6 GLIOMA CELLSZY. WEI¹, MM. FENG², B. YANG², ZY. YAN², BQ. WANG² and XY. BU²

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Glioma is the most common primary tumor in the brain, accounting for about 40~50% of intracranial primary tumors. Most chemotherapeutic drugs have difficulty in penetrating the blood-brain barrier, and their clinical applications are greatly limited. We evaluated the effects of methylmercury-L-cysteine (MeHg-L-cys) and methylmercury chloride (MMC) on apoptosis of C6 glioma cells. L-type amino acid transporter (LAT1) was used to investigate the targeted transport function and cytotoxicity of MeHg-L-cys in glioma. MeHg-L-cys enhanced the ability of targeting glioma cells and reduced the adverse reactions to normal brain tissues. Therefore, it is significantly important to develop new anti-glioma drugs targeting the blood-brain barrier.

To the Editor,

Currently, surgery is still the preferable treatment for glioma followed by combined radiotherapy and chemotherapy to improve the patient's quality of life (1). Nowadays, chemotherapy drugs are divided into cytotoxic cell drugs and cell inhibitory drugs, according to their ability to kill tumor cells or prevent tumor cell proliferation, respectively (2). However, these drugs have multiple side effects, some of which have severe clinical consequences such as decreased immunity with secondary bacterial infections caused by resistant germs.

The use of biological differences between normal cells and tumor cells is one of the best options for alleviating adverse effects (3). Based on the structure, physical and chemical characteristics of methylmercury chloride (MMC), both the cytotoxic characteristics of brain targeted accumulation and multi-target cytotoxicity have been investigated

(4). LAT1 is not expressed in normal astrocytes, which are highly expressed in glioma cells. In addition, malignant glioma cells present high uptake of methionine. The MeHg-L-cys conjugate is a competitive analogue of methionine with a LAT1 transporter substrate. In this study, the cytotoxic effects are investigated of MeHg-L-cys conjugates targeting LAT1 glioma cells, and a new avenue for the clinical treatment of gliomas is opened.

MATERIALS AND METHODS*Reagents*

Glioma cells of C6 rats were provided by Jilin University experimental animal center.

The following materials were used. Methylmercury chloride (MMC) (Merck, Germany); L-cysteine (Merck, Germany); Homemade methylmercury-L-cysteine conjugate (MeHg-L-cys); four methyl pyrazole salt (MTT) (Sigma,

Key words: brain glioma, L type amino acid transporter, targeting cytotoxicity

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USA); Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA); penicillin (Huabei pharmaceutical factory); Streptomycin (Dalian Merro pharmaceutical factory); Bovine serum albumin (BSA) (Zhejiang Tianhang Biological Technology Co. Ltd.); Chloralhydrate (Zhengzhou He Tian Biological Technology Co., Ltd.); Propidium iodide (PI) (Sigma, USA); Trypsin (Sigma, USA); Two methyl sulfoxide (DMSO) (Sigma, USA); Glial fibrillary acidic protein (GFAP) (DakoDenmarkA/S, Denmark.); AnnexinV-FITC (Biyuntian reagent company); (SCA-165DSCO2 incubator (Japan); Hfsafe-1500 biological safety cabinet (Shanghai force Scientific Instrument Co., Ltd.); Model Model 550 automatic microplate reader (Bio-Rad, USA); Automatic ice maker (Scotsman, Italy); Inverted microscope (Olympus Corporation, Japan); Lab centrifuge (Beijing lab centrifuge Co. Ltd.); -86°C ultra low temperature refrigerator (Thermo Fisher Scientific (Chinese) Co. Ltd.); Mettler Toledo PG4002-S electronic balance (Shanghai Fu Zhong Biological Science Co Ltd.); Automatic double water distiller (Shanghai Asia Pacific Technology Glass Company.); Liquid nitrogen biological vessel (Chengdu, YDS-30-125); water bath temperature control instrument KW-2A (Beijing Changyuan experimental equipment factor); Constant temperature oven (Shanghai medical instruments Co., Ltd.); Flow cytometry (Beckman, USA); Magnetic resonance instrument 1.5 T (Simens 1.5 TAVANTU) and C6 rat glioma cells were purchased from ATCC (Shanghai Rich Biological Technology Development Co., Ltd.).

Inhibitory effects of MeHg-L-cys and MMC on glioma cells of C6 rats

C6 rat glioma cells were cultured in DMEM medium containing 10% fetal bovine serum at 5% CO₂ atmosphere and 37°C. When the cells were in logarithmic phase (about 80%), they were digested with 0.25% trypsin and centrifuged to make single cell suspensions. The cell concentration was adjusted and inoculated in a flat bottomed culture dish with 96 holes. 90 µl of single cell suspensions were added to each hole. After culturing 24 h, they were added to 10 µL solution with concentrations of MeHg-L-cys and MMC 0, 2.5, 5, 10 and 20 µM. Each group contained 5 samples. The blank control holes were set. After 24 h, the absorbance of glioma cells of C6 rats was measured by MTT assay, and the effect of MeHg-L-cys and MMC on cell proliferation was observed.

Effects of MeHg-L-cys and MMC on apoptosis of C6 glioma cells

C6 rat glioma cells were inoculated into 24 pore culture plate flats with different concentrations of both MeHg-L-cys and MMC (0, 20, 10, 5 and 2.5 M). After 24-h culture, 0.25% trypsin was used for digestion. After 24 h, the above samples were collected, and the cell concentration was $(8-10) \times 10^4$. One mL of each solution was taken and 1000 g centrifugation was performed for 4-5 min. The supernatant was discarded, then 195 µl Annexin V-FITC binding liquid was added, and the suspension cell was gently shaken. 5 µl Annexin V-FITC was added and mixed. The solution was incubated at 20-25°C for 8-10 min without light. 1000 g centrifugation was performed for 4-5 min and the supernatant was discarded. Then, 190 µl Annexin V-FITC binding liquid was added to the suspension cells and they were gently shaken. Finally, 10 µl of iodine staining solution was added and mixed. The 1×10^4 cells were measured by FACS and analyzed by Cellquest software.

Effects of MeHg-L-cys and MMC on cell cycle in C6 glioma cells

C6 glioma cells were treated as described above, and cells were collected after 48 h of culture. After PBS washing, 100 mL RNA enzyme (0.1 mg = mL⁻¹), 100 L (containing 0.01% TritonX100), and PI (0.05mg = mL⁻¹) were added to each pore. The solution was incubated at 4°C for 30 min in the dark. The solution was measured by fluorescence-activated cell sorting (FACS) (type of flow cytometry) and analyzed by Hod Flit software.

Statistical analysis

The experimental results were expressed by mean $\pm$ standard deviation ($\bar{x} \pm s$), while SPSS 18.0 software was used for statistical processing. Homogeneity between groups was used, and was statistically significant if $P < 0.05$.

RESULTS

Effect of MeHg-L-cys on the survival of C6 rat glioma cells

The effects of different concentrations of MeHg-L-cys and MMC on cell survival in C6 rat glioma cells are shown in Fig. 1. C6 glioma cells

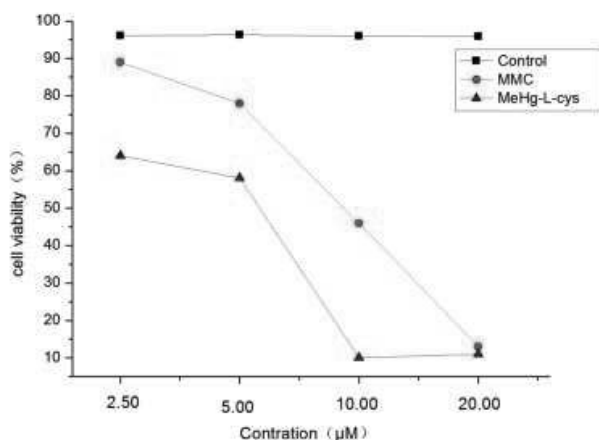


Fig. 1. The inhibitory effect of MeHg-L-cys on the survival of C6 rat glioma cells based on MTT assay.

were exposed to both MMC and MeHg-L-cys at concentrations of 0, 2.5, 5, 10, 20 μM . After 24 h, cell survival rate decreased significantly and it was observed that it was dose-related: the survival rate decreased as the drug concentration increased. This indicated that MMC and MeHg-L-cys could inhibit the survival of C6 glioma cells *in vitro*. The survival rate of the MeHg-L-cys conjugate group was lower than that of the MMC group ($P < 0.05$).

Effect of MeHg-L-cys on apoptosis of C6 rat glioma cells

The effects of different concentrations of MeHg-L-cys and MMC on cell apoptosis in C6 rat glioma cells are shown in Fig. 2. C6 glioma cells were exposed to both MMC and MeHg-L-cys at concentrations of 0, 2.5, 5, 10, 20 μM . After 24 h, cell survival rate decreased significantly and was dose-related. The rate of apoptosis of the treated groups was significantly higher than that of the control group ($P < 0.05$). These results indicate that MMC and MeHg-L-cys can induce apoptosis in C6 glioma cells.

Effect of MeHg-L-cys on cell cycle of C6 rat glioma cells

C6 glioma cells were exposed to different concentrations of MeHg-L-cys and MMC, and the effects on cell cycle are given in Table I and Fig. 3. C6

glioma cells were exposed to both MMC and MeHg-L-cys at concentrations of 0, 2.50, 5, 10 and 20 M. The increase of the dose resulted to the continuous increase of cell ratio of G2/M phase, and showed a dose-dependent correlation. The percentage of cells in G0/G1 phase was significantly higher than that of the control group ($P < 0.05$), and the percentage of cells in S phase was significantly lower than that of the control group ($P < 0.05$). This suggests that MMC and MeHg-L-cys can arrest C6 glioma cells in G0/G1, thus reducing their cell ratio into S phase.

DISCUSSION

It has been reported that LAT1 is expressed in human glioma cells and is not expressed in normal brain glial cells, and also that the level of LAT1 expression increases depending on the degree of malignancy of the tumor (5). Studies have shown that malignant glioma cells have high uptake properties for methionine (statistically significant levels of uptake), and the amount of intake is positively correlated with the degree of malignancy of gliomas (6-8). Methionine is a transport substrate for LAT1 while MeHg-L-cys conjugate is a competitive analogue of LAT1 transport substrate methionine. It facilitates targeted transport of MMC to glioma cells, and enhances its ability to target the brain glioma cells and reduce the normal brain tissue adverse

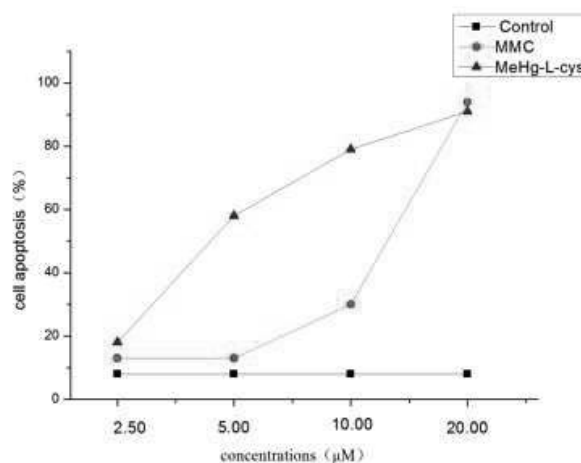
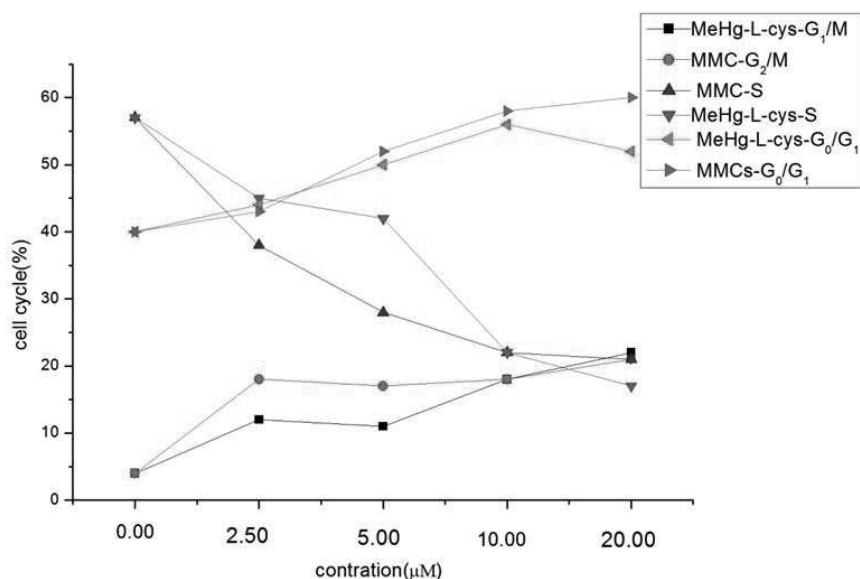


Fig. 2. MeHg-L-cys induced apoptosis on C6 rat glioma cells (FISH method).

Table I. Effect of MeHg-L-cys on cell cycle in C6 glioma cells.

	Dose group (μM)	G_0/G_1 phase (%)	S phase (%)	G_2/M phase (%)
MeHg-L-cys	0.00	39.25 $\pm$ 0.22	55.59 $\pm$ 0.59	5.23 $\pm$ 0.55
	2.50	43.61 $\pm$ 0.6	44.36 $\pm$ 0.44	12.03 $\pm$ 0.21
	5.00	50.34 $\pm$ 0.48*	38.95 $\pm$ 0.44*	10.71 $\pm$ 0.3
	10.00	56.49 $\pm$ 0.5*	22.38 $\pm$ 0.53*	21.13 $\pm$ 0.92*
	20.00	54.56 $\pm$ 0.61*	17.5 $\pm$ 0.49*	27.94 $\pm$ 0.8*
MMC	0.00	39.25 $\pm$ 0.22	55.52 $\pm$ 0.59	5.23 $\pm$ 0.55
	2.50	43.58 $\pm$ 0.46	36.81 $\pm$ 0.15	19.61 $\pm$ 0.6
	5.00	53.8 $\pm$ 0.74*	27.59 $\pm$ 0.5*	18.62 $\pm$ 1.07*
	10.00	59.85 $\pm$ 0.38*	22.67 $\pm$ 0.31*	17.50 $\pm$ 0.4*

* $P < 0.05$ compared to control group

**Fig. 3.** Effect of MMC and MeHg-L-cys on cell cycle of C6 glioma in rats by FACS.

reactions (9). Based on this, we investigated the cytotoxicity of MeHg-L-cys conjugates by targeting LAT1 to glioma cells, opening up new avenues for the clinical treatment of gliomas. Targets for other metallotoxic anticancer drugs have also been investigated showing new horizons to position (10,

11). LAT1 was highly expressed in glioma cells. This study investigated the enhancement of targeted transport and cytotoxicity of MeHg-L-cys conjugate by LAT1. MMC induces apoptosis of glioma cells, blocks cell proliferation and has anti-tumor activity.

It can be concluded that MMC and MeHg-L-cys

inhibit the proliferation of C6 glioma *in vivo*, which may be induced by the apoptosis of glioma cells. Small doses of MMC and MeHg-L-cys have the potential to cure brain gliomas. This study demonstrates the potential of MeHg-L-cys conjugates to promote the targeting of MMC to brain malignant glioma cells via LAT1.

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LETTER TO THE EDITOR

PERIOPERATIVE NURSING INTERVENTION ON PATIENTS UNDERGOING
LAPAROSCOPIC GASTRIC STROMAL TUMOR RESECTIONLS. ZHANG¹, J. ZHANG¹ and XM. ZOU²

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This study aimed to investigate the effectiveness of perioperative nursing intervention on patients undergoing laparoscopic gastric stromal tumor resection. Sixty patients with gastric stromal tumor were selected from our hospital and evenly divided into group A and group B. Patients in both groups underwent laparoscopic resection. Patients in group A were given conventional nursing intervention before and after surgery, while those in group B were given comprehensive nursing intervention. Various indicators were compared between the two groups. The amount of bleeding of group B was less than that of group A, and the first anal exsufflation of group B was also earlier than that of group A; the differences had statistical significance ($p < 0.05$). Patients in group B felt less pain than patients in group A; except for 72 h after surgery, difference of pain degree between group A and B had statistical significance in other periods ($p < 0.05$); the number of cases with complications and categories of complications of group B were less than those of group A, and the difference had statistical significance ($p < 0.05$). The efficacy satisfaction of group B was also higher than that of group A, and the difference was statistically significant ($p < 0.05$). Perioperative nursing intervention is beneficial and positive and has bright development prospects.

To the Editor,

Gastric stromal tumor, which is frequently seen in the digestive tract, stems from gastric mesenchymal tissue, and excessive proliferation of immature fusiform cells is the main cause for the occurrence of gastric stromal tumor (1) for which surgical treatment is preferred (2). With the constant advancement of social science and technology, surgical techniques are also constantly developing. Laparoscopic surgery is performed using micro cameras. Differing from other invasive surgical treatment, laparoscopic surgery is minimally invasive (3). An increasing number of doctors tend to select laparoscopic surgery for gastric stromal tumor resection because of the advancement

of laparoscopic surgical instruments and increasingly proficient operation techniques (4). Pelletier et al. (5) compared the effectiveness of laparoscopic surgery and traditional open surgery in gastric stromal tumor resection through literature evaluation and found that laparoscopic surgery was much safer and effective. Through observing the postoperative indicators of patients who underwent laparoscopic gastric stromal tumor resection, Dressler et al. (6) found that laparoscopic gastric stromal tumor resection had little influence on postoperative life and patients recovered in a shorter time. After treating a 74-year-old patient with laparoscopic wedge resection, Stevanović et al. (7) found that various

Key words: laparoscope, nursing intervention, gastric stromal tumor, clinical efficacy

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indicators were good and thereby considered it as an effective therapy. This study focused on the clinical effectiveness of perioperative nursing intervention on patients undergoing laparoscopic gastric stromal tumor resection. Through comparing the indicators of patients who were given either comprehensive nursing or conventional nursing, it was found that the clinical effectiveness of group B was superior to that of group A, indicating that the implementation of comprehensive nursing intervention was correct.

MATERIALS AND METHODS

Study subjects

Sixty patients who were confirmed with gastric stromal tumor were selected from the 2nd Affiliated Hospital of Harbin Medical University, Heilongjiang, China and evenly randomly divided into group A (a control group) and group B (an observation group). All the patients underwent laparoscopic resection. To distinguish the effects of different nursing interventions, group A and B were given different nursing intervention means. In group A, the ratio of males to females was 17:13 (age: 41-62 years). In group B, the ratio of males to females was 18:12 (age: 39-65 years). Patients in the two groups had no other diseases. In group A, there were 8 cases of benign gastric stromal tumor (tumor size between 1.2 cm and 2.6 cm), 3 cases of borderline tumor (tumor size between 2.3 cm and 2.7 cm) and 19 cases of malignant tumor (tumor size between 7.9 cm and 9.6 cm). In group B, there were 10 cases of benign tumor (tumor size between 1.4 cm and 2.9 cm), 2 cases of borderline tumor (tumor size between 2.1 cm and 2.9 cm) and 18 cases of malignant tumor (tumor size between 8.7 cm and 10.1 cm). No liver metastasis or surrounding organ invasion were observed by upper abdominal enhanced CT examination before surgery. The baseline data of the two groups had no statistically significant differences ($p > 0.05$), therefore, the results were comparable.

This study was reviewed and approved by the medical ethics committee of the Second Affiliated Hospital to Harbin Medical University, Heilongjiang, China. All the subjects signed informed consent and expressed the willingness of cooperation.

Preoperative nursing

After admission, nurses evaluated the disease

condition and psychological situation of the patients, asked them for information such as medication history, and checked the existence of other complications. Moreover, examinations for blood routine indexes and coagulation time were performed, and blood matching and blood packs were prepared. Electrocardiography and chest X-ray examination were also carried out; surgery was not allowed if the patients had heart disease.

For preparation of the gastrointestinal tract, food containing rich nutrients was suggested, especially semi-liquid food. Food was forbidden, and the intestinal tract was cleaned using purgative prescription the day before surgery. Gastric lavage and clysis were performed the night before surgery, and clysis was also performed in the morning of the day of surgery.

Surgery

Patients in both groups underwent laparoscopic gastric stromal tumor resection. After general anesthesia, patients took a supine position. Then an incision was cut on the upper edge of the umbilical region. With deep aponeurosis reserved, the skin edge was pulled, the aponeurosis was punctured and then cannula, ultrasound knife and laparoscope were inserted. After the position of lesions was confirmed, gastric wall tissues were pulled upward and a linear cutting stapler was used to cut stromal tumor and part of normal tissues along the edge of the lesions under the laparoscope. Hemostasis and suture were performed after resection (8).

Postoperative nursing

Group A was given conventional nursing intervention including observation of disease condition and daily nursing (9-11).

Close attention was paid to the vital signs of the patients. The placement of the tubes was checked. The patients were asked whether they had abdominal distension and pain or not. Drugs were given when there was unbearable pain. The changes of wounds on the abdomen were closely observed. The occurrence of bleeding and fever was notified to doctors for immediate treatment.

The patients lay on the back for 6 ~ 8 hours after daily nursing. Moreover, they were asked to turn their heads to either side to prevent emesis and asphyxia. They were supplied with oxygen for 3 ~ 5 hours. The position of the

patients was changed timely.

Group B was given comprehensive nursing intervention including preoperative gastrointestinal nursing, postoperative tube nursing and psychological and diet nursing.

Preoperative gastrointestinal nursing: the patients were asked to eat more digestible food with high protein before surgery, semi-liquid food two days before surgery, liquid food one day before and sodium phosphates on the night prior to surgery. Food and water were forbidden 12 h before surgery.

Postoperative canal nursing: A stomach tube was inserted and fixed after operation to reduce the pressure on intestines and stomach. The color and volume of drainage liquid were observed. Doctors were notified if there was abnormality. The position of the drainage pack should not be too high. The bladder and perineum were kept clean during urinary canal indwelling. Before transfusion, the puncture site was disinfected and the surgical adhesive membrane was changed. Sterile gauzes were used for fixation at the end of transfusion.

Psychological and dietary nursing: The patients were given psychological guidance by means of chatting and distraction to establish peaceful psychological status and increase confidence before surgery. They were encouraged through communication also after surgery. Food and water were forbidden after the operation; nutrition was supplemented by i.v. drip. Warm water was allowed if gastrointestinal peristalsis improved. The patients were provided with rice soup if no abnormal reaction occurred and then liquid water; however, irritable food such as soybean milk, dessert and raw or cold foods were forbidden. The diet recovered after two or three weeks.

Efficacy indicators

Nursing efficacy was evaluated by analyzing the amount of intraoperative bleeding, postoperative anal exsufflation, pain score, complications and satisfaction of the two groups. The pain score was between 1 and 5 points; higher score indicated stronger pain. Satisfaction degree was classified to great satisfaction, general satisfaction and dissatisfaction. The computational formula for the satisfaction degree was: satisfaction degree = (number of cases of great satisfaction + number of cases of general satisfaction)/total number of patients.

Statistical analysis

Data were statistically analyzed using SPSS. ver. 19.0. Quantitative data were expressed as mean $\pm$ standard deviation. There was a statistical difference if $p < 0.05$; difference was considered as statistically significant if $p < 0.01$.

RESULTS

Intraoperative bleeding amount and postoperative first anal exsufflation

Table I demonstrates that the bleeding amount of group B was significantly less than that of group A, and there was a statistical difference ($p < 0.05$). The postoperative first anal exsufflation of group B was significantly earlier than that of group A, indicating that the recovery of group B was superior to that of group A; there was a statistical difference between the two groups ($p < 0.05$).

Results of pain score

Fig. 1 was drawn after integrating the scores by

Table I. Comparison of bleeding amount and first anal exsufflation time

Indicator/group	Group A	Group B	p
Intraoperative bleeding amount (ml)	189.48 $\pm$ 17.61	146.18 $\pm$ 16.21	<0.05
Postoperative first anal exsufflation time (day)	2.4 $\pm$ 1.6	1.4 $\pm$ 0.6	<0.05

SPSS ver. 19.0. Fig. 1 demonstrates that the feeling of pain of patients in group A was much stronger than that in group B, two times that of group B at 24 h after surgery, indicating that the feeling of pain of patients in group B was obviously relieved by comprehensive nursing. There was still a remarkable difference between group A and B at the 6 h, 12 h and 48 h after surgery ($p < 0.05$). The difference of feeling of pain between group A and group B 24 h postoperative was statistically significant ($p < 0.01$); there was no statistical difference at 72 h postoperative ($p > 0.05$).

Complications

Table II demonstrates that 2 patients in group B and 11 patients in group A had complications.

Surgery-induced complications mainly included anastomotic bleeding, anastomotic fistula, anastomotic obstruction and pulmonary infection. Anastomotic bleeding was the major complication in group A, and there was only one case of anastomotic bleeding and one case of pulmonary infection in group B. Integration of the data based on SPSS ver. 19.0 suggested a statistical difference between the two groups ($p < 0.05$).

Satisfaction of patients

The satisfaction of nursing was investigated after several weeks. The satisfaction degree of groups A and B was 40% and 93.3%, respectively. This indicated that the satisfaction of patients in group B with comprehensive nursing was significantly higher

Table II. Number of cases with complications and categories of complications

Group	Number of cases with complications	Anastomotic bleeding	Anastomotic fistula	Anastomotic obstruction	Pulmonary infection
Group A	11	5	2	1	3
Group B	2	1	0	0	1
p value	< 0.05				

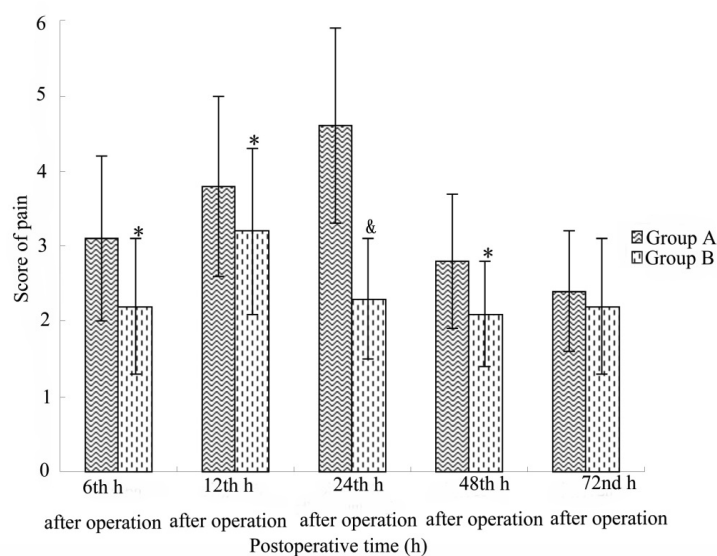


Fig. 1. Comparison of score for pain. * indicates $p < 0.05$ compared to group A; & indicates $p < 0.01$ compared to group A.

than that in group A with conventional nursing, and there was a statistical difference ($p < 0.05$).

DISCUSSION

With the development of laparoscopic surgery equipment, this form of surgery has been increasingly trusted by doctors and patients. Based on this, other advanced technologies such as endoscopy combined with laparoscopy have emerged (12). Mueller et al. (13) performed laparoscopic wedge resection on cardia and gastric antrum lesions of patients and obtained favorable therapeutic effects. Through investigating the National Comprehensive Cancer Network and European Society for Medical Oncology, Samardzic J et al. (14) found that laparoscopic surgery was featured by shorter hospital stay and less pain. This study investigated the efficacy of perioperative nursing intervention on patients undergoing laparoscopic surgery. Nursing is an elaborate work which plays a significant role in the daily recovery of patients. Standards for conventional nursing intervention, which is the most basic, includes cleanliness, tidiness, comfort, safety, quietness and no complications. Standards for comprehensive nursing intervention which is relatively systematic include mental regulation in addition to the standards of conventional nursing. In this study, the surgery method and nursing method (observation of disease condition, daily nursing, preoperative gastrointestinal nursing, postoperative canal nursing and psychological and dietary nursing) are described in detail. The intraoperative bleeding amount, postoperative first anal exsufflation, pain, complications and satisfaction degree of the two groups were studied. Maintaining normal blood volume is the key for reducing intraoperative bleeding. Therefore, the requirements for the skills of surgeons are strict (15). Earlier postoperative first anal exsufflation suggests faster recovery of intestines and stomach. Understanding pain of patients through its evaluating using scores is one of the functions of comprehensive nursing intervention (16). The number of cases with complications and categories of complications are of great significance for reflecting clinical efficacy. In the current study,

the number of patients with complications in group B was significantly less than that in group A which shows that comprehensive nursing could reduce intraoperative bleeding and incidence of complications and was positive to wound healing and psychological comfort (17). It was found that the patients who received comprehensive nursing had less bleeding, earlier first anal exsufflation, milder pain, less complications and high satisfaction of the therapeutic effect. Hence, it is concluded that perioperative nursing intervention is greatly beneficial for the recovery of patients. Comprehensive nursing in combination with laparoscopic gastric stromal tumor resection is recommended. Comprehensive nursing intervention is worth promoting for its great development prospect.

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LETTER TO THE EDITOR

THE ROLE OF SUBCUTANEOUS ADIPOSE TISSUE IN PSORIASIS

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It is widely accepted that obesity is a systemic comorbidity factor in psoriasis. At the same time, there is rapidly growing evidence that the adipose tissue is not only systemically but also locally involved in the pathophysiology of psoriasis and in response to successful anti-psoriatic treatment.

To the Editor,

It is widely accepted that obesity is an important factor among the systemic comorbidity factors in psoriasis. Analysis of 16 observational studies with a total of 2.1 million participants revealed a pooled odds ratio for obesity among the patients with psoriasis of 1.66 compared with non-psoriatic subjects; the corresponding ratio for patients with severe psoriasis was even as high as 2.23. Obesity has also a negative impact on the efficacy of systemic anti-psoriatic treatment (1). In addition, there is a high prevalence of metabolic syndrome in patients with psoriasis (2), the existence of which strongly correlates with the psoriasis area and severity index in these subjects (3). Moreover, several studies have demonstrated that weight loss may be a useful preventive and adjuvant therapy in psoriasis (4).

At the same time, there is rapidly growing evidence that the adipose tissue is not only systemically but also locally involved in the pathophysiology of psoriasis (5-7). Different experimental results support this viewpoint. Psoriatic skin of K5.hTGF β 1 transgenic mice demonstrated a pronounced dermal white adipose tissue (WAT) layer in lesional skin (8), which was strongly reduced after PUVA treatment, demonstrating a correlation with clinical

skin improvement. Furthermore, the local levels of adiponectin (having anti-inflammatory properties) in human skin and in subcutaneous WAT located underneath the psoriatic lesions were shown to be significantly lower in patients with psoriasis than in control subjects (9). Additionally, the expression of the microRNA (miR) member miR-26b-5p was shown to be strongly upregulated locally in sWAT underneath the lesional psoriatic skin (10). In turn, the upregulation of miR-26b is connected to adipocyte differentiation (11) and was earlier associated with inflammation in human WAT (12). Interestingly, miR-26b was shown to be regulated by different adipokines (13) known to be produced in patients with psoriasis. Recent findings that weight gain alters expression of the adiponectin receptor 1 (14) suggest a possible mechanism connecting systemic and local effects of the adipose tissue in psoriasis. In the mouse model of imiquimod-induced psoriasis, ATDSC demonstrated immunosuppressive activity (15). Intravenous application of autologous adipose tissue-derived stromal cells (ATDSC) resulted in significant reduction of Psoriasis Area and Severity Index (PASI) in a psoriasis patient not responding to either etanercept or infliximab (16).

A recent publication of Dattola et al. (17) provides

Key words: obesity, adipose, psoriasis

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additional evidence that WAT located underneath the psoriatic lesions is directly involved in psoriasis. These authors investigated sonoelastographically the WAT underneath the psoriatic plaques in 60 patients with PASI>5. Measurements were made at baseline and after one-month treatments provided with three different types of therapy; the strain ratio was calculated in a standard way comparing the strain in the muscle (reference tissue) to the strain in the adjacent WAT area. Whereas the strain imaging does not provide reliable information regarding the absolute values of the elastic modulus (18), this method offers the opportunity to compare the elastic moduli in different tissue areas as well as before and after treatment. Strain ratio in WAT tissue located directly under the non-lesional skin was significantly lower than the corresponding ratio for the WAT located under the psoriatic plaques (17). This means that the stiffness of the WAT spatially adjacent to psoriatic lesions is much higher than in the neighboring non-lesional skin, which must be connected with a local inflammation existing in this specific subcutaneous region. Effective psoriasis treatment significantly reduced the WAT stiffness under the plaques, making it comparable with the values typical for the non-lesional skin and demonstrating correlation with achieved improvement of the skin condition. Whereas these findings do not answer the question whether the superficial WAT located underneath the psoriatic plaques is etiologically responsible for the skin inflammation or is the result of this inflammation, they support the idea that the pathophysiology of the psoriasis is not limited to the epidermis and should involve the spatially restricted tissue composite containing the skin and the adjacent WAT.

The study of Dattola et al. (17) clearly demonstrated that anti-psoriatic treatment has an influence on WAT. The treatment in this study was not chosen for this purpose. The next step should be the use of a procedure that clearly influences WAT, and to investigate whether such an approach may improve psoriasis. One local approach could be radiofrequency capable of inducing a local structural modification of subcutaneous WAT (19).

The concept to include not only systemic but local effect of the adipose tissue in psoriasis is important

for clinical implications and probably will demand the significant modification of the treatment strategy. This mechanism should be further investigated experimentally.

CONFLICT OF INTEREST

ILK is the managing partner of Wellcomet GmbH. No methods or devices of Wellcomet were used in this publication. Wellcomet GmbH provided support in the form of salaries for ILK, but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The commercial affiliation of ILK with Wellcomet GmbH does not alter our adherence to all policies on sharing data and materials. UW declares no competing interests.

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LETTER TO THE EDITOR

MALNUTRITION IS PREVALENT IN PATIENTS WITH CARDIORENAL SYNDROME AND NEGATIVELY INFLUENCES CLINICAL OUTCOME

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Cardiorenal syndrome (CRS) describes the concurrent failure of cardiac and renal function, each influencing the other. Malnutrition and cachexia frequently develop in patients with heart failure or kidney failure. However, no information is currently available on the prevalence of malnutrition in CRS patients. We studied CRS patients admitted to an internal medicine ward during a 5-month period and evaluated their clinical characteristics and nutritional status. Malnutrition risk was assessed by using the validated screening tool NRS-2002 whilst body composition was assessed by bioimpedance analysis and muscle function was measured by handgrip (HG) strength. Cardiac mass was also recorded. Length of stay, hospital readmission and 6-month mortality were registered. During the study period, 22 CRS patients were studied. Twenty patients were diagnosed with either CRS type 1 or CRS type 5. In CRS patients, fat-free mass showed a trend toward representing a protective factor for 6-month mortality (OR=0.904; $p=0.06$). Also, fat-free mass correlated with HG strength and cardiac ejection fraction. Malnutrition risk was diagnosed in 45% of the patients, whereas 8 patients met the definition of cachexia. Even without statistical significance, CRS patients with malnutrition had lower BMI (Body Mass Index) ($p=0.038$) and fat-free mass ($p=$ n.s.). However, CRS malnutrition was associated to higher 6-month mortality ($p=0.05$), and appears to negatively influence the outcome in CRS (OR= 9; $p=0.06$). Our results show that malnutrition is prevalent in CRS patients and influences the clinical outcome. The assessment of nutritional status, and particularly body composition, should be implemented in daily practice of patients with CRS.

To the Editor,

Cardiorenal syndrome (CRS) is defined as the concurrent and progressive failure of kidney and heart functions. Patients with chronic kidney failure or chronic heart failure frequently develop disease-related malnutrition, which negatively impacts on morbidity and mortality (1). Consequently, it is likely that patients with chronic CRS may also show signs of malnutrition as proposed by ESPEN (2) and impaired outcome.

Our objective was to evaluate cachexia among patients admitted to our Internal Medicine ward with CRS. Twenty-two patients were enrolled in the study during 5 consecutive months. As no significant differences were observed between acute and chronic patients in relation to metabolic and nutritional parameters, we discussed their characteristics as a single group (Table I). According to Nutritional risk screening (NRS)-2002 (3), 10 patients resulted at nutritional risk (score ≥ 3 ; 45% of sample). The study

Key words: cardiorenal syndrome, cachexia, malnutrition

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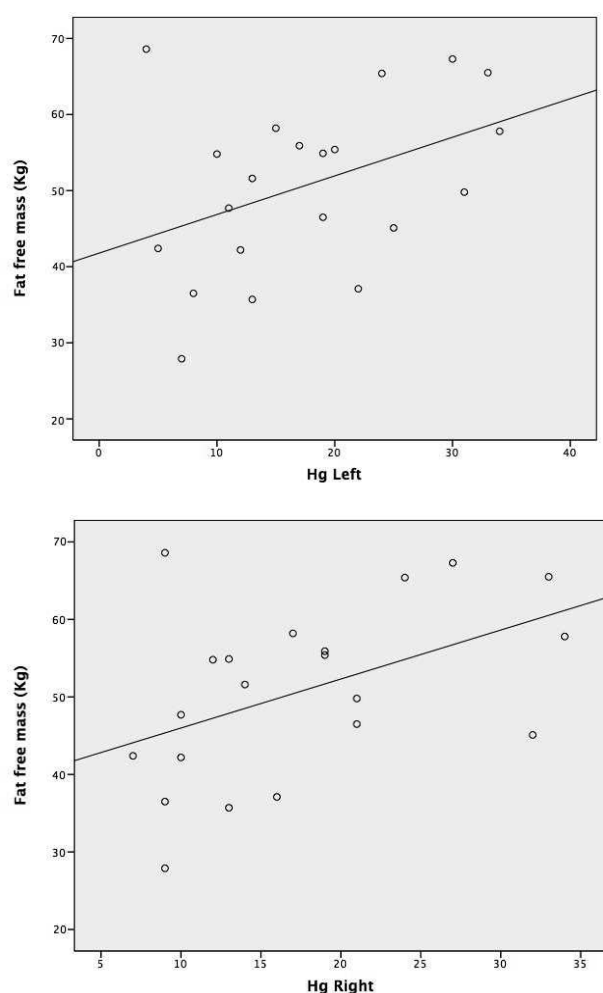


Fig. 1. Correlation between fat-free mass (Kg) of CRS patients ($n = 22$) and HG strength (Kg), left hand ($r = 0.505$, $p = 0.023$; upper panel) and right hand ($r = 0.472$, $p = 0.035$; lower panel)

was approved by the Local Ethics Committee.

The patients defined as malnourished (36% of sample) had lower Body Mass Index (BMI) ($p = 0.038$), reduced fat-free mass (no statistical significance) and higher 6-month mortality ($p = 0.05$) compared to well-nourished. Finally, malnutrition showed a trend toward representing a clinically relevant negative prognostic factor for 6-month mortality ($OR = 9$; $p = 0.06$).

Fat free mass significantly correlated with either left or right hand-grip (HG) strength ($r = 0.505$, $p = 0.023$ and $r = 0.472$, $p = 0.035$) and cardiac ejection fraction ($r = 0.484$, $p = 0.036$). Fat free mass as

measured by bioimpedance analysis (BIA) was not significantly different between patients with NRS-2002 score < 3 and those > 3 (Fig. 1).

It is well known that CRS is present among patients admitted to an internal medicine ward (4), and for the first time we found that malnutrition is frequently present in CRS and exerts a negative influence on patient mortality. These results should prompt a routine assessment of nutritional status in CRS patients since clinical outcome could be ameliorated by delaying or preventing wasting.

The mechanisms linking CRS and the development of malnutrition have not been fully elucidated. Cicoira et al. proposed that the state of cachexia frequently found in patients with chronic heart failure or chronic kidney failure contributes to the renal and cardiac failures, respectively. This cardio-renal cachexia syndrome is characterized by activation of immune and neuroendocrine systems, which sustain wasting and negatively affect heart and kidney functions (5). In this light, our results extend the relevance of the proposal by Cicoira et al. In fact, the vast majority of the patients in our study met the definition of CRS-1 or CRS-5. In particular, CRS-1 is determined by any of the acute heart failure syndromes in turn causing acute kidney injury. CRS-5 is determined by a systemic disorder causing both renal and cardiac dysfunction (1). Nevertheless, cachexia was prevalent among these patients.

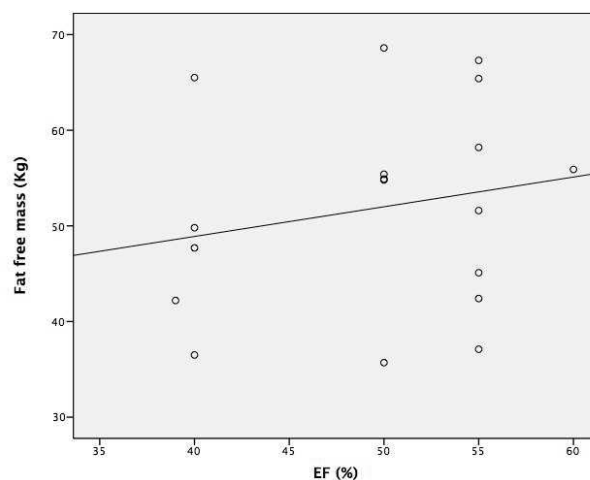


Fig. 2. Correlation between fat-free mass (Kg) of CRS patients ($n = 22$) and ejection fraction (EF, %; $r = 0.484$, $p = 0.036$)

Table I. Baseline clinical characteristics of the 22 patients with CRS diagnosis.

	Median value	Max	Min
Creatinine (mg/dl)	1.5	7.8	0.8
eGFR_{MDRD} (ml/min/1,73m ²)	44	75	8
Azotemia (mg/dl)	55.35	308	32
Hemoglobin (g/dl)	10.5	16.5	8.1
Uric Acid (mg/dl)	7.2	11.6	4.7
Albumin (mg/dl)	3.4	4.3	1.6
CRP (µg/ml)	3250	208800	500
BMI (Kg/m ²)	26.25	45.3	17.5
Body surface area (m ²)	1.88	2.29	1.36
NRS-2002	2	5	1
HG right hand (Kg)	16.5	34	7
HG left hand (Kg)	18	34	4
Phase angle	5.6	8.6	3.3
Fat free mass (Kg)	51.6	68.6	27.9
Fat free mass (%)	65.6	97.6	48.5
Fat free mass index (Kg/m ²)	17.71	23.02	11.61
Ejection fraction (%)	50	60	39
Cardiac mass (g)	215.9	389	97.3
Cardiac mass/BSA (g/m ²)	117.5	228.83	49.78

eGFR: estimated Glomerular Filtration Rate; MDRD: Modification of the Diet in Renal Disease

The presence of malnutrition in CRS-1 patients could be explained by the increased release of pro-inflammatory cytokines during acute heart failure. Finally, in our study, we found a significant correlation between fat-free mass and cardiac ejection factor. It is conceivable that the increased inflammatory response triggered by acute heart decompensation could also negatively impact on protein muscle metabolism.

Almost half of the patients were at risk of malnutrition as assessed by NRS-2002. Interestingly, we could not find significant difference between at risk and not at risk CRS patients with regard to BMI, HG, fat-free mass, 6-month morbidity and mortality. Despite the small number of patients enrolled, nutrition risk screening expresses the likelihood of a negative clinical outcome related to nutritional and clinical parameters.

In our study, the most frequent admission causes were dyspnea and fever, which suggest that the increased energy expenditure for respiration and the inflammatory response played a role in the development of cachexia.

A major result of our study is the elucidation

of the possible protective role of fat-free mass on CRS patient mortality. The impedance analysis provides prediction rather than measurement of body composition (6). The fat-free mass includes muscle mass but is not equal to muscularity, representing a valuable surrogate of muscle mass (7). Our study extends the clinical relevance of preserving muscle mass during disease, showing a trend toward significance for a protective role of fat-free mass in CRS patients.

The small sample size enrolled in our study could not allow for statistical significance, which may also explain the marginal relevance of malnutrition as negative prognostic factor. On the other hand, the clinical relevance of the present data is supported by the consistency of results in pointing to fat-free mass as a determinant of outcome. Moreover, our patients were studied during hospitalization for acute illness or worsening of chronic illness, which is not the ideal time for assessment. Finally, we could not assess whether the reported abnormal parameters observed during the acute phase are sustained, recovered or worsened.

The mechanisms by which muscularity influences survival have not yet been clarified. In cancer patients, low muscle mass and concomitant large fat mass may yield to inaccurate calculation of chemotherapy dose (8). Similarly, low muscle mass in CRS patients could alter the distribution volume of drugs acutely or chronically prescribed which, in turn, may reduce their efficacy or even increase side effects. Considering the large prevalence of wasting in patients suffering from chronic diseases (9), this hypothesis deserves further investigation.

Our study shows that malnutrition is prevalent in patients with CRS, even when acute-onset CRS types are considered. CRS malnourished patients have lower BMI and smaller muscularity than non-malnourished. More importantly, our data suggest that a correlation may exist between malnutrition and outcome in this clinical setting. Whether preserving fat-free mass in CRS patients may improve long-term clinical outcome remains to be tested in prospective clinical trials. Nevertheless, assessment of nutritional status, and particularly body composition, should be implemented in the daily practice of patients with CRS.

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LETTER TO THE EDITOR

OMALIZUMAB A NEW PROSPECTIVE: A NASAL POLYPOSIS

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Omalizumab, a monoclonal antibody against IgE, may be effective on nasal polyps, but its use is not currently authorized to treat that disease. We report the cases of three patients who were given omalizumab for asthma after undergoing nasal surgical polypectomy. Although such procedure is frequently followed by polyp recurrence, none of the three patients developed this complication, and in one subject the regression of initial polyp return was registered after starting omalizumab. Our data support the hypothesis that omalizumab may be useful to treat nasal polyposis.

To the Editor,

It is estimated that 48% of patients with chronic rhinosinusitis complicated by nasal polyposis (CRSwNP) suffer from comorbid asthma (1-2). The treatment of nasal polyposis is based on the administration of topical corticosteroids. In patients who are not responding to therapy, however, systemic corticosteroids are usually effective, but have significant side effects. Functional Endoscopic Sinus Surgery (FESS) is a good alternative, because it is effective in almost all cases; nonetheless, the recurrence of polyps is frequent and only partially prevented by the long-term administration of topical corticosteroids. According to the literature, despite the prophylaxis with corticosteroids, the need for a second surgical intervention occurs in 20-50% of cases within a few months (3-5), and in 40-60% within 18 months of surgery (6-8). For this reason, the treatment of nasal polyposis is a challenge for otolaryngologist always in search of new techniques and new medical therapies that can reduce relapses of polyps.

Omalizumab is a humanized anti-IgE monoclonal antibody currently indicated for the treatment of severe uncontrolled asthma and chronic spontaneous urticaria (9-10). Some studies suggest that it may also be effective in other immunological diseases, both mediated and non-mediated by IgE (11-15). Since chronic rhinosinusitis complicated by nasal polyposis (CRSwNP) is one of these pathologies, it is reasonable to hypothesize that omalizumab may be utilized to prevent post-surgical recurrences of nasal polyposis.

This letter reports the uncomplicated clinical course and, in one case, the partial regression of the recurrence of nasal polyps, observed in three patients, affected by uncontrolled asthma and allergic rhinitis to *Dermatophagoides pteronissinus*, who had undergone the surgical removal of nasal polyps and were successively treated with omalizumab for severe asthma.

Three patients who had previously undergone FESS for nasal polyposis were subjected to a

Key words: Omalizumab, nasal polyposis, chronic rhinosinusitis

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36-month treatment with omalizumab. Dosage and frequency of administration were based on the patient's weight and on total IgE serum levels. Any previous therapy for asthma (LABA, inhaled and oral corticosteroids) was not abruptly discontinued, but adjusted according to the patient's symptoms. Among the routine controls carried out during the treatment with omalizumab, nasal endoscopy was performed at the beginning, and then every six months. This provided the opportunity to regularly inspect the nasal cavities and the orifices of the paranasal sinuses, in order to detect any recurrence of nasal polyps.

Patient 1, a 65-year-old male (total IgE baseline: 197 IU/ml, Specific IgE: 1.91 IU/ml, weight: 91kg), had undergone surgical polypectomy three times for recurrence, the last one six months before starting omalizumab treatment. He did not present nasal polyps at baseline nasal endoscopy, nor did he develop any recurrence during treatment.

Patient 2, a 72-year-old female (Total IgE baseline: 212 IU/ml, Specific IgE: 5.31 IU/ml, weight: 68kg), had undergone surgery for nasal polyps twice, the last time 30 months before omalizumab. No polyp recurrence was recorded at the start or during the treatment.

Finally, patient 3 was a 56-year-old male (Total IgE baseline: 310 IU/ml, Specific IgE: 5.39 IU/ml, weight: 93kg) who had undergone surgical nasal polypectomy 30 months earlier and in whom polyp recurrence had been already identified 12 months after surgery, although no further measures had been taken. Baseline nasal endoscopy confirmed the presence of nasal polyps that protruded from the ostium of the maxillary sinus, bilaterally. In the following months, however, a clear reduction in the size of the polyps was observed, particularly during the endoscopic controls performed 24 and 36 months after starting omalizumab administration.

The patients reported no systemic adverse effects or local side effects during treatment with omalizumab. All patients gave informed signed consent to the use of the data.

These few preliminary data seem to support the hypothesis that omalizumab may help prevent post-surgical recurrence of nasal polyps (16), which could

prove to be a helpful aid for otorhinolaryngologists in selected patients. Controlled clinical studies are needed in future in order to test this innovative hypothesis.

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LETTER TO THE EDITOR

THE ROLE OF LIPOFILLING IN RECONSTRUCTIONS WITH DERMAL REGENERATION TEMPLATE: CLINICAL AND HISTOLOGICAL ASSESSMENT

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Skin and soft tissue reconstruction represents one of the most debated issues of plastic surgery. The advent of regenerative medicine has shown new pathways with the use of lipofilling and dermal regeneration templates. The aim of this study was to investigate the histological and clinical modifications occurring after lipofilling in the areas previously reconstructed with Integra® and an autologous thin dermal-epidermal graft. Histological and immunohistochemical analysis were performed on nine patients to compare skin before and after lipofilling. Pre- and post-operative examinations (POSAS, VAS scale) were carried out as well as taking clinical photographs. The authors detected an overall clinical and histological improvement in all cases. Data obtained from POSAS and VAS scale showed a statistically significant ($p < 0.05$) improvement concerning all variables investigated before surgery. The biopsies revealed qualitative modifications with hematoxylin-eosin and Masson trichrome stain. Immunohistochemistry with CD31 antibody also demonstrated quantitative changes with an increased number of vessels. The photographs enabled to compare the clinical situation before and after lipofilling with better aesthetic outcomes. Lipofilling gave good functional and aesthetic results in the areas treated with Integra® and autologous thin dermal-epidermal grafts.

To the Editor,

Skin and soft tissue reconstruction after traumas, following neoplasm excision or due to malformations with loss of cutaneous tissue represents one of the most debated issues of plastic surgery. In the last few decades, the advent of regenerative medicine has shown new pathways with the use of fat grafting and dermal regeneration templates (1, 2). Nowadays, Integra® dermal regeneration template is the most widespread matrix used to reconstruct losses of skin tissue (3).

The aim of this prospective study was to investigate the histological and clinical modifications which occurred after lipofilling in the areas previously reconstructed with Integra® and a thin autologous dermal-epidermal graft.

MATERIALS AND METHODS

Study design

The study was conducted from November 2014 to June 2015 in the Plastic and Reconstructive Surgery Department

Key words: regenerative medicine, lipofilling, Integra, dermal-epidermal grafts, soft tissue reconstruction, biomaterials/technology

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of Foggia University Hospital. The participants were selected from 52 patients with losses of skin tissue who had reconstructions with Integra® between 2009 and 2013. After a first assessment, the following subjects were excluded from our study:

- Patients who had undergone removal of highly invasive malignant tumors, such as sarcoma or melanoma;
- Patients who relapsed during the follow-up year, after undergoing removal of malignant skin tumors;
- Patients aged <18 or ≥ 80;
- Patients with significant comorbidities, such as severe hypertension, poorly controlled diabetes mellitus, rheumatic or autoimmune diseases;
- Patients who lacked adequate amounts of subcutaneous adipose tissue at the possible donor areas (i.e. abdomen, buttocks, hips, thighs).

Pre and post-operative examinations

The patients who fulfilled the inclusion and exclusion criteria underwent a lipofilling procedure in order to improve the aesthetic outcomes, symptoms and functionality of the reconstructed areas. Before lipofilling and after a follow-up period of 90-120 days, the following tests were carried out in order to evaluate the clinical and the histological changes which had occurred:

- Patient and Observer Scar Assessment Scale (POSAS) for scar evaluation;
- Visual Analogue Scale (VAS) for pain assessment, providing a range of scores from 0-10 (100 mm scale);
- Skin wedge biopsies, to study the histological changes. The first sample was taken from the border area treated with Integra® before lipofilling and the second one from the same area after fat grafting. A sample of the skin from the surrounding untreated area served as a control (data not reported). Specimens were preserved by formalin fixation. The samples were processed into paraffin and stained with hematoxylin-eosin to show the architectural features of tissues, collagen deposition and vascularity, Masson trichrome stain for collagen evaluation and immunohistochemical technique with CD31 antibody (Ventana-Roche, monoclonal JC70 mouse) to study vessels;
- Clinical photographs, to follow the clinical evolutions.

Examiners who were unaware of the surgical procedure, conducted pre- and postoperative assessments. The same board-certified surgeon, who was not a member of the operating team, always completed the Observer scale. To obtain reliability, vessels were counted separately by two pathologists and the mean value was calculated. The above-mentioned cautions were adopted in order to increase the objectivity and accuracy of results.

Surgical procedure

The same surgeon performed lipofilling and skin biopsies. Under local anesthesia and sedation, the lipofilling procedure was executed according to the Coleman technique (4).

The first phase consisted of fat harvesting. Two 2-mm long incisions were made in the lower quadrants of the abdominal region. The abdomen was chosen as it is an easily accessible soft adipose tissue reservoir. The tissue was infiltrated with Klein solution, refrigerated at 4°C, consisting of saline, lidocaine, HCO³ and epinephrine. The solution is able to loosen the tissue and minimize the bleeding, therefore facilitating fat harvesting and reducing postoperative pain. Using a blunt cannula of 2 mm in diameter, connected to a 10 ml Luer Lock syringe, fat was manually suctioned with a 1:1 ratio of the amount of infiltrated solution to the tissue harvested. In the second phase of fat purification, the obtained tissue was centrifuged at 3000 rounds per min for 3 min in order to separate the adipocytes containing middle layer from the oils (supernatant), serum (subnatant) and hematic cells suspended in the plasma at the bottom. In the third and last phase, fat injection was performed. The fat tissue was transferred to 1 ml syringes and transplanted subcutaneously into the recipient area using 0.5-1 mm diameter cannulas to increase the engraftment. Compressive dressings on the harvested region and analgesic therapy were carried out.

All the patients signed an informed consent in order to undergo lipofilling, pre- and postoperative skin biopsies, to fill POSAS and VAS scale and to authorize the photographs for scientific purposes. The research was conducted in accordance with Guideline for Good Clinical Practice and the ethical principles originating in the Declaration of Helsinki.

Statistical analysis

The assessment of significant differences among the

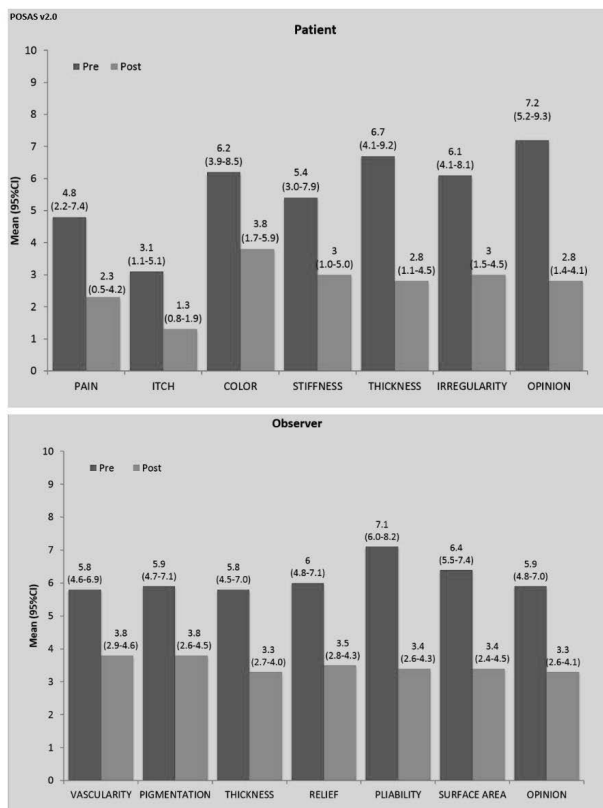


Fig. 1. POSAS v2.0, Patient and Observer Scar Assessment Scale, mean values with 95% CI. Data obtained from POSAS filled in by patients and surgeons showed a statistically significant improvement ($p < 0.05$) after lipofilling, concerning all the variables investigated before surgery.

mean values of continuous variables examined by POSAS and VAS scale was performed using *t*-test for independent samples. The threshold for statistical significance was established at a p value < 0.05 and for every variable 95% confidence intervals (95%CI) were calculated. All analyses were performed using software STATA-MP 10.1 for Mac OS X.

RESULTS

Nine patients (17.3%) who presented with losses of skin tissue, with different etiology and in different body areas, operated no less than one year earlier, were included in the study group. Eleven (21.2%) patients were excluded according to the exclusion criteria, seventeen (32.7%) refused and fifteen (28.8%) had died.

All nine patients underwent lipofilling once. Fat

was always taken from the lower quadrants of the abdominal region. The average quantity of infiltrated fat per session was 24.3 ± 21.7 cc. Mean time from the treatment performed with Integra® was 3.3 ± 1.5 years. Out of the study group, six patients (66.7%) were male. The average age was 56.9 ± 15.6 ; the mean Body Mass Index (BMI) was 26.9 ± 3.5 .

Five subjects (55.5%) were operated in order to improve areas already reconstructed after oncologic demolition, all located on the face. Three patients (33.3%) underwent the operation because of limb trauma, of whom two patients (22.2%) presented with losses of skin tissue at the plantar region of the foot, and one (11.1%) of the hand. Finally, one patient (11.1%) was operated because of congenital giant nevus.

The postoperative state (72 hours) was uneventful in all patients. At thirty days after the operation, four subjects (44.4%) presented with sore harvesting areas and one patient reported bruising.

Data obtained from POSAS scale filled by patients and surgeons showed a statistically significant improvement ($p < 0.05$) after lipofilling, concerning all the variables investigated before surgery (Fig. 1). Data obtained from VAS scale presented to patients showed a statistically significant decrease ($p < 0.05$) in pain after lipofilling. A statistically significant difference in the mean value of the score obtained from VAS scale before (4.8; 95%CI: 2.2-7.4) and after lipofilling (1.8; 95%CI: 0.7-2.8; $p < 0.05$) was detected.

After 90-120 days from lipofilling, a general qualitative improvement was observed in all samples stained by hematoxylin-eosin, with an increased vascularization and regenerative phenomena, such as formation of new collagen and a decrease in fibrosis (Fig. 2, A-B).

Even skin biopsies stained with Masson trichrome revealed an overall qualitative improvement in all cases. The images showed the presence of newly formed immature collagen after lipofilling (Fig. 2, C-D). Skin biopsies, processed with CD31 antibody (Ventana-Roche, monoclonal JC70 mouse), resulted in an improvement in the vascularity (Fig. 2, E-F). Immunohistochemistry showed the following quantitative changes:

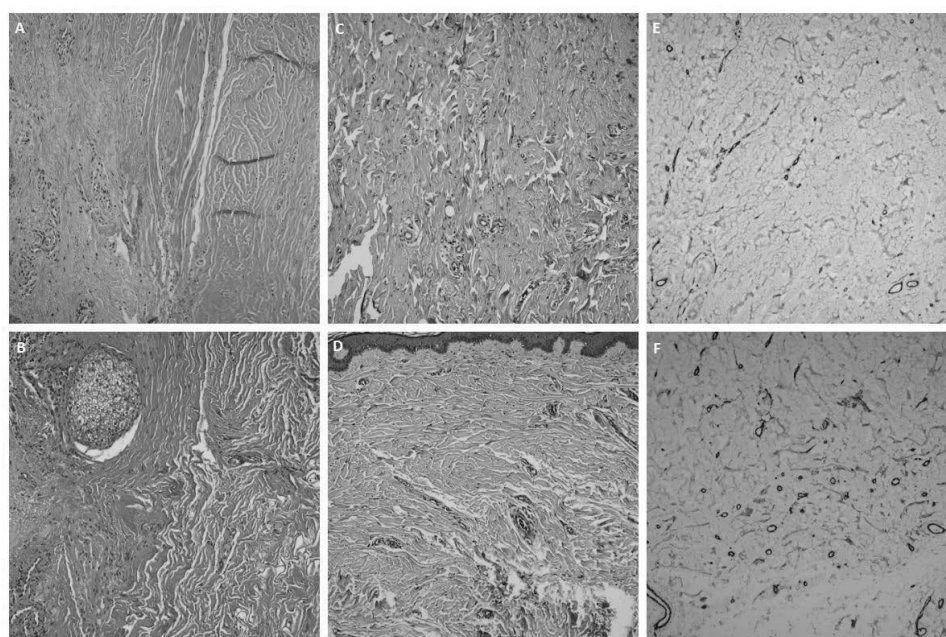


Fig. 2. *A)* Skin treated with Integra® and a thin dermal-epidermal graft (HE X10). *B)* Skin after a follow-up period of 90 days from lipofilling. Skin biopsies stained with hematoxylin-eosin revealed improved vascularization and a decrease of fibrosis after lipofilling (HE X10). *C)* Skin treated with Integra® and a thin dermal-epidermal graft (Masson trichrome stain X10). *D)* Skin after a follow-up period of 90 days from lipofilling. Skin biopsies showed the presence of newly formed immature collagen after lipofilling (Masson trichrome stain X10). *E)* Skin treated with Integra® and a thin dermal-epidermal graft (IHC: CD31 X10). *F)* Skin after a follow-up period of 90 days from lipofilling. Vascularization is improved and blood vessels are tripled after lipofilling (IHC: CD31 X10).

- An increase in number of vessels after lipofilling was detected in each patient of the study group after surgery;
- A statistically significant difference in the mean value of the number of vessels before (25.8; 95%CI: 21.3-30.2) and after lipofilling (45.8; 95%CI: 40.1-51.4; $p < 0.0001$).

Photographs allowed the comparison of the clinical situation before and after lipofilling. Three clinical cases belonging to each main diagnostic group were selected: one patient with congenital giant nevus (Fig. 3, A-B), one patient with residual scarring after oncologic demolition (Fig. 3, C-D) and one case of limb injuries (Fig. 3, E-F).

DISCUSSION

Our study focused on the histological and clinical modifications which occurred after lipofilling in

the areas previously reconstructed with Integra® and a thin autologous dermal-epidermal graft. An overall histological improvement was detected in all the cases between 90 and 120 days after lipofilling. The clinical results supported histology. The increased vascularization and regenerative effects could explain the excellent aesthetic and functional outcomes obtained.

Integra® is made up of two layers, a silicone one in the upper part, and type I collagen and glycosaminoglycans in the lower. One of its most important advantages is the possibility to perform a thinner autologous dermal-epidermal graft (0.05-0.15 mm vs 0.48 mm). However, Integra® has some limitations, such as aesthetic outcomes not always satisfying, possibility of hypo or hyper-pigmentation, tendency towards retraction, inadequate cover of deep planes (5).

Lipofilling, defined as autologous fat transfer,

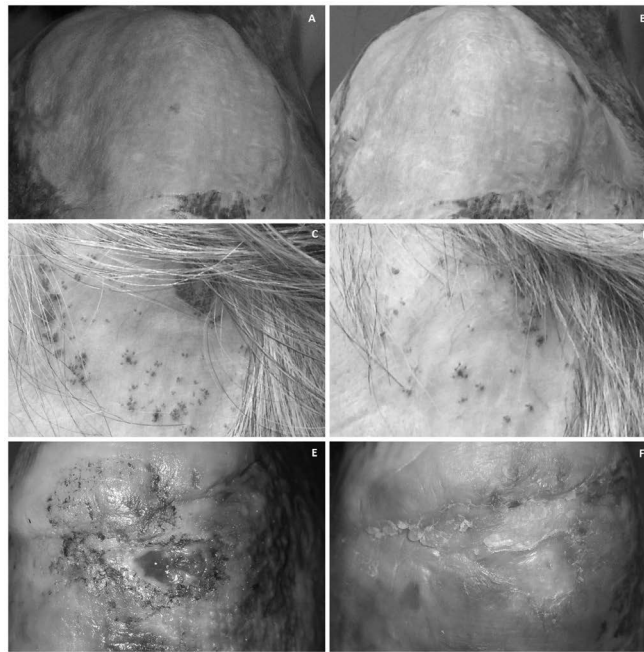


Fig. 3. Case 1. A 30-Year-old female with congenital giant nevus in treatment. **A)** The right shoulder had a first reconstruction with Integra® and an autologous dermal-epidermal graft. Before lipofilling the patient presented with a thin and retracting scar that affected the functionality of the limb. **B)** After a follow-up period of 90 days from lipofilling we detected a morpho-functional improvement.

Case 2. A 60-year-old female with residual scar of the left temporal region after oncologic demolition. **C)** The area was previously treated with Integra® and a thin dermal-epidermal graft. Before lipofilling the patient presented with an atrophic scar. **D)** After a follow-up period of 90 days from lipofilling it is possible to notice better aesthetic outcomes.

Case 3. A 47-year-old male with trauma of the left heel. **E)** The area had a first reconstruction with Integra® and a thin dermal-epidermal graft. Before lipofilling the patient presented with a complex clinical situation with high tendency to relapse. **F)** After a follow-up period of 90 days from lipofilling better tissue quality and symptom improvement were detected.

has developed into a standard procedure in plastic surgery, with an increasing number of indications in the aesthetic and reconstructive field (6, 7). Many microstructural studies have shown that the adipose tissue contains an extracellular matrix, composed of collagen, laminin, fibronectin, growth factors and cellular components (8, 9). The Adipose-derived stem cells (ASCs) and progenitor cells have been shown to play a key role in promoting wound healing (10) and in regulating vascular density, granulation process and collagen deposition (11). Clinical improvements in patients concern skin structure, pore size, elasticity, color and scar size, as revealed by Klinger et al. who demonstrated a tissue regeneration enhancing process promoted by fat injection for cases of severe burn outcomes (12).

Other studies have combined the use of ASCs

with dermal substitutes. Meruane et al. suggested in an animal study that the seeding of ASCs onto a dermal substitute improves skin regeneration and tissue integration by increasing vascularity and collagen synthesis (1). Lafosse et al. developed a biological dressing made of ASCs on a human acellular collagen matrix and showed that it could represent a promising and safe therapy for non-healing wounds (13).

Our study aimed to suggest a “three-step protocol” including Integra®-skin graft-lipofilling for the treatment of injuries with loss of skin tissue. Based on our experience, lipofilling is a technique easy to perform and able to ensure a morpho-functional improvement in all the tested body areas. The higher satisfaction implied a better compliance and motivated all the patients to undergo the postoperative

biopsy, which was fundamental in understanding the dynamic phenomena provoked by lipofilling. The most important histological data of our preliminary study was improved vascularization. The increase in the number of vessels led to a new balance to the skin, contributing to these good morpho-functional results (14, 15). Nevertheless, further clinical and histological investigations must be carried out in order to clarify the role of the aforementioned effect.

The patients presented with complex clinical situations and many of them had been undergoing a high number of visits and advanced dressings for years. With a larger sample, it would be possible to investigate other parameters, such as the analysis of costs compared with classic methods, and study how high the impact on working and social life is.

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LETTER TO THE EDITOR

DIAGNOSIS OF A NEONATAL OPHTHALMIC DISCHARGE, *OPHTHALMIA NEONATORUM*, IN THE “MOLECULAR AGE”: INVESTIGATION FOR A CORRECT THERAPY

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An early double case of acute *Ophthalmia neonatorum* in 3-day-old twins is reported. Culture of eye swabs showed a wide bacterial polymorphism, in which common bacteria, such as *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Corynebacterium ulcerans* and other *Enterobacteriaceae*, coexisted with atypical *Mycoplasmataceae* and *Chlamydiaceae* from resident cervical-vaginal maternal microbiota. The neonates were in an apparently healthy state, but showed red eyes with abundant greenish-yellow secretion, mild chemosis and lid edema. The maternal cervical-vaginal ecosystem resulted differently positive to the same common cultivable, atypical bacteria culturally and molecularly determined. This suggested a direct maternal-foetal transmission or a further foetal contamination before birth. An extended culture analysis for common bacteria to atypical ones was decisive to describe the involvement of *Mycoplasmas* (*M. hominis* and *U. urealyticum*) within the scenario of the *Ophthalmia neonatorum* in a Caucasian couple. The introduction of a routine PCR molecular analysis for *Chlamydiaceae* and *N. gonorrhoeae* allowed to establish which of these were present at birth, and contributed to determine the correct laboratory diagnosis and to define an adequate therapeutic protocol obtaining a complete resolution after one year for culture and atypical bacteria controls. This study suggests to improve the quality of laboratory diagnosis as unavoidable support to a correct clinical diagnosis and therapy, in a standardized modality both for swabbing and scraping, to check the new-born microbial programming starting in uterus, overtaking the “cultural age” to the “molecular age”, and to revise the WHO guidelines of “SAFE Strategy” for trachoma eye disease, transforming it into “SAFES Strategy” where the “S” letter is the acronym of “Sexual ecosystem and behavioural valuation/education”.

Key words: *Ophthalmia neonatorum*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, *Chlamydiaceae*, *Mycoplasmataceae*, cultural and molecular PCR research, conjunctival microbiota, trachoma, SAFE(S) strategy, maternal cervical-vaginal ecosystem, molecular age

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To the Editor,

Ophthalmia neonatorum (ON) is the major acute purulent ocular manifestation during the neonatal period in the underdeveloped and industrialised countries in the World (1). ON is a conjunctivitis that occurs in the first 6 weeks of life, affecting 1.6% to 12% of all newborns and is caused by a chemical, bacterial and/or viral process.

N. gonorrhoeae (Ng), rated up to 100% ON causative until the second half of XIX century, accounts now for less than 1% of all reported cases, but in absence of adequate prophylaxis 30-42% of babies born by vaginal delivery to infected mothers will develop ON within 48-120 hours of birth. The transmission rate is higher in mothers with chlamydial concomitant infection (1).

C. trachomatis (Ct), identified as newborn inclusion conjunctivitis from epithelial cell cytoplasmic basophilic inclusion bodies (Stargardt-Lindner or Halberstaeder-Prowazek), accounts for up to 40% of all cases of ON and the risk of acquiring it during delivery increases up to 75% (2). The incubation period varies from 1 to 2 weeks and the prevalence is higher in spring and summer. A mild conjunctivitis with a scanty watery discharge to a severe mucopurulent one with chemosis and membrane formation is the onset scenario. Loss of vision is very rare, and some cases can heal spontaneously without complication but, if left untreated, superficial neovascularisation and conjunctival scarring can occur, inducing cicatricial entropion and trichiasis.

Mycoplasmas are the smallest and simplest self-replicating atypical bacteria, differing from others in that they have very small genome-size allowing them to live inside the host cells as saprophytic state. *Mycoplasma hominis* (Mh), *Mycoplasma genitalium* (Mg) and *Ureaplasma urealyticum* (Uu) are facultative anaerobe, which grow slowly by fission binary and produce "fried egg" colonies on agar plates that can take up to 3 weeks to develop, and are difficult to culture.

Viral conjunctivitis also can be transmitted during delivery. Adenovirus may induce conjunctivitis with petechial haemorrhage and lymphadenopathy. Herpes simplex virus 1 and 2 might be diagnosed late

as they present 6-14 days after birth with vesicles or ulcers at eyelid borders and are commonly treated empirically as for chlamydial or gonococcal infection. It is generally recognized that *Chlamydiaceae* (*C. pneumoniae* (Cp) and Ct) and *Mycoplasmas* (Mh and Uu), as sexually transmitted infections, are atypical microorganisms able to survive in infected human cell and monocytes (3), from which they can be transmitted, entering the blood circulation and/or by means of conjunctival, pharyngeal, intestinal, prostatic and cervical inflamed mucosae without showing any early clinical manifestation. The time of onset and the persistence of the symptoms are related the individual genetic tissue susceptibility and on self-modulating expression chlamydial capacity (4). As a rule, child, mother and sexual partner(s) should be treated for the causative germ or virus molecularly detected. Atypical bacteria, such as *C. trachomatis* and *Mycoplasmas* (here indicated all together as CMHU), are present in 50-75% of the asymptomatic and 20-25% of symptomatic population, recurring variously at different severities of chronic diseases (5). Thus, ocular chlamydial infection, evolving from acute or subacute to chronic phase (6-7), can show several grades of clinical appearance related to different ethnic population, due to different genetic predisposition, probably derived from different eating and environmental conditions (8). Again, chronic chlamydial cervicitis in genetically susceptible subjects, can evolve and produce several more complications, such as Fitz-Hugh-Curtis's syndrome, up to cervical cancer (9). Among the possible chronic complications of benign prostatitis caused by CMHU infections, it is necessary to underline the chlamydial and mycoplasmal nemasperm DNA fragmentation with the consequent couple infertility (7). Several studies have already demonstrated that *Chlamydiaceae* are potentially implicated in the most severe ocular (10) and other diseases, e.g. chronic cardio-vascular, neurological and pulmonary diseases. Lastly, many other studies have clarified the key role of *Chlamydiaceae* (*C. pneumoniae* and *C. trachomatis*) and *Mycoplasma* (*M. hominis* and *U. urealyticum*) infections in severe diseases that, in genetically predisposed subjects, can evolve after decades into

several cancer typologies (11). On the basis of these recent and revised results obtained by molecular procedure, we used the molecular PCR routinely for Chlamydiaceae and *N. gonorrhoeae* researches, to confirm the correct identification of *S. pneumoniae* and to evaluate the HLA-B₂₇ genotype for arthritis susceptibility.

MATERIALS AND METHODS

Patients

Three patients, a mother and her twins, were enrolled in the study. The twin sisters, from an Italian Caucasian couple, were 3 days old when they underwent the first visit in the consulting room in the Emergency Service of the Eye Clinic (COCR) of the University of Chieti, Italy. No ocular hygiene had been carried out before the sampling.

Clinical specimens

The “red eyes” clinical manifestation was covered with an abundant greenish-yellow secretion which was collected from the inner cantus and under the conjunctiva by cotton swabs for cultural analyses. Two mL blood samples from both neonates were collected at the birth into EDTA tubes from the umbilical cord for biochemical tests and used for HLA-B₂₇ test. All material was immediately processed for fresh microscopic valuation, and cultural and molecular study. The cellular materials were collected by scraping for post-therapeutic checks (12).

Microscopic valuation, microbiological and molecular analyses

The secretion was immediately dispersed into 1 mL of sterile saline solution. A drop of each sample was evaluated by optical microscope (OM). The same preparation was dried in a thermostat at 37°C for Gram staining (Gs) and another slide was used for May-Grunwald Giemsa staining (M-GGs). Each pH measure was carried out on aliquots of 300 µL by digital portable pH-meter. An aliquot of 1 µL was dispersed on chromID CPS and Columbia CAN plates, respectively for aerobic and anaerobic microorganisms. Total Microbial Charge (TMC) and the relative presence of each species was expressed as percentage of TMC (considered as 100%) (8). Ocular culture was considered significant at >10⁵ CFU/mL. A *Streptococcus pneumoniae* (Sp) colony

underwent PCR analysis to confirm the eventual misidentification of *Streptococcus pseudopneumoniae* as true *S. pneumoniae*. All microbiological products were purchased from bioMerièux and bacterial identifications were performed by Slidex Strepto-kit and API Coryne Gallery, while Mycoplasma IST2 system was used for Mycoplasma quantification. Ct- and Ng- DNA extractions from each sample were performed in duplicate by PCR (BioAesis srl, Jesi, Italy), while Cp-DNA extraction and HLA-B₂₇ tests were carried out following procedures already reported in our precedent studies (12).

The clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki and Oviedo. Written informed consent enclosed in the clinical record was obtained from the mother who replied to a questionnaire prepared by experts on atypical infections. The file was submitted and amended by the Ethics Committee (CdE) of the Italian Ophthalmological Society (SOI), protocol no. 023/S/2016R.

RESULTS

All clinical microbiological indications, culture and molecular results are summarized in Table I. pH ocular conjunctival secretion resulted 8.1 for the 1st neonate and 8.6 for the 2nd neonate. The microscopic evaluation of ocular material from each sister revealed the presence of approximately 800 and 1500 leucocytes/hpf and of squamous epithelial cells (sec) 100-150 and 150-300 sec/hpf, respectively, for the 1st and the 2nd neonate. M-GGs showed a high presence of monocytes and neutrophils, resulting respectively (~30%) and (~50%), in both the preparations, mediating 3 distinct counts/100 elements. In the polymorphic scenario, Gs staining showed no G-positive lanceolate bacteria (1-5/hpf). The ocular material of the 1st sister presented a TMC: 5.7 x 10⁵ CFU/mL, while that of the 2nd sister showed a TMC: 6.3 x 10⁵ CFU/mL. The bacterial distribution consisted in *C. ulcerans* (Cu): 60%, *E. coli* (Ec): 25%, *E. faecalis* (Ef): 8%, *S. pneumoniae* (Sp): 5%, *K. pneumoniae* (Kp): 2% for 1st neonate and Cu: 80%, Ec: 8%, Ef: 7%, Sp: 3%, Kp: 2% for the 2nd neonate. Molecular tests for *C. pneumoniae* and *N. gonorrhoeae* resulted negative neonatal secretions of both sisters, while the ones for *C. trachomatis* were

Table I. pH, optical microscopic fresh valuation, total cultural charge and percentage of each cultural bacteria are reported together with atypical bacteria to catch the correct interpretation of the true pathological agents.

Patient	Ocular ecosystem 1 st neonate	Ocular ecosystem 2 nd neonate	Cervical-vaginal ecosystem mother
Ecosystem pH ref. range (6.93 +/- 0.24)*	8.1	8.6	6.8 fertil. age ref. range: 3.8 - 4.5
OM Freshly valuation	~ 800 leucocytes/hpf	~1500 leucocytes /hpf	~3000 leucocytes /hpf
M-GG's staining	Neutrophils: 40-50% Monocytes: 20-30%	Neutrophils: 40-50% Monocytes: 20-30%	Neutrophils: ~ 80% Monocytes: ~ 10%
100-150 sec/hpf	100-150 sec/hpf	150-300 sec/hpf	~ 500 sec/hpf
G (+) lanceolate bacteria	1-5/hpf	1-5/hpf	Rare (1-3/2-3 hpf)
TMC	5.7 x 10 ⁵ CFU/mL	6.3 x 10 ⁵ CFU/mL	>1.0 x 10 ⁶ CFU/mL
Bacterial percentage	Cu : 60% Ec : 25% Ef : 8% Sp : 5% Kp : 2%	Cu : 80% Ec : 8% Ef : 7% Sp : 3% Kp : 2%	Cu : 75% Ec : 15% Ef : 6% Sp : 2% Kp : 2%
Mycoplasmas (CCU/mL)	Mh: 1.0 x 10 ⁴ CCU/mL Uu: 1.0 x 10 ⁵ CCU/mL	Mh: 1.0 x 10 ⁵ CCU/mL Uu: 1.0 x 10 ⁴ CCU/mL	Mh: > 1.0 x 10 ⁵ CCU/mL Uu: > 1.0 x 10 ⁵ CCU/mL
C. trachomatis (PCR)	Present	Present	Present
C. pneumoniae (PCR)	Absent	Absent	Absent
N. gonorrhoeae (PCR)	Absent	Absent	Absent

*from Norn MS. *Acta Ophthalmol* 1988; 66(5):485-9.

both positive. Moreover, the tests for *M. hominis* and *U. urealyticum* were both positive in each sister: the 1st neonate presented Mh: 1.0 x 10⁴ CCU/mL and Uu: 1.0 x 10⁵ CCU/mL, while the 2nd one showed Mh: 1.0 x 10⁵ CCU/mL and Uu: 1.0 x 10⁴ CCU/mL. The mother's cervical-vaginal ecosystem showed a 6.8 pH value (ref. range of fertile age: 3.8 - 4.5) in a setting of vast polymorphic forms and variable Gram staining. She presented a TMC: > 1.0 x 10⁶ CFU/mL, with a following cervico-vaginal ecosystem distribution: Cu: 75%, Ec: 15%, Ef: 6%, Sp: 2%

and Kp: 2%. The bacterial cultural growth detected the presence of mycoplasmas: *M. hominis* and *U. urealyticum* were both > 1.0 x 10⁵ CCU/mL. Only Ct-DNA was positive for Chlamydiaceae, while the Ng-DNA test of cervico-vaginal source resulted negative. Neonates and the mother were positive for HLA-B₂₇ tests.

Test-cure for normal and atypical bacteria and therapeutic follow-up.

We designed a specific therapeutic protocol

Table II. The first cycle (four months) for the annual planned protocol of anti-inflammatory (a-IT) and antibiotic (ATB) therapies, observing one month of pause before the complete cultural and molecular check.

a-IT and ATB	1 st week	2 nd week	3 rd week	4 th week
I ^a cycle	CAF/HC ointment 3 times/8 h/day	AZT drop 3 times/8 h/day	N/D gel 3 times/8 h/day	1 st cultural check
a-IT and ATB	5 th week	6 th week	7 th week	8 th week
II ^a cycle	TC/SMT ointment 3 times/8 h/day	ERY syrup 2 times/day	CAF/TC ointment 3 times/8 h/day	TC drop 3 times/8 h/day
a-IT and ATB	9 th week	10 th week	11 th week	12 th week
III ^a cycle	AZT drop 3 times/8 h/day	CAF/HC ointment 3 times/8 h/day	TC drop 3 times/8 h/day	N/D gel 3 times/8 h/day
a-IT and ATB	13 th week	14 th week	15 th week	16 th week
cultural and molecular check	No therapy	No therapy	No therapy	2 nd control visit, cultural and molecular check

CAF/HC: Cloramphenicol/Hydrocortisone ointment 1%+0,5%; AZT: Azithromycin drop 1.5%; N/D: Netilmicin/Dexamethasone gel 3mg/mL+1mg/mL; TC/SMT: Tetracycline/Sulphamethiazole ointment 1%+5%; ERY: Erythromycin syrup; CAF/TC: Chloramphenicol/Tetracycline ointment 1%+0,5; TC: Tetracycline drop 1%.

based on the continuous succession of short cycles of different antibiotics with the purpose of evading the ability of atypical bacteria to resist to adverse conditions and in order to make them unable to re-emerge in the hosts for a long time afterwards although with different clinical evidence. Not being possible to know the patient's compliance, the test-cure was based on the clinical manifestations and laboratory results only.

In Table I the annual complete antinflammatory (a-IT) and antibiotic (ATB) treatments are reported. A cultural check was performed on the last day of the 4th week. At the 1st control visit in the consulting room of COCR for clinical evaluation, the neonates showed an apparently good recovery of the ocular condition and normal intraocular pressure (IOP). They presented only a yellowish secretion, constituted by very poor epithelial cellular debris in respect to acute phase of ON. On OM observation,

we found 10-15 sec/hpf and 15-20 leucocytes/hpf in both neonates. From the common cultural results a very low TMC emerged, resulting in 3.5×10^4 for the 1st neonate and 4.7×10^4 CFU/mL for the 2nd one. The bacterial percentage was 85%, 10% and 5% for Cu, Ef and Sp, respectively. Ec and Kp enterobacteria strains were absent in both neonates. Second and third antiinflammatory and antibiotic treatments were administered from 5th to 12th week. At the 6th week erythromycin syrup twice/die (Table II) was introduced. Milk enzymes and nourishment with glutathione was always administered. During the 4th month no therapy was administered for cultural and molecular check, carried out on the last day of month. At the 2nd control visit in COCR, the neonates underwent Schirmer's (Schirmer I°) and Break Up Test (BUT) tests. ST I° were 18 mm and 19 mm, while BUT values were 13" and 14", respectively, for the 1st and 2nd neonates; IOP was normal. This

proved a sufficient lachrymal functionality and barely sufficient stability of the lachrymal film, without adverse pharmacological effects. On the one hand, if a physiological ocular functionality had been recovered, on the other hand the ocular clinical condition presented only a glassy mucoid ocular secretion. The sampling of this check was performed by conjunctival scraping, collecting 5-7 epithelial cell/hpf. The cultural check showed a further decreasing of TMC, highlighting a Cu monoculture of 4.7×10^3 for the 1st neonate and 7.2×10^3 CFU/mL for the 2nd one, while the presence of Uu (1.0×10^3 CCU/mL) for both was observed. The Chlamydial check was negative for both of them. After this result, we continued the therapy following the same protocol, changing the ATB to weekly intervals, from 5th to 11th month, as suggested by Dukers-Muijters et al. (13). The last ATB change was performed, substituting ERY syrup with a triturated pill of 500 mg AZT. Checks for Mycoplasma and Chlamydia were carried out at the end of 12th month and again gave negative results, while only rare Cu colony were present. The sampling of this check was performed by conjunctival scraping collecting 1-5 epithelial cell/hpf. Both neonates were undergoing COCR follow-up. As she was breast-feeding, the mother would have to wait for the seminal fluid result of the husband to begin together with the chlamydialicidal and mycoplasmicidal antibiotic cycles after the nursing ended. The neonates were enrolled in a follow-up program.

DISCUSSION

In this study, in accordance with the Center for Disease Control and Prevention (CDC) guidelines, the mother followed an erythromycin- and ampicillin-based prophylactic therapy directed against *S. Agalactiae* and others genetically sensible. We could rule out the parasitical and viral aetiologies based on clinical pictures. At the same time, at first chemical-physical characteristics, the geographic area and the good health of the mother had led us to perform culture testing for *Mycoplasmas* (Mh and Uu), that resulted positive in all sources investigated. In this precocious (2 days) acute inflammatory

scenario of the ON, we hypothesized a probable terminal impairment of trophoblast function (14). We quantified the specific cell typology (Table I) optical microscopic and questioned maternal anamnesis. Therefore we introduced routinely the PCR molecular methodology from the first check to assure the correct microbial assessment into ocular ecosystem. We further analysed the three samples for Ng-DNA by PCR to exclude any possible prophylaxis failure due to a missed diagnosis according to the molecular retrospective study carried out by Krivochenitser et al. (15). The high pH values of ocular secretory suspension of 8.1 and 8.6 (reference range of 6.93 ± 0.24) contributed positively to this correct decision as the *Mycoplasmas* (Mh and Uu) make the culture medium strongly alkaline. An indirect additional confirmation of this was given by 6.8 high pH value of the maternal vaginal ecosystem (reference range of 3.8-4.5 in fertile women), in spite of an elevated level of oestrogenic hormones. For the first time, we described the *Mycoplasma* ocular presence from ocular material collecting a conjunctival secretion rich in monocytes, neutrophils, epithelial and bacterial cells. All this permitted us to correctly collect the adequate cellular material for Chlamydiaceae molecular research. Between the Chlamydiaceae, the *C. trachomatis* positivity in all ecosystems investigated, swabbing instead of scraping, demonstrated our correct view on this debatable point, as our results contrasted with the other results reported in the medical literature. With regard to this, we can assert that, when chlamydial infection produces an abundant discharge, "swabbing" is the best methodology during the first clinical appearance of an acute infection, as it occurs in the acute *Ophthalmia neonatorum*. Conversely, "scraping" is the best procedure in chronic conjunctivitis (CC) chlamydial infection (7, 12), and in different chronic inflammation (8). If not correctly diagnosed and promptly treated with adequate chlamydialicidal and mycoplasmicidal antibiotics and antiinflammatory therapies, these microorganisms could spread from the inflamed areas to other human mucous membranes and tissues, through both lachrymal-nasal-pharyngeal colonization (1) and the systemic circulation. In this

way, if genetically predisposed, a human being can become the unique container of elementary bodies (EBs) which can persist in an asymptomatic steady-state for decades before causing different clinical manifestations. Several common bacteria, varying from *S. aureus*, *S. epidermidis*, *H. influenzae*, *S. pneumoniae*, *S. viridans*, *M. catarrhalis*, to *K. pneumoniae* and other *Enterobacteria* (here reported as total common bacteria: TCB) found in ocular infection, have been implicated in acute and chronic conjunctivitis, but these old studies were conducted before the “molecular age”. Typical examples of these studies described a sudden outbreak of infective conjunctivitis, attributed to *S. pneumoniae* by a cotton swab culture, result in HLA-B₂₇ negative subject, but excluded the molecular research of Chlamydiaceae. One of these studies was carried out without chlamydial testing. It described the case of a 79-year-old woman who suffered from chronic pharyngitis and worsening symptoms (dysuria), attributed to *S. pneumoniae*. Another typical example of an “old study conception” was recently reported, in which *Chlamydia trachomatis* and *Neisseria gonorrhoea* testing were ruled out, attributing the ON clinical manifestation to *K. pneumoniae*. The justification of this exclusion was attributed “to healthy full-term infant born in the United States who has received prophylaxis”, although the antibiotic prophylaxis provides poor protection against hypothetical *C. trachomatis* conjunctivitis.

Taking this into account, it will always be possible for a leak to occur in the efficient system of prevention in the USA and in other countries if the molecular technology were omitted by similar justifications. The use of the PCR or different molecular technology was recommended by CDC for sexually transmitted diseases. To test the cure of *N. gonorrhoeae* and *C. trachomatis*, many studies are carried out excluding ON and neonatal CC. All this entails an assessment of the TCB to determine acute and chronic infective ocular diseases. On the contrary, the non-use of the molecular technologies underrates the Chlamydiaceae as true causal agents of different ocular clinical pictures and produces false diagnoses, therefore, chlamydial infection could lead to much more severe ocular diseases (10)

and/or to different pathologies already mentioned in asymptomatic and symptomatic patients belonging to the ethnic population genetically susceptible.

In conclusion, our study, despite being made up of just two cases of precocious acute conjunctivitis, aims to reassert a few unavoidable key points as shown below, in order to establish a new guideline to improve the quality of laboratory diagnosis and to assure the first correct therapy from the onset

- a) To establish the different sampling modality in acute and chronic clinical manifestation of any sudden asymptomatic infective disease on the basis of a correct anamnestic specific questionnaire elaborated and collected by an expert team in atypical infections.
- b) To standardize the cellular sampling collected by swabbing or scraping, dispersing it in a fixed volume (1 mL) of sterile saline solution to assess the TMC and the percentage of each common bacteria.
- c) To assess the physico-chemical state, carry out microscopic characterization of respective specific samplings, and choose pre-fixed culture medium to assure the major covering for TMC.
- d) To include the mycoplasmas (present as usual cervical-vaginal microbiotas), Chlamydiaceae and Ng-DNA, considering the different local geographic areas and ethnic predisposition and to assess the maternal cervical-vaginal ecosystem for the same bacteria, also in asymptomatic patients. The ecosystem modification may have a profound impact not only on the health of women but also of their newborns. Microbial programming, begun in utero, is thus a central component for the development of a balanced mucosal immune system in the newborn (16).
- f) To revise the guideline of “SAFE Strategy”, transforming itself in SAFES Strategy (17), where the “S” is the acronym of “Sexual ecosystem evaluation”.

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LETTER TO THE EDITOR

EXTRACORPOREAL SHOCKWAVE THERAPY ON MUSCLE TISSUE:
THE EFFECTS ON HEALTHY ATHLETES

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The aim of this study is to investigate the effects of extracorporeal shock wave therapy (ESWT) on muscle rheological and functional properties in a population of young athletes. Thirty-two football and basketball players were recruited and randomized into two groups. The athletes underwent three sessions of therapy administered every five days to the thigh muscles. The treatment consisted of ESWT (electromagnetic generator, Energy Flux Density=0.03 mJ/mm²) or a placebo treatment bilaterally on the quadricep and femoral bicep muscles. Monitoring was carried out at recruitment (T0), at the end of treatment (15 days, T1) and at 30 days (T2) with myometric evaluation (measuring elasticity, stiffness and muscular tone) and electromiography exam (recording the Motor Unit Amplitude Potential values). The results showed a significant increase in the treated athletes in the elasticity (lateral vastus muscle, $p=0.007$), in muscular tone (femoral rectus, $p=0.031$) and in muscular recruitment (the lateral vastus, $p<0.005$; medial vastus muscle, $p=0.055$). These results could represent a translational interpretation of the known biological effect on connective tissue: an increase in blood flow, oxygenation, metabolic process activation and proliferative effect. The effects found may represent the justification for verifying the usefulness of using of shockwave therapy to reduce muscular fatigue and improve performance during the sport season.

To the Editor,

Muscular use in sport is a continuous dynamic process characterized by repeated muscle recruitment up to the threshold fatigue (1). Exercise-

induced changes in muscle cell function adapt the tissue to the demands of increased physical work. During intense and lasting physical exercise skeletal muscle is damaged and subsequently inflamed.

Key words: shockwaves, muscle, fatigue, injury, athletes

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The inflammation itself is how the body reacts in order to repair the exercise-induced damage (2). An important factor that contributes to muscular fatigue is the reduction in the availability of oxygen needed for muscular contraction due to the fact that physical activity increases consumption of oxygen in the blood (3).

Biostimulation has been described as a process that provides beneficial effects on muscular activity during athletic activity. Massages, low intensity exercises, criotherapy alternating between cold and hot water baths, neuromuscular stimulation, stretching and lasertherapy are used to aid recovery between training sessions as well as to prevent injuries and to improve athletic performance (4).

Extracorporeal shock wave therapy (ESWT), was first developed in the early 1980s. This acoustic stimulation is used in the treatment of tendinopathy and fracture delay (5). Recently, this therapy has also been used in muscular diseases related to injuries, fibromyalgia, myositis ossificans and hypertonia amongst others (6-7). A preliminary clinical experience on a small study group verified the effects of shock waves (SW) on muscular skeletal performance in golfers and weightlifters (7). The hypothesis of the researchers regarding the improvement in performance is that the acoustic mechano-transduction of shock waves is able to influence the extra-cellular matrix via receptors and sensory substances. Taking the above in consideration, the aim of our study is to investigate the effects of SW on properties in the muscular tissue of young athletes.

MATERIALS AND METHODS

We carried out a double-blind, placebo-controlled randomized clinical trial. At baseline, subjects were randomized 1:1 to receive shockwave treatment or placebo. Treatments were allocated according to a sequential randomization list generated using Stata MP12 statistical software. We generated codes to ensure that 50% of our participants received the active treatment and 50% received the placebo treatment. From February 2017 to June 2017 we recruited 32 athletes, 5-a-side football players or basketball players. All participants provided

written informed consent and the study was approved by the Local Ethics Committee. The inclusion criteria were: male, age range from 18 to 35, who had played at a semi-professional level for at least 1 year and who trained at least once a week. The athletes continued their training program throughout the follow-up period.

The exclusion criteria were: incapable of understanding and lacking a history of musculoskeletal injury to the hips or knees in the previous 2 months; leg asymmetry > 1 cm; participation in less than 80% of the scheduled team physical training and sports sessions in the previous 3 months; signs or symptoms of any disease (i.e., myopathies, cardiologic, neurologic, inflammatory, pulmonary, metabolic and oncologic disorders); contraindications to SW treatment (epilepsy, coagulopathies, pacemaker, neoplasia, high temperature, infections); use of any nutritional supplement or pharmacologic agent; drug assumption such as GABA-ergic, benzodiazepine, anticoagulants, muscle relaxants, botulinum toxin (BTX-A) infiltrations.

Epidemiological data were collected (age, weight, height, smoking habit, sport practice). The athletes underwent a myometric evaluation and electromiography exam at T0 (before treatment), at T1 (15th day) and T2 (30 days from recruitment). The subject recruitment was conducted by the same physician who had 12 years of clinical and teaching experience in rehabilitation for musculoskeletal disorders. The identification of sites for treatment and monitoring processes (myometric evaluation and electromiography examinations) were conducted by the same physician, who had 10 years of experience in this field for patients affected with neurological disease as well as for sportsmen and women. Both physicians were blinded to the treatment administered. The randomization was conducted by another physician, who had no interaction with any physicians or patients involved in this clinical trial until the end of the study. All treatments were performed by the same physician, who had 6 years of clinical and rehabilitation experience for tendon pathologies.

Identification of evaluation and treatment site of muscle

The middle of the muscle belly of the medialis vastus, lateralis vastus, femoral biceps and femoris rectus bilaterally were identified by anatomical guide (8). Clinical measurements included direct measurement with

a tape measure: for the femoral biceps the point was at the midpoint of a line between the fibula head and the ischial tuberosity; for the medialis vastus it was four fingerbreadths proximal to the superior-medial angle of the patella; for the lateralis vastus it was over the lateral surface of the thigh, one handbreadth above the patella; for the femoris rectus it was on the anterior surface of the thigh, midway between the superior border of the patella and the anterior superior iliac spine. These points were marked with a dermograph pen and were used as landmarks for instrumental evaluations and treatment (Fig. 1).

Myometric evaluation with Myoton

The Myometer (MyotonPro®, Tallinn, Estonia) was used to examine bilaterally muscle properties of the lateralis vastus, medialis vastus, rectus femoris, and femoral biceps muscles. This device measures tone (Hz), elasticity (DecrLog) and stiffness (N/m) of muscle. The testing end of the MyotonPro® was placed perpendicular to the middle of the tested muscle belly. Measurements were carried out on T0, T1 and T2.

During the tests, the subject lay relaxed on an

examination couch. For measurement of the anterior muscles, the subject lay supine with the knees slightly flexed and supported by a cushion underneath. For the posterior muscles, a prone position was adopted. Three consecutive measurements (with a time interval of 1 s between each) were made at each site, and the average value was used for further analysis. On the following days, the measurements were repeated exactly as on day 1 and the sites were identified according to the initial landmark. Measurements were made during short (3–5 s) submaximal contractions at 80% of the previously determined maximum voluntary contraction (MVC) (with 1–2 min rest between contractions). The MVC and the submaximal forces were registered by a spring balance attached to a strap around the ankle (above the lateral malleolus) and running parallel to the ground. Feedback regarding the force level was given by the investigator. Initially the investigator invited the subject to perform the most vigorous muscle contraction possible and which he could perform only once against the resistance of his hand and this corresponds to the maximum voluntary contraction. After a period of pause, the investigator invited to affect the new sub-maximal contractions and

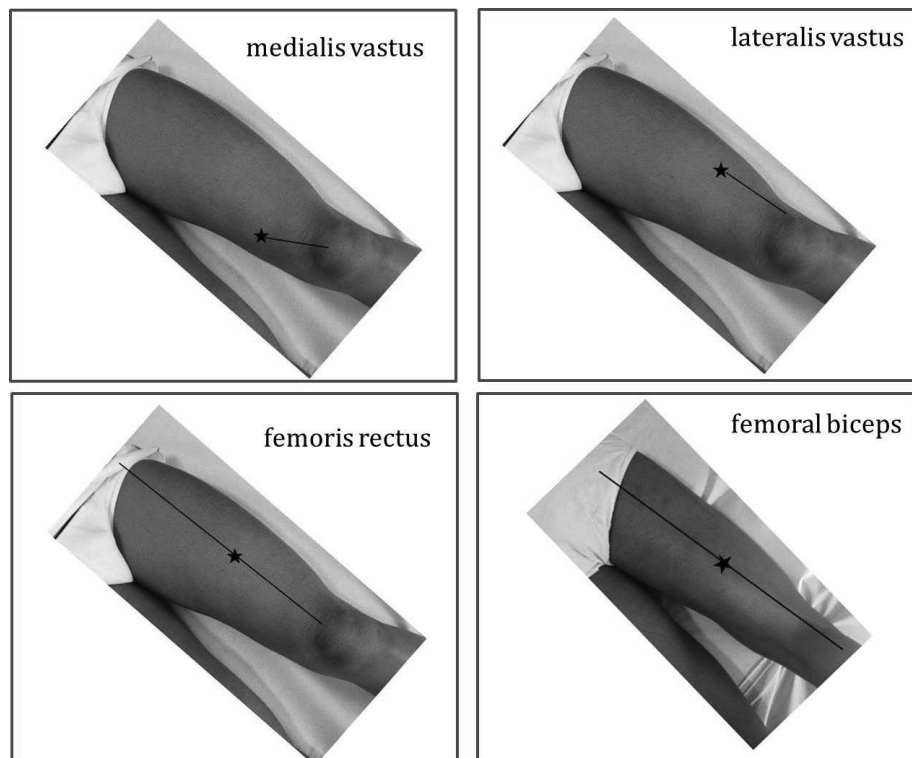


Fig. 1. The image demonstrates the detection of the instrumental monitoring point and treatment for each muscle in the study.

this time should have greater ease in resisting. Reliability of this measurement was confirmed in literature (9).

Surface electromyography (sEMG) and EMG electrode placements

For monitoring the muscle activity, EMG activity was measured using the surface EMG methods (10). The myoelectric signals were acquired by surface EMG system (Nicolet Viasys Viking, Natus Medical Incorporated, San Carlos, CA, USA) at the sampling frequency of 1024 Hz, band width of 20 to 500 Hz and common-mode rejection ratio greater than 80 dB. Bipolar EMG electrodes were placed with 1 cm inter-electrode distance. Placement of EMG electrodes for the 8 selected muscles were in accordance with the recommendation reported previously. We analyzed amplitude of Motor Unit Amplitude Potential (MUAP) (microV).

Participants initially performed voluntary contraction, crunch and reverse crunch in crook-lying position against manual resistance to their maximum effort for 3 repetitions respectively, and EMG data at 80% maximal voluntary isometric contraction (MVIC) were collected. Each repetition lasted for 5 s with a 10-s rest interval. For exercise trials under the natural core activation (NCA) condition, participants were instructed to perform the hip exercises naturally without any instruction or correction specified by the examiner.

Extracorporeal shock wave treatment

The ESWT consisted of 3 sessions, with an average frequency of 5 days, delivering 500 impulses at EDF = 0.03 mJ/mm² for each muscular belly of the medialis vastus, lateralis vastus, rectus femoris and femoral biceps, bilaterally to the lower limbs. The choice was based on our previous experiences in the treatment of muscle diseases (myositis, painful muscle in epicondylitis) in agreement with biological rationale to induce a vascularizing effect (11). The treatment point corresponded to the same part used for the EMG and Myoton evaluations. We applied the ESWT in direct contact with the skin at 4 sites on each limb. An ESWT generator with ultrasonographic probe (Storz, MiniLith, Switzerland) was used for the SW treatment. The ultrasonographic gel was inserted between the skin plane and the probe. The subject was in a prone position for the treatment of the femoral biceps and in a supine position for the remaining three muscles. The same

procedures were performed to administer the placebo but without effective irradiation, since the probe was not in direct contact with the skin.

Data collection and statistical evaluation

For each athlete, using a standardized form, the epidemiological parameters (age, weight, height, smoking habit, sporting activity and role, number of sport session per week) were collected. Furthermore, tone values, elasticity and stiffness values were evaluated with Myoton and the electromyographic evaluation of the medialis vastus, lateralis vastus, femoris rectus and femoral biceps were registered bilaterally. Compiled forms were put into a database built with Office Excel software and analyzed using Office Excel and Stata MP12 software. Continuous variables were expressed as mean, standard deviation and range, categorical variables as proportions. The normality of the continuous variables was evaluated and, for those not normally distributed, a standardization model was constructed. To compare the continuous variables, ANOVA test for repeat measurements (parametric), Wilcoxon test (non-parametric) and Friedman test (non-parametric) were used. Fisher test was used to compare the proportions. For all tests used, a value of $p < 0.05$ was considered significant.

RESULTS

The study sample consisted of 32 subjects, 16 (50%) of whom belonged to the treated group and 16 (50%) to the placebo group. The average age of the sample was 24.5 ± 4.1 years (range: 18-32 years); statistically significant differences emerged between the two groups ($t=3.1$; $p=0.005$). The average weight of the sample was 79.9 ± 12.1 kg (range: 66–115 kg), the average height was 180 ± 8.7 cm (range: 166-199 cm) and the average BMI 24.5 ± 2.8 kg/m² (range: 20.1-33.6 kg/m²), without any statistically significant differences between the two groups ($t=1.2$; $p=0.246$). There were 6 smokers in all, and no statistically significant differences were found between the treated group and placebo group ($\chi^2=0.1$; $p=1,000$). Five-a-side soccer was played by 16 subjects (50%) and the remaining 16 (50%) played basketball; no statistically significant difference emerged between the two groups ($\chi^2=0.9$,

Table I. Mean and standard deviation of the three parameters [Tone (Hz), Elasticity (DecrLog) and Stiffness (N/m)] of the muscular belly evaluated by the Myoton, group and time of valuation.

Parameter	Treated group			Placebo group		
	T0	T1	T2	T0	T1	T2
Right medialis vastus						
Tone (Hz)	17.7±3.0	16.5±2.5	17.1±4.2	19.0±4.1	18.9±3.8	18.3±3.4
Elasticity (DecrLog)	1.1±0.1	1.1±0.2	1.1±0.2	1.0±0.2	0.9±0.2	1.0±0.2
Stiffness (N/m)	434.6±134.9	378.9±134.3	390.3±161.8	505.7±174.0	502.9±162.1	461.5±167.6
Left medialis vastus						
Tone (Hz)	17.8±2.5	17.9±3.8	18.6±4.8	18.2±3.2	18.0±3.2	18.0±3.5
Elasticity (DecrLog)	1.2±0.2	1.2±0.2	1.1±0.3	1.2±0.2	1.1±0.2	1.1±0.2
Stiffness (N/m)	428.4±103.8	440.6±163.1	447.3±175.3	438.1±121.1	433.2±122.8	429.3±120.0
Right lateralis vastus						
Tone (Hz)	22.0±4.0	21.5±3.2	22.1±4.3	21.6±4.3	21.2±4.3	21.1±4.2
Elasticity (DecrLog)	0.9±0.2	1.0±0.2	0.9±0.2	0.8±0.2	0.8±0.1	0.8±0.2
Stiffness (N/m)	573.5±156.0	513.9±116.0	565.9±170.7	526.2±149.0	534.4±167.6	520.0±165.3
Left lateralis vastus						
Tone (Hz)	21.8±3.1	20.9±3.7	21.6±3.1	21.9±3.5	21.7±3.6	21.7±3.8
Elasticity (DecrLog)	1.0±0.2	1.0±0.2	0.9±0.2	0.8±0.1	0.8±0.1	0.8±0.1
Stiffness (N/m)	562.4±129.0	501.6±129.7	529.0±97.8	556.6±135.0	546.3±138.1	553.4±146.2 ₂
Right femoris rectus						
Tone (Hz)	17.7±2.8	18.2±2.9	18.1±2.3	17.6±2.5	18.1±2.5	17.9±3.1
Elasticity (DecrLog)	1.2±0.2	1.2±0.2	1.1±0.1	1.2±0.3	1.2±0.3	1.2±0.2
Stiffness (N/m)						

Tone (Hz)	17.1±2.9	17.1±2.8	18.5±3.3	19.2±4.3	18.1±4.2	18.2±4.4
Elasticity (DecrLog)	1.2±0.2	1.2±0.2	1.2±0.2	1.2±0.2	1.2±0.2	1.1±0.2
Stiffness (N/m)	333.8±93.1	332.3±105.2	375.7±99.4	373.6±140.0	374.8±156.0	360.4±145.3
Right femoral biceps						
Tone (Hz)	18.7±2.4	17.7±2.4	20.0±3.1	20.0±2.7	20.1±2.9	20.0±2.7
Elasticity (DecrLog)	1.2±0.2	1.2±0.2	1.2±0.2	1.2±0.1	1.2±0.1	1.2±0.2
Stiffness (N/m)	434.5±114.0	381.9±98.0	445.5±107.5	428.6±109.7	430.9±115.4	427.5±106.4
Left femoral biceps						
Tone (Hz)	17.7±2.7	19.3±3.7	19.5±3.2	20.2±2.5	20.2±2.4	20.1±2.4
Elasticity (DecrLog)	1.2±0.2	1.3±0.2	1.2±0.2	1.2±0.1	1.2±0.1	1.1±0.1
Stiffness (N/m)	368.6±105.0	418.5±121.3	438.3±126.1	425.0± 78.0	424.6±84.0	425.8±82.4

$p=0.425$). On average, the athletes trained 3.3 ± 1.0 days/week (range: 1 to 5); no statistically significant differences were found between the groups ($t=1.2$; $p=0.246$). The distribution of the sample with regards to sport practiced and role, showed that the greatest proportion of football players were defenders (43.8%) and the greater proportion of basketball players were playmakers (40.6%). Table I shows the mean and standard deviation of the three parameters (tone, elasticity and stiffness) of the muscular belly evaluated by the Myoton, group and time of detection. Comparing with the ANOVA test the variables described in Table I, statistically significant differences were shown with regard to elasticity of the right medialis vastus ($F = 4.7$; $p = 0.04$) and left lateralis vastus ($F = 8.9$; $p = 0.007$). There was also a statistically significant difference between the times for stiffness of right medialis vastus ($F = 4.2$; $p = 0.022$) and tone of right lateralis

vastus ($F = 59.4$; $p = 0.000$). Finally, a statistically significant difference was found between the groups and times for the variables of left femoris rectus tone ($F = 3.8$; $p = 0.031$). Table II shows the mean and standard deviation of electromyographic values of muscular bellies. There was a statistically significant difference between the groups for the variables related to muscular recruitment of right medialis vastus (to the limits of statistical significance; $F = 4.1$; $p = 0.055$), right lateralis vastus ($F = 5.0$; $p = 0.035$) and left lateralis vastus ($F = 9.7$; $p = 0.005$). Comparing the times, we report a statistically significant difference for the muscular recruitment value variables of right medialis vastus ($F = 4.2$; $p = 0.022$), left lateralis vastus ($F = 3.7$; $p = 0.031$) and right femoral rectus (to the limits of statistical significance; $F = 34.9$; $p = 0.053$). No statistically significant difference was found in regard to the relationship between group and time ($p>0.05$).

DISCUSSION

To our knowledge, this study is the first to analyze the acute effects of ESWT on muscle performance in healthy athletes. Briefly, we observed that SW stimulation on muscular bellies determines a significant increase in elasticity (left lateralis vastus) and muscular tone (left femoris rectus). The EMG results show an increase in muscular recruitment of the left and right lateralis vastus and the right medialis vastus.

The hypothesis regarding tone and elasticity improvements described in our study is related to a good blood supply which may increase oxygen supply, energy substrates and decrease carbon dioxide

and catabolites. Therefore, metabolic processes increase, avoiding overload, pain and limitation of contractile strength. Moreover, metabolic/trophic stimulation on tissues may induce a proliferative effect on connective and protein components. Muscle tissue repair processes include growth factors such as pro-fibrotic transforming growth factor (TGF)- β 1 and stromal cells. Physiological tissue repair aims at restoring the mechano-protective properties of the extracellular matrix (12). Matrix straining and stiffening lower the threshold for TGF- β 1 activation by increasing the mechanical resistance to cell pulling (13).

Mechanical stimulations, locally applied to the muscle, trigger the activation of muscle spindle

Table II. Mean and standard deviation of electromyographic values of MUAP (microV) of muscular bellies for group and time of detection.

Muscular belly	Treated group			Placebo group		
	T0	T1	T2	T0	T1	T2
Right medialis vastus	440.9 $\pm$ 208.7	549.0 $\pm$ 262.1	487.4 $\pm$ 144.3	338.9 $\pm$ 94.8	435.3 $\pm$ 304.2	352.6 $\pm$ 88.0
Left medialis vastus	423.4 $\pm$ 177.4	528.4 $\pm$ 297.8	390.9 $\pm$ 161.2	338.7 $\pm$ 84.8	367.2 $\pm$ 135.3	373.1 $\pm$ 130.3
Right lateralis vastus	434.1 $\pm$ 152.2	559.3 $\pm$ 204.4	503.5 $\pm$ 199.2	396.4 $\pm$ 146.6	369.1 $\pm$ 110.2	351.2 $\pm$ 109.7
Left lateralis vastus	477.2 $\pm$ 284.2	587.4 $\pm$ 181.1	635.6 $\pm$ 285.4	353.9 $\pm$ 92.2	350.8 $\pm$ 105.8	386.9 $\pm$ 163.2
Right femoris rectus	471.6 $\pm$ 191.6	621.3 $\pm$ 371.2	443.0 $\pm$ 117.4	475.8 $\pm$ 138.6	544.1 $\pm$ 195.2	466.4 $\pm$ 88.8
Left femoris rectus	442.5 $\pm$ 221.5	605.8 $\pm$ 275.9	498.9 $\pm$ 174.7	485.7 $\pm$ 211.4	463.5 $\pm$ 120.9	467.3 $\pm$ 158.2
Right femoral biceps	523.3 $\pm$ 206.7	552.0 $\pm$ 174.3	508.7 $\pm$ 294.4	505.1 $\pm$ 152.3	552.7 $\pm$ 163.9	519.7 $\pm$ 139.3
Left femoral biceps	578.4 $\pm$ 342.0	601.4 $\pm$ 263.5	515.5 $\pm$ 352.3	532.7 $\pm$ 211.8	536.6 $\pm$ 196.3	552.8 $\pm$ 197.9

receptors at the level of the directly stressed muscle tendon complex, the “vibration tonic reflection”. This particular mechanical behaviour induces facilitation in the excitability of spinal reflex (14). The perimysium seems capable of a response to mechano-stimulation with a myofibroblast facilitated active tissue contraction, thereby adapting passive muscle stiffness to increased tensional demands (15). In the muscle, this may be related to the increased oxygen demand and vascularization of the muscle itself, since the perimysium in muscular fasciae contains the highest proliferation of blood vessels and the increased thickness of the perimysium may provide a useful cushioning effect during muscular contraction. On the other hand, the increase in perimysial thickness has also been shown to correlate with a higher passive stiffness (16).

In conclusion, the result of the study allows to exclude the hypothesis that SW therapy on muscles may compromise sports activity. Indeed, we may hypothesize that a correct treatment program in the sports season, may reduce muscle overload and so enhance performance.

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