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EDITORIAL

USE, ABUSE AND MISUSE OF BIOMARKERS IN PAEDIATRICS

E. VENTURINI, C. TERSIGNI, E. CHIAPPINI, L. GALLI and M. DE MARTINO

*Department of Health Sciences, University of Florence,
Anna Meyer Children's University Hospital, Florence, Italy*

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Currently, a gold standard for distinguishing between infectious, inflammatory, auto-immune diseases and malignancy in infants and children is not available. The combination of biomarkers with clinical features and other diagnostic tests could help clinicians in the diagnostic process. Ideally, a biomarker should have high sensitivity, specificity, and predictive value, as well as being easily obtained also in preterm babies and infants, requiring a small amount of blood and being quickly measured. The available literature agrees on the fact that a “perfect” biomarker is not currently available in paediatric practice. Thus, clinicians must consider time by time the balance between marker characteristics and their sensitivity and specificity in different conditions. The development of new tests with higher sensitivity and specificity in distinguishing different pathological situations is auspicious. Moreover, future efforts should be focused on validating also in children the recently developed biomarkers including CD64, IL-27 and IL-8.

Many biomarkers are currently used for detecting different pathological conditions in paediatric practice. However, no single one can be considered as a gold standard for distinguishing between infectious, inflammatory, auto-immune diseases and malignancy. A biomarker is defined as “an indicator of normal biological processes, pathological processes, or pharmacological responses to therapeutic intervention that can be objectively measured” (1). Ideally, a biomarker should have high sensitivity, specificity, predictive value and must be easily obtained and quickly measured, also in preterm and infants (requiring small blood samples) (2). Currently, a biomarker with all these characteristics is not available. Therefore, the combination of these markers with the clinical features and other diagnostic tests is usually indicated (3). An increasing

interest has been shown on the development of novel biomarkers. However, the process needed to validate these biomarkers for clinical use requires complex steps (4).

The most studied biomarkers in the diagnostic processes are: procalcitonin (PCT), erythrocyte sedimentation rate (ESR), C reactive protein (CRP), serum amyloid A (SAA), interleukin-6 (IL-6) and alpha-2-macroglobulin (α 2-M) and white cell count (WCC). The main characteristics of the most used biomarkers are reported in Tables I and II.

MATERIALS AND METHODS

A literature search covering English language articles published between 1st January 2010 and 30th July 2015 and concerning children only was conducted using multiple

Key words: biomarkers, children, procalcitonin, C-reactive protein, erythrocyte sedimentation rate

Mailing address:

Prof. Maurizio de Martino, MD
Department of Health Sciences, University of Florence,
Anna Meyer Children's University Hospital,
Viale Pieraccini 24, I-50139, Florence, Italy
Tel.: +39 055 5662494 - Fax: +39 055 4221012
e-mail: maurizio.demartino@unifi.it

Table I. *Biomarker characteristics (2).*

Biomarker	Production site	Increase level time (after infection / inflammation stimuli)	Half-life	Normal value
Procalcitonin	Tiroid (C-cells) / neuroendocrine cells *	3-4 hours	22-35 hours	0.5 µg/L
Erythrocyte sedimentation rate	n/a	24-48 hours	Days or weeks (depending on half-lives of Ig subclasses or fibrinogen)	0-1 month: 0-2 mm/hours 1 month -12 years: ≤10-20 mm/hours >12 y: - male ≤15 mm/hours -female ≤20 mm/hours
C reactive protein	Liver	4-6 hours	19 hours	< 10 mg/L
Serum amyloid A	Liver (and also other tissues: kidney, lung, spleen, heart)	4-6 hours	35 +/- 28 hours	< 10 mg/dL
Interleukin-6	Neutrophils, monocytes-macrophages, fibroblasts, endothelial cells, smooth muscle cells, T-lymphocytes	< 1 hour	6 hours	1 pg/mL
White cells count	Bone marrow	6-24 hours	Circulating neutrophils: 6-8 hours	Neonates: 9,000-30,000 cells/microL Children: 4,000-11,000 cells/microL

**not univocally defined in pathological processes*

Table II. False-positive and false-negative of procalcitonin, erythrocyte sedimentation rate, C-reactive protein and white cell count (5-9)*.

	False-positive	False-negative
Procalcitonin	- Medullary carcinoma of the thyroid - Small-cells lung cancer - Renal failure	- Severe acute malnutrition
Erythrocyte sedimentation rate	- Anaemia - Intravenous immunoglobulin (IVIG) therapy - Macrocytosis - Elevated fibrinogen - Decreased albumin	- Elevated albumin - Decreased fibrinogen - Hyperviscosity - Microcytosis - Polycythaemia - Sickle cell disease - Haemolytic anaemia - Drugs (i.e. steroids, high dose salicylates, valproic acid, non steroidal anti-inflammatory drugs)
C reactive protein	- Obesity - Burns - Trauma (surgery, fractures)	- Severe acute malnutrition - Hepatic failure
White cell count	- Trauma (surgery, fractures) - Seizures - Drugs (i.e. steroids, beta agonist, lithium) - Splenectomy	- Bone marrow aplasia - Viral infections - Chemotherapy - Anticonvulsant and immunosuppressive medications

*not applicable for serum amyloid A and interleukin-6

keywords and standardized terminology in the Pubmed database. Limits were field [Title/Abstract] and language [English]. All relevant articles were then evaluated, and pertinent articles were included in this review. All reference lists were also reviewed to identify other relevant publications.

RESULTS

Pathophysiology

Procalcitonin. Physiologically, PCT level is undetectable in plasma because it is totally converted into calcitonin within the thyroid C-cells (6). On

the contrary, its level quickly increases in case of inflammation, becoming detectable in plasma within 3-4 hours. PCT site of production during inflammation is not clearly defined and the mechanisms involved in this process are poorly understood. Studies on animal models have shown that calcitonin mRNA is expressed during sepsis in many extra-thyroid tissues which lack secretory granules, causing an unregulated secretion of unprocessed PCT (10, 11). Recent findings showed that two different pathways might be involved in increasing PCT levels. The first, direct pathway, is mediated by the lipopolysaccharide or other microbial toxins in case of infection. The second one leads indirectly to an increased PCT level through inflammatory cytokines, such as tumour necrosis factor- α (TNF- α), interleukin (IL)-1, IL-2 and IL-6 (5). It was hypothesized that cytokines block the proteolysis of PCT to calcitonin in the endoplasmic reticulum (6). On the contrary, in case of viral infection, PCT production seems to be inhibited by interferon- γ release (5).

Erythrocyte sedimentation rate. ESR measures the distance through red blood cell sediment in a period of one hour in a vertical tube with anticoagulant (8). The determination of ESR should be carried out at room temperature on a fresh blood sample obtained no more than 2 hours previously (9). Its level is strictly related to fibrinogen levels and can be influenced by IgG levels, red cell morphology and concentration. Therefore, the presence of large asymmetric proteins positively charged, such as fibrinogen and IgG, causes the aggregation of negatively charged erythrocytes (8). These aggregates fall faster, increasing ESR levels. Moreover, ESR increases 24-48 hours after the onset of inflammation and remains high for days-weeks being influenced by the long half-life of IgG and fibrinogen (7-21 days and 100 hours, respectively) (9). Other factors (i.e. age, sex, race, body weight and medications) might modify ESR levels (9).

C reactive protein. CRP is produced by the liver in response to IL-1 β , IL-6 and TNF- α , enhancing the expression of adhesion molecule in the inflammation site (12). CRP increases 4-8 hours after the beginning of inflammation, doubling every 8 hours with a peak after 36-50 hours, and rapidly decreases at resolution

of the inflammatory process (8). The determination of CRP can be carried out on stored serum or plasma (9).

Serum amyloid A. SAA is an apolipoprotein produced by the liver, following the release of pro-inflammatory cytokines (IL-6, IL-1, TNF- α). SAA serum normal level is 3 mg/L. SAA quickly increases during pathological processes with a peak level after 9 hours from the start of inflammation. SAA is the serum precursor of amyloid A protein, the main responsible for amyloidosis deposit in chronic inflammatory disorders. However, its role in the inflammatory process is still not completely understood (12).

Interleukin-6. IL-6 is an inflammatory cytokine secreted during inflammatory states by neutrophils, monocytes, macrophages, fibroblasts, endothelial cells, smooth muscle cells and T-cells. The concentration of this cytokine in blood of healthy individuals is about 1 pg/mL but rapidly increases during infections or autoimmune disorders (13).

α 2-macroglobulin. α 2-M is a large glycoprotein member of α -macroglobulin superfamily with anti-protease activities. α 2-M is increased in patients with sepsis and inflammatory disorders (14). The primary function of α 2-M is the rapid inhibition of excess proteinases released during tissue injury, such as those liberated by neutrophils at the site of inflammation or those secreted by pathogens (15).

White cell count. Circulating leukocytes are produced by the bone marrow in response to specific growth factors and their normal levels vary with age. Leucocytosis is defined in case of a level above the 95% confidence interval of their median value (i.e. >13,000-15,000 for children between 4 and 16 years and >20,000 for neonates). An increased level of leukocytes with a neutrophil predominance can be related to bacterial and fungal infections but also to non-infectious causes such as tissue damage (trauma, hypoxia, surgery, burns), and inflammatory and autoimmune disorders (1-4, 16).

Biomarker modification in pathological conditions

Infections. In a meta-analysis performed on 1,883 children (aged <36 months) with fever without source, PCT showed a sensitivity of 0.83 (95%CI: 0.70-0.91) in diagnosing serious bacterial infection (16). Its

sensitivity resulted higher compared to CRP and WCC numbers, which were of 0.74 (95%CI: 0.65-0.82) and 0.58 (95%CI: 0.49-0.67), respectively (16). Therefore, the authors suggested that PCT is particularly helpful in ruling out severe infections, better than CRP and WCC numbers (negative likelihood ratio of 0.25, 95%CI: 0.15-0.40) (20). However, authors suggested to combine different markers with clinical and other diagnostic tests (17, 18).

The use of SAA in distinguishing infection/inflammation was assessed on 83 Brazilian children (19). There was no significant difference between the two groups. However, when the infectious group was subdivided into bacterial and viral infections, a significantly higher SAA level was seen in the first group (kappa concordance index of 0.73) (19).

Studies on children showed an increased level of IL-6 which correlates with the severity of septic shock (2, 20, 21).

Pneumonia. PCT has been used to identify pneumonia complicated with pleural effusion. A prospective study on 128 children with community acquired pneumonia (CAP), showed a median PCT level of 1.68 ng/mL (interquartile range [IQR]: 1.19-6.56) in complicated CAP compared to 0.69 ng/mL (IQR: 0.12-2.53) in uncomplicated ones ($p=0.021$) (22). A possible explanation could be a higher release of inflammatory cytokines in case of pleural inflammation (22). Interestingly, PCT has been recently used to guide antibiotic treatment of CAP (23-25). In an Italian randomised prospective study on 319 hospitalised children with uncomplicated CAP, the use of PCT as a guide for treatment, with a cut-off value of 0.25 ng/mL, led to a 15% reduction in antibiotic prescribing (23). On the contrary, a randomised controlled trial on 337 Swiss children showed that the use of PCT in driving therapy choices did not modify the antimicrobial prescription rate but reduced the treatment length (25). Authors hypothesised that this difference in the two studies could be mainly related to a low antibiotic prescription rate among Swiss paediatricians as well as to a more severe infection in the Swiss cohort during the study period (28).

Pyelonephritis. A 2015 Cochrane review analysed the use of PCT, CRP and ESR in the diagnosis of

acute pyelonephritis in children (26). ESR was found to be inaccurate in confirming the diagnosis of upper urinary tract infection (sensitivity: 0.87, 95%CI: 0.77-0.93; specificity: 0.48, 95%CI: 0.33-0.64). A low CRP level (<20 mg/L) was consistent with a lower risk of pyelonephritis. However, PCT seemed to be more specific compared to the other markers in ruling out urinary tract infection (specificity: 0.74, 95%CI: 0.55-0.87) (26). Moreover, PCT level was related with the severity of renal involvement (27).

Meningitis. Few recent studies assessed the use of PCT, CRP and WBC in the diagnosis of bacterial meningitis (28-31). The 2010 National Institute for Health and Care Excellence (NICE) guidelines recommend the use of CRP and WBC for the diagnosis of bacterial meningitis, whereas PCT was not indicated (32). On the contrary, the more recent NICE guidelines on fever in children between 3 months and 5 years of age suggest that PCT has a significantly better diagnostic performance than CRP or WBC in identifying sepsis, septic shock and meningitis. PCT is also excellent in discriminating between viral or bacterial and localised or invasive infections (31). In the same way, Scottish guidelines suggest the use of PCT in identifying children with invasive bacterial diseases (33). A meta-analysis carried out on 881 children with fever without source in developed countries found a sensitivity and specificity of PCT in diagnosing meningococcal meningitis of 0.89 (95%CI: 0.76-0.95) and 0.74 (95%CI: 0.40-0.92), respectively. Moreover, CRP and WBC showed a sensitivity of 0.74 and 0.50 and a specificity of 0.54 and 0.68, respectively (28). The authors concluded that PCT is currently the best predictor of bacterial meningitis, but, considering its sub-optimal sensitivity and specificity, it cannot be used alone (28-30). It is important to remember that an early determination of PCT can result falsely negative. Therefore, multiple determinations are required if the clinical suspicion subsists (30).

Autoimmune/autoinflammatory diseases. The acute phase reactants, like ESR and CRP, usually increase in rheumatologic disorders, being useless in differentiating between disease flares and infections (34, 12).

However, it is well known that ESR levels are

closely related to the activity of systemic lupus erythematosus, and an increased level of ESR and/or CRP is a minor diagnostic criteria for rheumatic fever (35). On the contrary, PCT levels are usually not related with rheumatologic disease activity, and their rise should be considered as a marker of an infectious process (34, 36-38). Even though, PCT level above 0.5 ng/mL was found in Kawasaki disease (KD) (39-41). In particular, PCT seems to be related with the severity of KD (39-41), being higher in case of complete KD and in case of coronary aneurysms (39, 40). Also ESR is elevated in KD and its level is protractedly high in patients treated with intravenous immunoglobulin for KD, because ESR levels are strictly related with IgG levels (9). Therefore, ESR should not be used for monitoring treatment outcome (9).

CRP and PCT levels were evaluated in 89 children with Henoch-Schönlein purpura (42). CRP resulted higher in these patients, whereas PCT was below the upper limit (median=0.08, range 0.05-1.02 ng/mL) (42).

SAA is increased during juvenile idiopathic arthritis and its level is related to the disease flare and to the number of active joints (43). Moreover, a higher SAA level in adult patients with Behçet disease seems to be related to the occurrence of complications (oral aphthosis, neurological impairment, ocular disease) (44). It must be considered that SAA level is influenced by several conditions (i.e. medications, body mass index, neurological disorders and diabetes mellitus) (45).

Two studies performed respectively on 22 and 105 children with periodic fever, aphthous stomatitis, pharyngitis, and cervical adenitis (PFAPA) showed a slow increase of ESR and CRP with a peak between the second and the fourth days of fever (46, 47). On the contrary, PCT did not increase during PFAPA recrudescence (47). Similarly, PCT level seems to remain within the normal limits (<0.5 ng/mL) during familial Mediterranean fever (FMF) attacks, being useful in distinguishing infections that can mimic FMF (48).

Cancer. The accuracy of PCT for diagnosing bacterial sepsis in oncological paediatric patients with febrile neutropenia was shown to be comparable to CRP (area under the curve: 0.75 versus 0.74) (49).

Chemotherapy and steroids seem to not modify PCT values (50). Moreover, ESR can be higher in lymphoma, leukaemia and solid tumours. In a study on 99 children (>10 years) with ESR ≥ 100 mm/h, 12.1% of the study subjects were diagnosed with malignancy (51).

Novel markers

Few novel markers, such as triggering receptor expressed on myeloid cells-1 (TREM-1), neutrophil cluster of differentiation-64 (CD64), soluble urokinase-type plasminogen activator receptor (suPAR), presepsin, cell-free DNA (cfDNA) and microRNAs, are under study for driving the diagnosis of infection (52).

CD64 is the high affinity receptor of Fc fragment of IgG. It is constitutively found on macrophages and monocytes. CD64 seems to be the most interesting marker which is over-expressed by polymorphonuclear (PMN) cells during the infection process (2). CD64 quickly increases after LPS stimuli, reaching a plateau level within 2 hours and returning to its basal level a few days after infection resolution. CD64 levels can be easily determined by flow cytometry from a blood sample (53). A recent meta-analysis, including overall 1,986 adult patients, evaluated CD64 expression as a marker of bacterial infection, showing a pooled sensitivity of 0.76 [95 %CI: 0.73-0.78] and specificity of 0.85 [95 %CI: 0.82-0.87] (54). The positive likelihood ratio and negative likelihood ratio were, respectively, 8.15 [95 %CI: 3.82-17.36] and 0.16 [95 %CI: 0.09-0.30] (11). This marker showed also a high sensitivity in early suspected sepsis in children and, interestingly, in preterm neonates (55-57). Despite that, CD64 seems to be increased also in inflammatory conditions such as Crohn's disease and PFAPA syndrome, correlating well with flare up phases (58, 59). The widespread use of genomics and proteomics led to the identification of other potential biomarkers of infection, such as IL-27 and IL-8. Serum concentration of IL-27 was compared to PCT in 101 children with systemic inflammatory response syndrome (SIRS) and in 130 with sepsis. At a cut-point value of ≥ 5 ng/mL, serum IL-27 predicted infection with a specificity and a positive predictive

value of more than 90%, performing better than PCT (60). A recent meta-analysis on 548 newborns, found for IL-8 a pooled sensitivity and specificity of 0.78 and 0.84, respectively, with a moderate accuracy (area under the curve (AUC)=0.8908) in neonatal sepsis (61).

DISCUSSION

The acute phase reactants could be precious diagnostic elements for physicians. Clinicians deserve a guide role in requesting investigations and should be able to choose in which situation it is appropriate to perform each test. The balance between marker characteristics (half-life, false positive, false negative) and their sensitivity in different conditions should be taken into account in the diagnostic process. Currently, the best markers available for detecting infectious diseases are PCT and CRP. Recently, these tests have been also used to guide antibiotic treatment with the aim of reducing bacterial resistances and costs (23, 25).

However, the available literature agrees on the fact that a “perfect” biomarker is currently not available (3, 17, 18, 28-30). Therefore, the combination of these markers, in conjunction with clinical examination and other diagnostic tests, is indicated (3). Future research should be addressed to the development of new tests with higher sensitivity and specificity in distinguishing the different pathological situations. Moreover, novel studies should validate, also in children, the use of the recently developed biomarkers (CD64, IL-27 and IL-8) (58-61). Finally, it is important to keep in mind that the physician’s decision should be guided by the clinical picture and not the biomarkers levels.

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EDITORIAL

IS VITAMIN E AN ANTI-ALLERGIC COMPOUND?

Al. CARAFFA¹, G. VARVARA², E. SPINAS³, S.K. KRITAS⁴, G. LESSIANI⁵, G. RONCONI⁶,
A. SAGGINI⁷, P. ANTINOLFI⁸, I. FRYDAS⁹, M.C. DE TOMMASO MORRISON¹⁰,
J. PIZZICANNELLA², E. TONIATO² and P. CONTI¹¹

¹University of Perugia, Perugia, Italy; ²Department of Dentistry, University of Chieti, Chieti, Italy; ³Department of Surgery and Odontostomatological Sciences, University of Cagliari, Italy;

⁴Department of Microbiology and Infectious Diseases, School of Veterinary Medicine, Aristotle University of Thessaloniki, Macedonia, Greece; ⁵Vascular Medicine Unit, Villa Serena Hospital, Città Sant'Angelo, Italy; ⁶Clinica dei Pazienti del Territorio, Policlinico Gemelli, Rome, Italy; ⁷Department of Dermatology, University of Rome Tor Vergata, Rome, Italy; ⁸University Hospital, Perugia, Italy; ⁹Aristotelian University, Thessaloniki, Greece; ¹⁰Salve Regina University, Newport USA;

¹¹Immunology Division, Medical School, University of Chieti-Pescara, Chieti, Italy

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Vitamin E is found in eight forms in nature which include four tocopherols (alpha, beta, gamma and delta) and four tocotrienols (alpha, beta, gamma and delta). The classic effect of vitamin E is to reduce and prevent oxygen damage to the tissue and is useful for the treatment of pain, inflammation and allergic reactions. In addition to antioxidant activity, vitamin E also has a number of different and related functions. It protects against cancer, improves immune response, lowers the incidence of infectious diseases, cardiovascular diseases and is protective in allergy and asthma risk, and other disorders. Vitamin E increases n-6 polyunsaturated fatty acid (PUFA) and decreases n-3 PUFA, an effect that diminishes asthma and allergic diseases. Moreover, vitamin E regulates vascular cell adhesion molecule-1 (VCAM-1)-dependent leukocyte migration through its oxidant and non-antioxidant effect. Furthermore, vitamin E modulates the endothelial function by altering VCAM-1-induced oxidative activation of endothelial cell PKC α . However, vitamin E is not consistently associated with asthma and/or allergy, and in some cases there are conflicting results on allergy and inflammatory diseases. The association of vitamin E and allergy appears to be very complex, and further study needs to clarify this dilemma.

In this report, we studied and provide an overview of the relationship between vitamin E and Type I allergies, asthma and inflammatory state. The study was based on our 30 years of laboratory experience. In addition, a Pubmed search was conducted to review the effect of vitamin E in allergy and inflammation.

It is well known that to have a well-functioning immune status a correct nutritional status is necessary

(1). Vitamin deficiencies are widespread, and are a major global health problem worldwide. The World Health Organization estimates that more than 2 billion people are deficient in the most important vitamins, with a major burden in developing countries. Deficiency of vitamins is a risk factor for increased incidence of severity in infectious illness, cancer, autoimmune diseases and inflammation,

Key words: vitamin E, inflammation, allergy, asthma, antioxidant

Mailing address:

Dr Pio Conti,
Affiliated Professor, Molecular Immunopharmacology and Drug
Discovery Laboratory, Tufts University, Boston, MA 02111, USA;
Professor of Immunology, Post Graduate School of Medicine,
University of Chieti-Pescara, Viale Unità d'Italia, 73 66100 Chieti, Italy
e-mail: pconti@unich.it

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including allergies and asthma (2). There is no doubt that vitamin E fortification can be effective for reducing inflammation and allergic diseases.

Vitamin E was discovered by Evans and Bishop in 1924 and isolated in 1936 as tocopherol (from the Greek meaning “to bring forth offspring” because it was noticed that this compound was important for the reproduction of rodents) and later become known as vitamin E. Vitamin E deficiency in animals causes fertility problems and muscle degeneration and it would appear that these effects can be reduced by vitamin E supplementation (3).

In 1931, Pappenheimer and Goettsh reported that vitamin E is important for prevention of encephalomalacia of chicks, and nutritional muscular dystrophy in rabbits and guinea pigs.

Vitamin E is not a single compound but is a generic name to describe eight forms of vitamin E found in nature, which include four tocopherols (T): alpha, beta, gamma, delta; and four tocotrienols (T3): alpha, beta, gamma, delta, where γ -tocopherol is the most common; while α -tocopherol, is the most biologically active form. Individual tocopherols and tocotrienols are named according to the position and number of methyl group on their chromanol rings (Fig. 1).

Vitamin E has antioxidant properties and is a key regulator in early step of DNA replication, enhances humoral and cell-mediated immunity, and is perhaps one of the vitamins to have received far more attention from researchers. In tumor cells, vitamin E may inhibit the cell cycle and cell differentiation and promote apoptosis, leading to cell death (4). In addition, it participates in cell cycle arrest and prolongs S phase contributing to sensitization of cancer cells to drugs (4).

The classic effect of vitamin E regards its capacity to inhibit the combining of body substances with oxygen, to stabilize polyunsaturated lipids, minimize lipid peroxidation damage and prevent undesirable oxidation reactions (antioxidant effects). A multiplicity of clinical signs occurs in animals suffering from vitamin E deficiency (5). Therefore, it can be useful to reduce or prevent oxygen damage to tissues, and vitamin E therapy can also be useful to treat pain and inflammation which occur during allergic reactions. Its absorption is related to fat digestion and is facilitated by bile and pancreatic lipase. Vitamin E passes through the placenta membrane and mammary gland as tocopherols, making it important for development of the embryo and the young after birth (6). Clinical studies and experimental animal models indicate that vitamin E

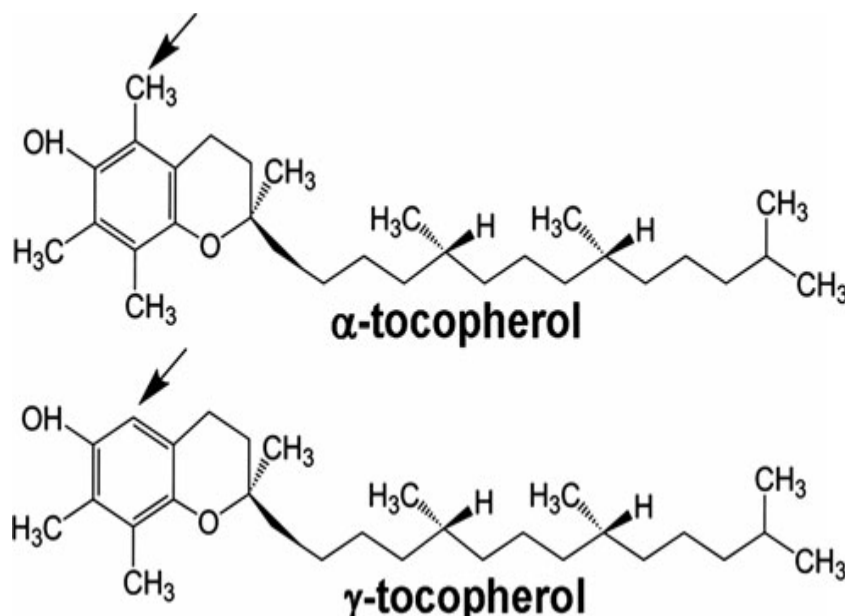


Fig. 1. *Alpha-tocopherol deferred from gamma-tocopherol for the presence of CH_3 group.*

and other anti-oxidants are protective in allergy and asthma risk (7-8).

Allergy and vitamin E

The classic term of “allergy”, coined by von Pirquet, referred to a state of alter reactivity, which includes both facets of the altered state: immunity (beneficial) and hypersensitivity (harmful). Later, Gell and Coombs classified immediate hypersensitivity reaction(s) as type I allergy.

About 40% of the population in industrialized countries are affected by allergic disease which has substantially increased over the past 50 years. Genetic and environmental factors play an important role in individual susceptibility and etiopathogenesis of this disease (9). It has been reported that unhealthy dietary patterns low in vitamins, and other antioxidants compounds, such as n-3 polyunsaturated fatty acids, may be contributing to the risk of allergy, asthma and other inflammatory diseases (10). Vitamin E and other anti-oxidants have known immune function effects and epidemiological associations with risk of allergic disease.

Vitamin E has a number of different but related functions, including intracellular antioxidant activity. It is thought that this vitamin has a basic functional importance on membrane integrity in virtually all cells of the body. Experimental data suggest a causative role of oxy-radicals in aging

processes, suggesting that vitamin E along with other antioxidants may have an important role in longevity. Vitamin E may also protect against cancer by acting as a protective reductant and by inhibiting malonyl-dialdehyde, a product of lipid peroxidation which increases tumor production. Vitamin E at high levels is well tolerated by animals and humans and results as one of the least toxic of vitamins. However, experimental animals given very high doses of this vitamin show prolonged blood clotting times due to hypoprothrombinemia. This effect may candidate vitamin E as an anti-coagulant therapeutic compound. Vitamin E significantly improves immune response, the reaction of vaccines and lowers incidence of infectious diseases. In addition it protects polyunsaturated fats from oxygenation and other compounds, such as vitamin A, from being destroyed.

Experimental animal model studies suggest that vitamin E may be useful in reducing lung damage caused by air pollution which provokes allergy and inflammation. However, in some cases, the effect of vitamin E deficiency conditions that occur in animals are not seen in humans. Diet has been widely implicated in the etiology of cardiovascular disease and other disorders, but nowadays it has been seen that the diet is also very important in allergic diseases, and its etiology is less clearly defined (11).

According to the recently published international

Table I. *Effects of vitamin E.*

<i>ENHANCES: Immune response, lymphocyte proliferation, DTH, IL-2 production, IFN-γ, α-2 and β-2 globulin fractions, T-cell-mediated function, cell-cycle-related proteins, the response to vaccines, IFN-gamma production by CD4+ and CD8+ T cells, apoptosis in cancer cells</i>
<i>DECREASES: PGE2 production, IL-6 and IL-1β secretion, inhibits COX activity, reduces NO production, causes cell cycle arrest, inhibits cancer cell differentiation, participates in killing cancer cells, inhibits the genes involved in cell proliferation, tumor growth, incidence of infectious diseases, incidence rate of common colds, progression of AD; and prevents clinical deficiency syndrome</i>

consensus, research efforts in the past decade have highlighted that decreasing antioxidant vitamins increases n-6 polyunsaturated fatty acid (PUFA), and decreases n-3 PUFA, an effect that augments asthma and allergic diseases (12). Therefore, there is an association between the effect of vitamins and/or diet and allergic diseases, including asthma.

As mentioned above, vitamin E has the capacity to scavenge reactive oxygen species (ROS) during lipid peroxidation and inhibit activation of protein kinase B *in vitro*, exerting an antioxidant and non-antioxidant immunomodulatory effects.

It has been reported that vitamin E regulates vascular cell adhesion molecule-1 (VCAM-1)-dependent leukocyte migration through antioxidant and non-antioxidant devices. During allergic diseases and inflammation, several mediators, including ROS, induce the expression of VCAM-1 on endothelial cells. VCAM-1 signal transduction and VCAM-1-dependent inflammation are blocked by antioxidants, one of which could be vitamin E. Vitamin E modulates endothelial function during VCAM-1-dependent transmigration by altering VCAM-1-induced oxidative activation of endothelial cell PKC α (13). It is possible that regulatory function in allergic responses is in part regulated by endothelial cell VCAM-1 activation of PKC α and leukocyte migration.

However, in some cases α -tocopherol has an inhibitory effect, whereas γ -tocopherol has a stimulatory effect, and treatments with γ -tocopherol provoke the inhibition of α -tocopherol. Therefore, these two tocopherols have opposite functions on VCAM-1 during leukocyte chemoattraction.

Vitamin E has beneficial effects by blocking the stimulatory effect of bacteria endotoxin or ozone on inflammatory neutrophils and reduces neutrophilia in burn and smoke inhalation injury in experimental animal models and human (14).

Vitamin E reduces NO production and has a direct effect on T-cell functions of naïve T cells, independent of its effect on macrophages. Dendritic cells treated with alpha-tocopherol may induce INF-gamma generation by CD4+ and CD8+ T cells with an inhibitory effect on tumor growth and possibly regression (4).

Human bronchial epithelial cell signaling activation results in NF- κ B activating pathway. IgE binds kB motifs and promotes several inflammatory genes such as TNF, IL-1 β , iNOS and COX-2, leading to their expression, reactions that can be inhibited by vitamin E (15). Moreover, vitamin E may increase delayed-type hypersensitivity (DTH) response, T-cell proliferation and IL-2 generation and decrease plasma lipid peroxide concentration and inflammatory PGE₂. The stimulatory effects of vitamin E on T-cell proliferation and IL-2 generation may be due to its inhibitory effect on PGE₂.

Furthermore, subjects receiving vitamin E supplementation have significantly reduced incidence rates of common colds and slower progression of atopic dermatitis (AD), as reported in many cases. However, the importance of the vitamin E for healthy development and function of the body is unquestioned.

Beckhause AA et al. reported that vitamin E, along with other antioxidant vitamins, are associated with lower odds of wheeze during childhood, but it has no effect on asthma and other atopic conditions (16). This result may be due to two different and opposing effects of alpha tocopherol and gamma tocopherol, since α -tocopherol blocks and γ -tocopherol increases airway hyperresponsiveness (17).

Vitamin E is not consistently associated with asthma or allergies and some authors have reported seemingly conflicting outcomes for vitamin E on allergic and inflammatory diseases.

Vitamin E exerts many complex effects on immunological pathways and inflammation. Because vitamin E is fat-soluble and some allergies (such as atopic dermatitis) are associated with a defect in the metabolism of lipids, it is still obscure to understand the mechanism of action of this vitamin in allergic diseases.

The association of vitamin E and allergy and/or asthma appears to be very complex, but the fact that they still provoke debate reflects the complexity of the subject, and the effect of vitamin E might differ between allergy and asthma.

The mechanisms through which vitamin E improves allergy and inflammatory conditions are currently under investigation. Clinical trials of

vitamin E interventions to prevent allergic diseases and asthma are advisable on the basis of existing evidence.

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PERIPHERAL BLOOD-DERIVED, $\gamma\delta$ T CELL-ENRICHED CELL LINES FROM GLIOBLASTOMA MULTIFORME PATIENTS EXERT ANTI-TUMORAL EFFECTS *IN VITRO*

V. MARCU-MALINA^{1,2}, D. GARELICK³, N. PESSES-YELOZ⁴, A. WOHL⁴,
L. ZACH⁵, M. NAGAR², N. AMARIGLIO^{6,7}, M.J. BESSER^{7,8}, Z.R. COHEN⁴
and I. BANK^{1,3,7}

¹Laboratory for Immunoregulation, Sheba Medical Center, Tel Hashomer, Israel; ²Institute of Hematology, Sheba Medical Center, Tel Hashomer, Israel; ³Department of Medicine F, Sheba Medical Center, Tel Hashomer, Israel; ⁴Department of Neurosurgery, Sheba Medical Center, Tel Hashomer, Israel; ⁵Institute of Oncology and Radiotherapy, Sheba Medical Center, Tel Hashomer, Israel; ⁶Cancer Research Center, Sheba Medical Center, Tel Hashomer, Israel; ⁷Sackler School of Medicine, Tel Aviv University, Israel; ⁸Ella Institute for Melanoma, Sheba Medical Center, Tel Hashomer, Israel

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The goal of this work was to assess the potential of T cells expressing $V\gamma 9V\delta 2+$ T cell receptors (TCR, $\gamma\delta$ T cells) present in peripheral blood (PB) mononuclear cells (MC, PBMC) of glioblastoma multiforme (GBM) patients to act as anti-tumoral agents. We found that $\gamma\delta$ T cell levels were decreased in patients' PB relative to a cohort of healthy donors (HD) (respectively $0.52 \pm 0.55\%$, $n=16$, vs $1.12 \pm 0.6\%$, $n=14$, $p=0.008$) but did not significantly correlate with postoperative survival ($R=0.6$, $p=0.063$). Importantly, however, the $\gamma\delta$ T cells could be expanded *in vitro* to consist $51 \pm 23\%$ of the cultured lymphocytes ($98\% CD3+$). This was achieved after 14 days of culture in medium containing the amino-bisphosphonate (ABP) Zoledronate (Zol) and interleukin (IL)-2, resulting in $\gamma\delta$ T cell-enriched lines (gdTCEL) similar to those of HD derived gdTCEL ($54 \pm 19\%$). Moreover, gdTCEL from patients and HD mediated cytotoxicity to GBM-derived cell lines (GBMDCL), which was abrogated by immune-magnetic removal of the $\gamma\delta$ T cells. Furthermore, low level interferon (IFN) γ secretion was induced by gdTCEL briefly co-cultured with GBMDCL or autologous - tumor-derived cells, which was greatly amplified in the presence of Zol. Importantly, IFN γ secretion was inhibited by mevastatin but enhanced by cross-linking of butyrophilin 3A1 (CD277) on a CD277+ GBMDCL (U251MG) or by pretreatment of GBMDCL with temozolomide (TMZ). Taken together, these data suggest that $\gamma\delta$ T cells in PB of GBM patients can give rise to gdTCEL that mediate anti-tumoral activities

Glioblastoma multiforme (GBM) is the most common malignant brain tumor in adults. The majority of patients die within 2 years and only 15% survive 5 years (1). The possible role of $\gamma\delta$ T cells as therapeutic reagents for GBM has been explored since the 1990s. These CD4-CD8- or CD8 $\alpha\alpha$ + T cells are a subset of

thymus-derived lymphocytes that express a T-cell receptor (TCR) composed of γ and δ chains rather than the $\alpha\beta$ TCR which is expressed by conventional CD4+ and CD8+ T cells (2). Initially, Fujimiya et al. noted both that $\gamma\delta$ T cells could be expanded from the peripheral blood (PB) of GBM patients using anti-CD3 and

Key words: $\gamma\delta$ T cells, glioblastoma multiforme, immunotherapy, brain tumors, survival

Mailing address:

Dr Ilan Bank,
Department of Medicine F,
Sheba Medical Center,
Tel Hashomer, 52621 Israel
Tel.: 97235409969 - Fax: 97235302114
e-mail: ibank@post.tau.ac.il

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interleukin (IL)-2 and that $\gamma\delta$ T cells killed autologous GBM in a manner augmented by IL-12 (3). Yamaguchi et al. also used anti-CD3 and IL-2 to expand $\gamma\delta$ T cells from PBMC of GBM patients, isolated the $\gamma\delta$ T cells from the cultures and confirmed their cytotoxicity to GBM (4). Later these authors revealed the additive effect of IL-15 in this regard (5). Bryant et al. by contrast, expanded cytotoxic $\gamma\delta$ T cells X 200-600-fold from the PB of GBM patients using a combination of anti-CD3, anti-CD2 monoclonal antibodies (mAb), IFN γ and IL-2 (6). Importantly, these authors demonstrated that cells prepared in this manner, impeded tumor growth when administered intracranially in xenogeneically tumor-transplanted mice (7).

In all these experiments, the $\gamma\delta$ T cell populations contained both V δ 2+ and V δ 1+ $\gamma\delta$ T cells, the two main subsets of $\gamma\delta$ T cells in human PB. Knight et al. demonstrated that V δ 1+ $\gamma\delta$ T cells lyse GBM targets specifically (8) whereas Siegers et al. recently reported that both V δ 1 and V δ 2 T cells expanded from the PB of healthy individuals can kill GBM (9).

However, in most healthy humans, the majority (50%-70%) of the PB $\gamma\delta$ T cells express V γ 9 and V δ 2 genes in the TCR (hence termed γ 9 δ 2T cells). γ 9 δ 2T cells are known to exhibit major histocompatibility complex (MHC)-independent cytotoxicity for a broad range of tumor types, including lymphoma, multiple myeloma, lung, breast cancer and GBM (10). Cytotoxic function is due at least in part to recognition, by the γ 9 δ 2TCR, of a complex of phosphorylated antigens (phosphoantigens) primarily isopentenyl pyrophosphate (IPP) that are metabolites produced in the mevalonate pathway of tumor cells that bind to butyrophilin 3A1 (BTN3A1, designated CD277) on target cells (11). This recognition leads to γ 9 δ 2TCR-mediated γ 9 δ 2T cell activation, and induction of cytotoxicity (11). γ 9 δ 2T cells in the PB of many, but not all, cancer bearing patients can be expanded *in vitro*, using either analogues of these phosphoantigens or aminobisphosphonates (ABP) as activating agents, together with IL-2. In the latter instance, ABP increases the intracellular concentration of IPP in monocytes (by blocking farnesyl pyrophosphate synthase), which, presumably by binding CD277, induces γ 9 δ 2 TCR-mediated activation of the PB γ 9 δ 2 T cells to express IL-2 receptors (12). When IL-2 is supplied exogenously to γ 9 δ 2 T cells in PBMC of a single unit of blood in the presence of ABP and IL-2, they expand to as many as 10^{10} cells (to consist 70-90% of the CD3+ T cells in the cultures).

These γ 9 δ 2 T cell-enriched cell lines (gdTCEL) have been used for autologous adoptive immunotherapy in cancer patients, achieving stable disease in ~40% of patients (10). Nakazawa et al. recently demonstrated the ability of such Zol activated γ 9 δ 2T cells isolated from healthy donors to kill GBM- derived cell lines (GBMDCL) (13) and Cimini et al. showed that Zol further augments the killing of GBMDCL by γ 9 δ 2 T cells (14). However, it has been previously suggested that the γ 9 δ 2 T-cell subset is depleted in the PB of GBM patients (6). Thus, given the potential, as indicated above of γ 9 δ 2 T cells to exert potent anti-tumoral effects, we undertook experiments to determine whether ABP-activated γ 9 δ 2T cells could be expanded robustly from the PB of GBM patients, whether T-cell lines enriched for these cells retain properties consistent with clinically relevant anti- tumoral potential, and how these properties could be further amplified.

MATERIALS AND METHODS

Human samples

The study was approved by the Institutional Review Board (Helsinki Committee) of the Chaim Sheba Medical Center, Ramat Gan, Israel. All patients participating in the study were treated in the Neurosurgery department of the hospital. Blood was taken on the day or one day after the surgery for biopsy or tumor dissection. Only patients with pathologically proven GBM were included. All patients and age-matched healthy volunteers signed an informed consent prior to blood sampling. Given that mean survival of GBM patients is 9 months, the patients were followed up prospectively for 2.5 years. This allowed for collection of survival data which was retrieved from the patients' files (14).

Cell lines

Glioblastoma cell lines (U251MG, U87MG and T98G) were a kind gift from Gad Lavie (Sackler School of Medicine, Tel Aviv University) (15), breast cancer cell lines (MDA-MB231 and MCF-7) and normal epithelial breast cell line HB2 were obtained from ATCC. Cell lines were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL, UK), 2 mM L-glutamine, 1 mM sodium pyruvate and 100 μ g/ml penicillin-streptomycin solution (Biological Industries, Beit Haemek, Israel). All cell lines used were tested and found negative for mycoplasma.

Primary GBM cell lines were established as following:

single cell suspension was prepared by mechanical dispersion. Tumor tissue was minced into 1-2 mm pieces, then bigger pieces sieved and forced through a 100 μ m cell strainer with a syringe plunger. Cells were washed and cultured in growth medium (DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, non-essential amino acids, 100 μ g/ml penicillin-streptomycin solution, gentamicin 50mg/ml and fungizone 1.25 μ g/ml).

Isolation and culture of PBMC

PBMC were isolated by Lymphoprep (AXIS-SHIELD PoC AS, Oslo, Norway) density centrifugation and cultured in growth medium (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL, UK), 2 mM L-glutamine and penicillin-streptomycin solution (100 μ g/ml) (Biological Industries, Beit Haemek, Israel)). Cultures were primed with 2 μ M of Zoledronate (Zol) (Novartis) and 100 international units (IU)/ml of recombinant human IL-2 (rhIL-2) (Boehringer-Mannheim) and maintained for 14 days; fresh medium with IL-2 was replaced every 3 days. For some experiments, in order to determine the role of the γ 9 δ 2T cells specifically in the killing of GBM, these cells were removed from the cultures by immuno-depletion using mAb V γ 9 and anti-mouse IgG micro-beads (Miltenyi Biotec) followed by passage through MACS columns according to the manufacturer's instructions.

Rearrangement analysis

The Clonal repertoire of the expanded γ 8T cells was analyzed using standardized Biomed 2 protocol as previously described (16). Briefly, TCR- γ rearrangements were amplified by polymerase chain reaction (PCR). For GeneScan analyses, V γ primers were fluorescently labeled and used for PCR. Fluorescence-labelled PCR amplificate (1 μ l of each) was added to a mixture of 12.5 μ l deionized formamide and 0.5 μ l GeneScan 500TM Rox internal lane standard (PE Applied Biosystem, Weiterstadt, Germany).

Characterization of cell subsets by flow cytometry

Fluorescently labeled isotype control mAb (BD Biosciences Pharmingen) or mAb to CD4, CD8, CD3, (BD Biosciences Pharmingen), and to V γ 9 (clone IMMU360), V δ 2 (clone IMMU389), Pan- γ 8TCR (Immunotech), anti-CD27 (Clone M-T271; BD Biosciences) and CD45RA (clone ALB11; Beckman Coulter) were used for staining as previously described (15). Lymphocytes were gated according to typical forward and side scatter plots using

a BD FACS calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) and analysis of the gated cells was performed using FlowJo software.

Functional assays

For the lysis assay target cells were labeled with 2.5 μ M of 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular probes) for 10 min at room temperature, then CFSE was quenched with the same volume of FBS and washed with growth medium twice. Labeled target cells were plated together with effector γ 8T cells at effector to target (E:T) ratio of 5:1 into round-bottomed 96-well plate overnight with or without 2 μ M of Zol. Next morning cells were harvested and stained with propidium iodide (PI). Specific lysis was measured with flow cytometry analysis of PI positive cells among CFSE labeled population. Background absorption of PI by the target cells without effectors was subtracted from the experimental samples. For the IFN γ secretion assays, target and effector cells were plated at E:T ratio of 1:1 and were co-cultured for 48 h. Then supernatants were collected and analyzed in duplicates using the ELISA max Deluxe Sets (Biolegend, San Diego, CA) according to the manufacturer's instructions. In some experiments target cells were pre-incubated with 20 μ M of mevastatin for 1 h prior to co-incubation with effector cells for the killing or cytokine secretion assays. Anti-CD277 (anti-BTN3A1) mAb 20.1 (a kind gift from Daniel Olive, Marseille, France) or mouse IgG were added to the target cells 1 h prior to addition of γ 82T cells.

To test effects of TMZ, U251MG cells were incubated with TMZ (20 μ M) (Sigma-Aldrich) or DMSO (vehicle of TMZ) for two days prior to addition of γ 82T cells with or without 2 μ M of Zol. Specific lysis was measured by Propidium iodide (PI) staining of CFSE labeled target cell after overnight incubation with effectors. For IFN γ secretion assays: U251MG were pre-incubated with TMZ (20 μ M) for 5 days and then γ 82T cells were added with or without Zol. After 48 h supernatants were collected and analyzed for IFN γ .

Statistical analysis

Significance of variation between groups was evaluated using a nonparametric 2-tailed Student's t-test. Correlation between groups of data was measured using a two-tailed Pearson analysis. Correlation of survival with blood cell types was performed only for patients who died during follow-up.

RESULTS

Patient population

The clinical characteristics of the patients (n=16) participating in the study are summarized in Table I. Tumors were larger than 4 cm in 87% (n=14) of patients at diagnosis, and most common tumour location was the frontal lobe, followed by the parietal, temporal and then occipital lobes. Gross tumor resection was performed in 88% (n=14) of the patients whereas two underwent biopsy alone, due to the anatomic location of the tumors. None of the patients had partial tumor resection. The majority (62%, n=10) were treated with concomitant chemo-radiotherapy (with TMZ). Four patients did not receive any adjuvant treatment at our institute, of whom one died shortly after biopsy and before further treatment could be started. The remaining three patients were lost to follow-up. Within the two and a half years of the clinical study period (1/2011 – 8/2013) ten patients died (62%): nine due to progressive GBM and one from complications of orthopaedic surgery.

$\gamma\delta$ T cells in the peripheral blood

To compare $\gamma\delta$ T cells in the GBM patients and the control healthy donors (HD), PBMC were analyzed by flow cytometry. The percentage of $\gamma\delta$ T cells in the lymphocytes of the GBM patients drawn peri-operatively was significantly lower than in the cohort of HD (respectively $0.52\pm0.55\%$, n=16, vs $1.12\pm0.6\%$, n=14, $p=0.008$) (Fig. 1A). There was no significant difference between mean age of males and female (66.3 ± 10 vs 57.9 ± 9.5 years, $p=0.12$) or the percentage of $\gamma\delta$ T cells among males and females in the patient cohort (0.355 ± 0.187 vs 0.612 ± 0.678 , respectively, $p=0.385$). The percentage of the cells in the GBM cohort declined significantly with patient age among males but not females ($R=-0.86$, $p=0.029$, vs $R=-0.39$, $p=0.294$) (Fig. 1B), consistent with the known age-dependent decrease of these cells (17). Since PB white blood cells were previously shown to correlate with survival in GBM, it was of interest to correlate subsets with survival in our cohort (18). Analysis of the relation of white blood cells in the PB to the length of post-operative survival (time to death), revealed no significant correlation between the number of lymphocytes (L) in the PB and survival whereas, as previously reported, there was a highly significant negative correlation between the number of polymorphonuclear (PMN) cells ($R=-$

0.77 , $p=0.0088$). The number of monocytes (M) also correlated negatively but non-significantly with survival ($R=-0.583$, $p=0.077$) (18). The number of $\gamma\delta$ T cells in the PB showed a non-statistically significant trend to positively correlate with duration of post-surgical survival ($R=0.602$, $p=0.063$).

Expansion of $\gamma\delta$ T cells ex vivo

We tested whether the $\gamma\delta$ T cells in PB of GBM patients could be expanded significantly *ex-vivo* (10). Fig. 2A represents dot plots of $\gamma\delta$ T cells staining prior to and following 14 days of culture with Zol and IL-2 in one example. After expansion, >95% of the cells in the gdTCEL were CD3+, and the distribution of CD4+, CD8+ and of CD3-/CD56+ NK cells was similar in GBM and HD gdTCEL. Surprisingly, $\gamma\delta$ T cells from both patients and HD expanded quite similarly, i.e. 464 ± 463 (range 12-1581) and 124 ± 118 (range 13-424)-fold, resulting in $12\pm7\times10^6$ and $13\pm8\times10^6$ cells, respectively, per 10 ml of PB, with similar percentages of $\gamma\delta$ T cells among the CD3+ cells in the gdTCEL ($51\pm23\%$ in GBM patients and $54\pm19\%$ in HDs) (Fig. 2, B and C). Staining with mAb to CD27 and CD45RA revealed that the $\gamma\delta$ T cells were of the effector memory CD27-CD45RA- (range 6-90%) or central memory CD27+ CD45RA- (range 1-99%) phenotypes with a similar distribution in patients and HD (Fig. 3, A and B). Repertoire analysis of the γ genes expressed in cultures from HD (n=3) and the GBM patients (n=3) revealed a polyclonal CDR3 length distribution (data not shown).

Anti-tumor functionality of gdTCEL

Preliminary experiments indicated that a low content of $\gamma\delta$ T cells (e.g. CP3 with only 21% $\gamma\delta$ T cells in the gdTCEL) was associated with only low level killing (<15% at ET ratio 10:1, data not shown). Therefore, we limited further functional analysis of possible significant anti-tumoral functionality in cytotoxicity and IFN γ release assays to gdTCEL containing >50% $\gamma\delta$ T cells. Fig. 4A shows representative results from 7 such GBM patients. As can be seen, when cells from the gdTCEL were co-cultured overnight with targets of GBMDCL, the lysis of CFSE labeled U251MG, T98G and U87MG GBMDCL, at effector to target ratio as low as 5:1, was significantly higher than that of fresh PBMC used as a negative control ($p<0.01$) (Fig. 4A). Killing was slightly augmented by concomitant

Table I. *Summary of clinical characteristics of the patients diagnosed with GBM.*

Variable	No. (%)
Age years	
30 ≤age ≤40	1 (6)
40 <age ≤50	1 (6)
50 <age ≤60	7 (44)
60 <age <65	2 (13)
Age ≥65	5 (31)
Mean	60.9
Sex	
Male	8 (50)
Female	8 (50)
No. of lesions on presentation	
Single	14 (87)
Multiple	2 (13)
Tumour size	
< 4cm	2 (13)
≥ 4cm	14 (87)
Tumour location	
Frontal	7 (37)
Parietal	4 (21)
Temporal	3 (16)
Occipital	3 (16)
Basal Ganglia	1 (5)
Thalamus	1 (5)
Extent of resection	
Complete	14 (88)
Incomplete	0
Biopsy	2 (12)
Adjuvant treatment	
Combined radiotherapy and chemotherapy	10 (62)
Radiotherapy only	2 (13)
Chemotherapy only	0
None	4 (25)
No. of patients at the end of the research period	
Deceased	10 (62)
Alive	3 (18)
Lost to follow up	3 (19)

exposure of the tumor cells to Zol. To test the specific role of the $\gamma 9\delta 2$ T cells in the gdTCEL-mediated killing, $\gamma 9\delta 2$ T cells were removed from the cultures by magnetic separation using mAb to V $\gamma 9$. The non-depleted and $\gamma 9\delta 2$ T cells depleted gdTCEL derived cells were incubated overnight in the presence of Zol with CFSE labeled U251MG cells and T98G cells at a 2:1 killer:target ratio. Lysis by $\gamma 9\delta 2$ TCR+ depleted gdTCEL was strongly reduced (Fig. 4B). Thus, $\gamma 9\delta 2$ T cells within the gdTCEL appeared to be the prime mediators of cytotoxicity against GBM cells.

As a further measure of the functional effects

mediated by gdTCEL, we measured IFN γ in supernatants after a 24-h co-culture with the GBMDCL. Although only low levels of IFN γ were measured in the co-culture supernatants, higher levels were measured in the presence of Zol, in particular in co-cultures including U251MG and U87MG (Fig. 4C). In addition, as shown in the bars on the right in Fig. 4C (representing the mean of independent experiments in which tumor cells and autologous gdTCEL from three different patients were co-cultured) the patient-derived gdTCEL secreted significantly higher levels of IFN γ that increased further in the presence of Zol

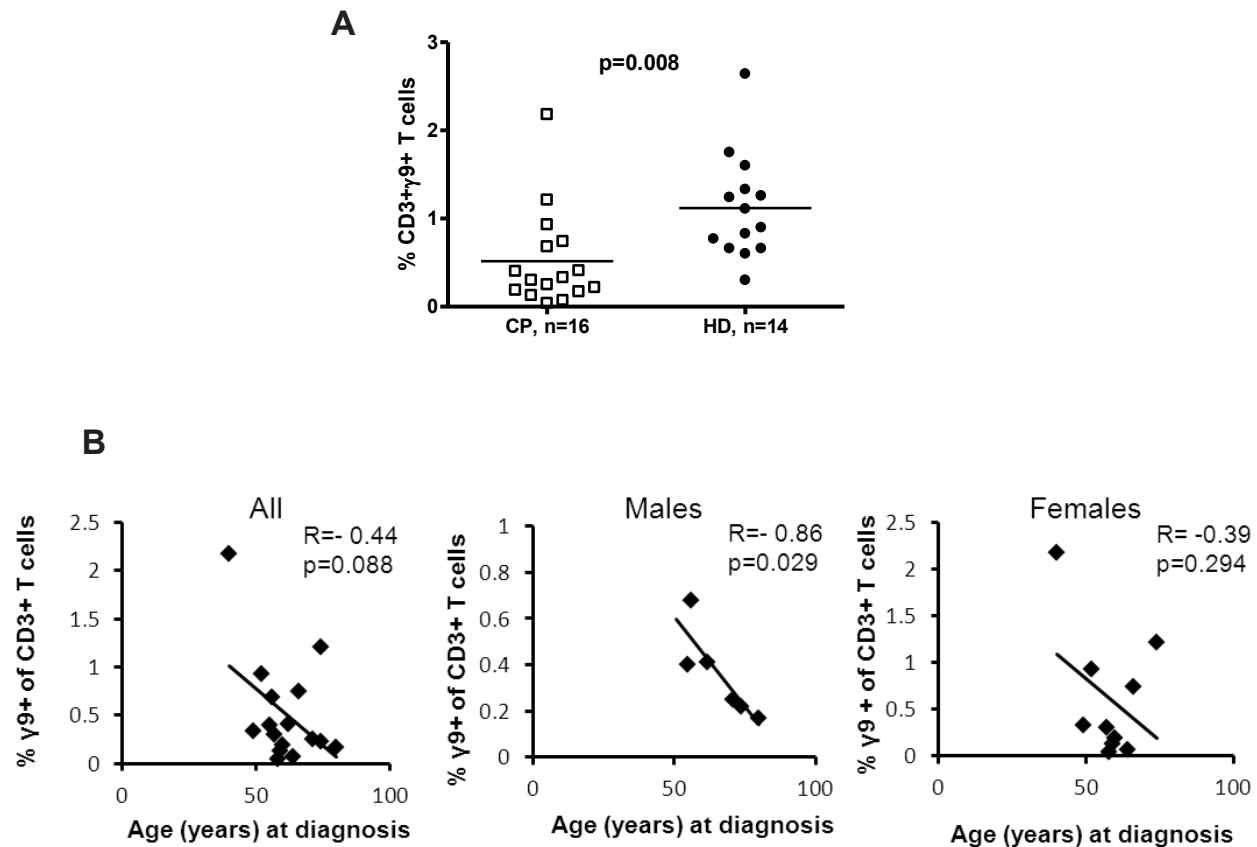


Fig. 1. $\gamma 9\delta 2$ T cells in the GBM patients versus HDs and its correlation with age and survival of GBM patients. PBMC from GBM patients (CP) and healthy donors (HD) were stained with fluorescently labeled CD3 and $\gamma 9$ specific antibodies and analyzed by flow cytometry. **A)** Percent of CD3+ $\gamma 9$ + T cells among total lymphocytes at the day of the blood collection in GBM patients versus HDs. **B)** Correlation dot plots of percent of $\gamma 9\delta 2$ T cells among total CD3+ T cells and age of GBM patients at time of the diagnosis. Data shown is for all patients studied and for males and females separately. Correlation values were calculated using two-tailed Pearson test.

in the presence of autologous malignant cells than in the presence of GBM-derived U251MG, T98G and U87MG cell lines. To determine whether gdTCEL from GBM patients (n=10) respond preferentially to GBM-derived cells, we compared their IFN γ secretion to that of HD (n=5) in co-cultures with either GBMDCL, breast cancer (BC) or normal breast epithelium-derived cell lines. (Fig. 4D). The highest and lowest levels of IFN γ were measured in co-culture with U87MG cells and normal breast epithelium respectively, and there was no significant difference between responses of HD and GBM-derived gdTCEL to the cell lines tested.

Effects of mevastatin and a mAb to CD277 on IFN γ secretion

We next examined the role of $\gamma 9\delta 2$ TCR-mediated pathways of activation in the induction of IFN γ secretion

during gdTCEL/GBMDCL co-cultures. Specifically, we asked whether perturbing IPP or CD277, which, when in complex, are recognized by $\gamma 9\delta 2$ TCR on target cells, would influence IFN γ secretion by the gdTCEL (19). Since IPP is produced in the mevalonate metabolic pathway, we examined the effect of inhibition of the mevalonate pathway upstream of IPP, by mevastatin. Mevastatin strongly inhibited IFN γ secretion during co-cultures of gdTCEL with U251MG cells, in particular, in the presence of Zol, as shown in Fig. 5A, representing 4 independent experiments (19). To explore the role of CD277 in gdTCEL/GBMDCL interactions, we first analyzed its expression on the GBMDCL by flow cytometry, using anti-CD277 mAb 20.1. This analysis revealed low levels of CD277 expression on U251MG and U87MG but none on T98G cells (data not shown). Increasing concentrations of the

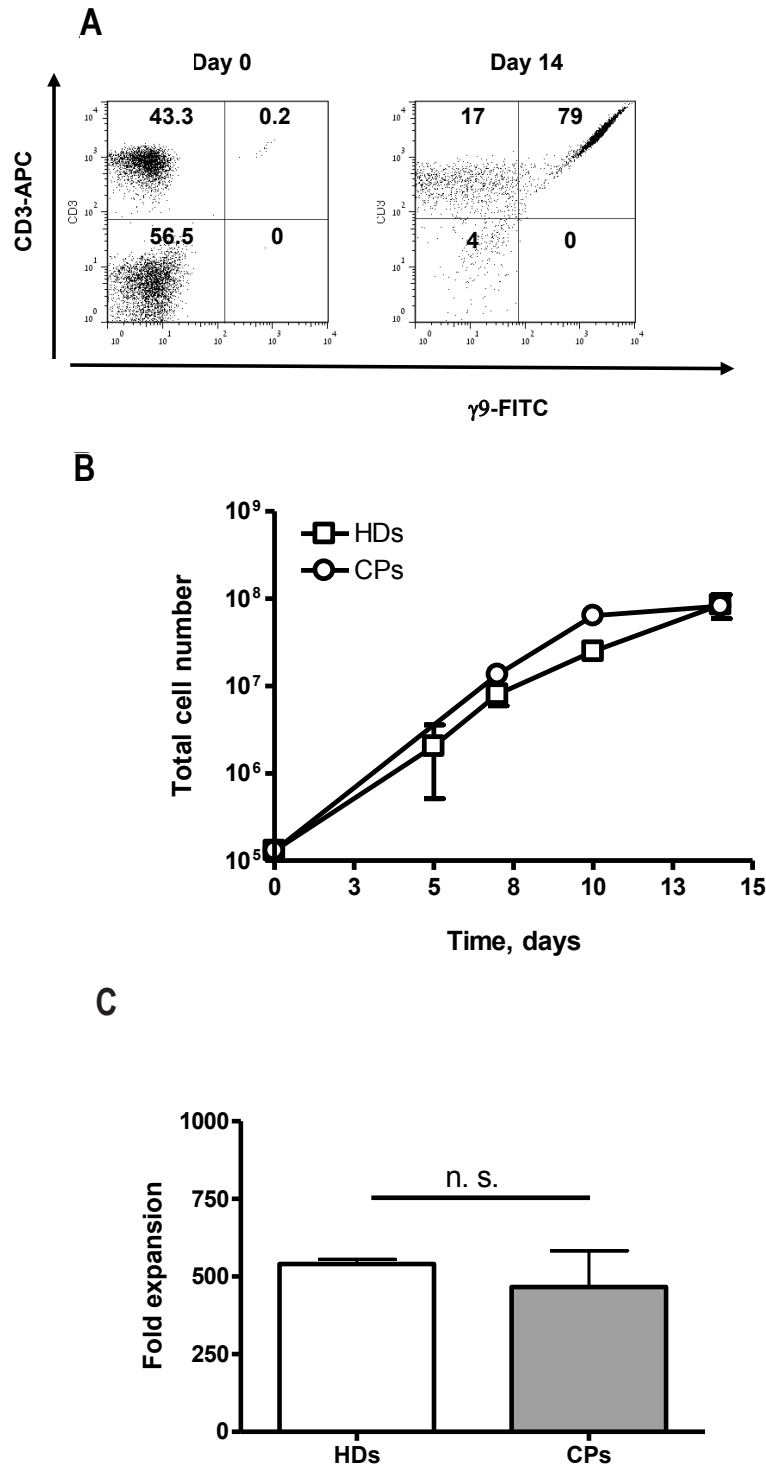


Fig. 2. Expansion of $\gamma 9\delta 2$ T cells from the peripheral blood of GBM patients. **A)** Representative dot plots of flow cytometry analysis of PBMC from a GBM patient stained with mAb to $\gamma 9$ and CD3, immediately after isolation from the peripheral blood (Day 0) and following culture with 2mM of Zol and 100 IU/ml of IL2 on day 14 (Day 14). Graphs plotting numbers of $\gamma 9\delta 2$ T cells (standard errors from the mean are indicated by vertical lines) over the culture period and fold numerical expansion of the $\gamma 9\delta 2$ T cells after 14 days indicated in parenthesis for the patient (labelled Cancer Patients, CP) and healthy donor (HD) cohort are shown in **B** and **C** respectively. ns: not statistically significant.

agonistic anti-CD277 mAb 20.1, but not of control IgG, when added to the target U251MG cells 1 h prior to addition of HD-derived gdTCEL, markedly enhanced their secretion of IFN γ , which was low in the absence of Zol, and further enhanced the effect of Zol alone (Fig. 5B).

Combination with chemotherapy

When employed in the clinical setting γ 9 δ 2T cell-mediated adoptive immunotherapy is likely to be used in combination with chemotherapy. Therefore, we tested how treatment with the commonly used chemotherapeutic drug TMZ affects sensitivity of a

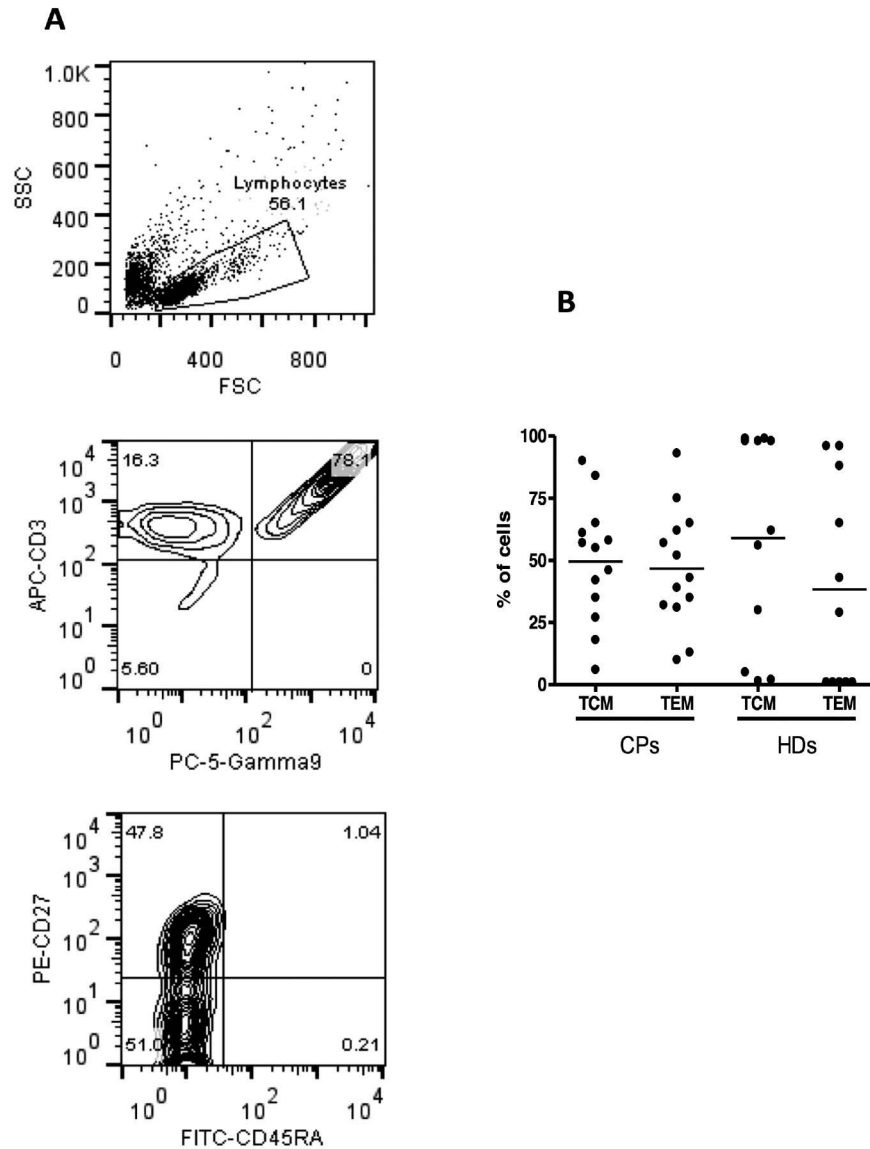


Fig. 3 Memory phenotype of the ex-vivo expanded γ 9 δ 2T cells in the gdTCEL. **A)** Flow cytometry analyses of gdTCEL population are shown. Lymphocytes in the gdTCEL were gated based on typical forward and side scatter characteristics (upper dot plot). The CD3 $^{+}$ γ 9 $^{+}$ double positive population (middle plot) was further analyzed for their expression of CD45RA and CD27 (lower plot). CD45RA-CD27 $^{-}$ cells were defined as effector memory T cells and CD45RA $^{+}$ /CD27 $^{+}$ cells- as central memory T cells. Antigens and their fluorochromes are indicated on the X and Y axis of each plot. Percent positive cells in each quadrant are indicated. **B)** Summary of the results of individual analysis for the GBM patients (CP) and the healthy donors (HD). Each dot represents results of one individual. The y axis represents percent positive cells among the γ 9 δ 2T cells in each culture. On the x axis, are indicated the nature of the cells; T cells: TEM (effector memory, CD27 $^{-}$ CD45RA $^{-}$) TCM (central memory, CD27 $^{+}$ CD45RA $^{-}$) for CP or HD-derived in vitro expanded γ 9 δ 2T cells.

GBMDCL to gdTCEL. Exposure of target U251MG cells to either TMZ solubilized in DMSO or DMSO alone, for 3 days did not induce lysis of U251MG. As expected, elevated lysis was, however, observed in co-cultures with gdTCEL (from HD) both in the absence and the presence of added Zol in a dose-dependent manner (Fig. 6A). Moreover, at E:T ratio of 5:1 killing was significantly greater in cells that had been exposed to TMZ. Furthermore, we found that pre-incubation of U251MG cells with TMZ solubilized in DMSO for 5 days significantly increased IFN γ secretion by gdTCEL contacting the U251MG cells (in the presence > absence of Zol), to levels greater than those observed upon contact with cells that had been exposed to DMSO alone (Fig. 6B). These results suggest that previous exposure to TMZ may increase the susceptibility of U251MG cells to killing by gdTCEL and enhance IFN γ secretion by gdTCEL contacting this target.

DISCUSSION

Although previous reports have studied $\gamma\delta$ T cells in GBM patients, the data presented in this paper show, for the first time to our knowledge, that, among 16 patients with GBM, a lower percentage of these cells, relative to healthy individuals, is present in the PB during the peri-operative period (19). Importantly, we found that despite this deficiency, the remnant cells can be efficiently expanded *ex vivo*, to create $\gamma\delta$ 2T cell-enriched gdTCEL that can kill GBM cell lines. Moreover, the gdTCEL secreted high levels of IFN γ when co-incubated with both GBM tumor cells and autologous tumor in the presence of the ABP Zol, in a manner that was inhibited by mevalonate, and augmented by cross-linking BTN3A1. When tumor cells are pre-treated with the anti-GBM reagent TMZ, both killing and IFN γ secretion are further enhanced in a gdTCEL-dependent manner.

Our finding that GBM patients have a significantly lower percentage of $\gamma\delta$ 2T cells in PB than healthy individuals complement those of Bryant et al. who demonstrated a decrease of total $\gamma\delta$ T cell in GBM patients at both the preoperative and early postoperative stages compared with healthy controls (6). Although not statistically significant, there was a trend toward a positive correlation between counts of $\gamma\delta$ 2T cells in the patients' PB and their post-operative survival which is reminiscent of the previous published data (6). This contrasted with an inverse relationship between

total number of PMN of the patients and post-surgical survival again consistent with previous reports (18). Since neutrophils have recently been shown to exert a suppressive effect on $\gamma\delta$ 2T cell proliferation, it is tempting to speculate that the increased risk in GBM patients with high PMN counts may be, at least in part, related to negative effects exerted by PMN on $\gamma\delta$ 2T cells *in vivo* (20).

The results of our *in vitro* experiments are consistent with numerous indications that activated $\gamma\delta$ 2T cells carry a strong anti-tumoral potential as both cytotoxic effectors and as high level secretors of IFN γ . In this regard, our data points out, for the first time to our knowledge, that even depleted $\gamma\delta$ 2T cells remaining in the PB of GBM patients immediately prior to surgical treatment, maintain virtually the same potential to proliferate in response to Zol, and give rise to gdTCEL with similar anti-tumoral potential as those derived from HD (Fig. 3). Notably, this conclusion is based on the data shown in Fig. 4, which represents results for patients in whom the gdTCEL contained >50% $\gamma\delta$ 2T cells. Indeed, our limited data, suggests that the anti-tumoral potential of gdTCEL containing lower percentages of $\gamma\delta$ 2T cells e.g. CP3, 8 and 16 may have a reduced effect. Since tumors have been shown to regress or stabilize after adoptive immunotherapy with *ex-vivo* expanded allogeneic gdTCEL, our data thus suggests that autologous *ex-vivo* activated gdTCEL could be obtained for treatment of the majority of GBM patients as well, at least in those exhibiting robust *ex-vivo* expansion (10). For example, in the animal model used by Bryant et al. $\sim 10^6$ $\gamma\delta$ T cells had a major therapeutic effect when introduced intracranially to mice carrying a xenogeneic human GBM tumor (7). We estimate that the corresponding cell number for intracranial human therapy would entail $\sim 10^9$ cells. By extrapolation from the number of cells in the gdTCEL of patients this number of cells could have been manufactured from the PB of 9 of the 16 GBM patients we studied.

In this regard, our results indicate that the classical Zol+IL2 expansion protocol used here produced gdTCEL from both GBM patients and HD which lysed GBM-derived cells and produced IFN γ in response to GBM cell lines, in particular in the presence of Zol (Fig. 4). Most importantly, gdTCEL cultured from the GBM patients strongly reacted to autologous tumor cells (Fig. 4B). *Ex-vivo* expanded $\gamma\delta$ T cells from healthy

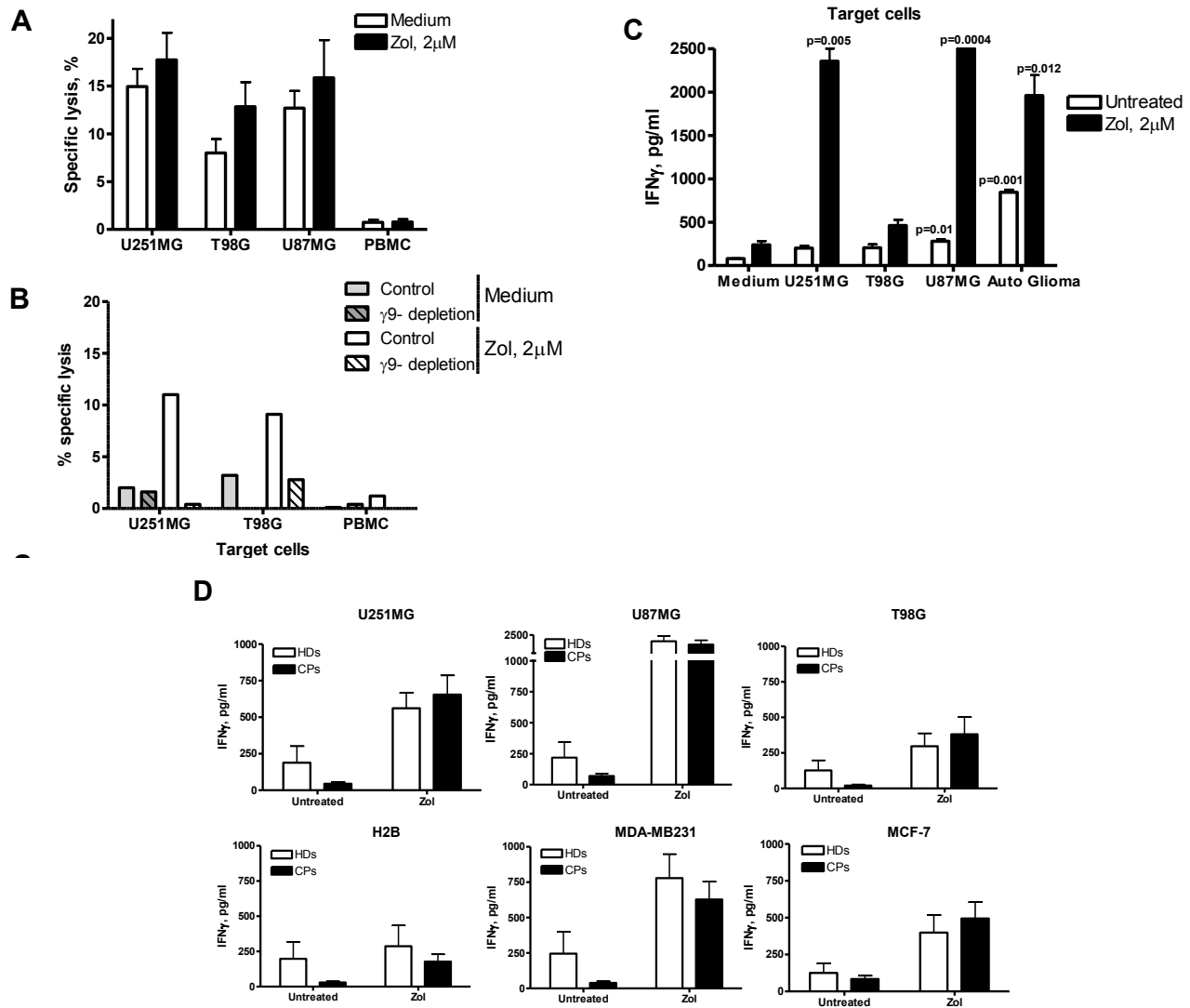


Fig. 4. Functionality of ex-vivo expanded gdTCEL. **A)** CFSE labeled target cells of glioblastoma multiforme derived cell lines or freshly isolated peripheral blood mononuclear cells (PBMC), shown on the X axis, were incubated with $\gamma 9\delta 2$ T cell enriched (>50% of the CD3+ T cells) 14 day cultures of PBMC triggered with Zol and IL-2 (gdTCEL) overnight at an effector to target (E:T) ratio of 5:1 without or with 2 μ M Zoledronate (Zol) as indicated. Specific lysis, defined by % propidium iodide positively staining CFSE+ cells was recorded. Bars represent mean $\pm$ SEM of specific lysis of GBM cells by gdTCEL from 7 different GBM patients. **B)** Cells of a gdTCEL from a GBM patient containing 55% $\gamma 9$ + T cells were labeled with FITC labeled antibody to V $\gamma 9$ then with anti-FITC beads, or with anti-FITC beads only (control). Labeled cells were depleted using MACS MS columns. The $\gamma 9$ depleted population contained no detectable V $\gamma 9$ + cells whereas control cells contained 55% $\gamma 9$ + T cells as assessed by flow cytometry (data not shown). $\gamma 9$ -depleted and control cells were incubated overnight with indicated target cells shown on the X axis at E:T ratio 2:1 using the methodology described above. **C)** Cells from gdTCEL were incubated with cells of GBM derived lines, or cells of briefly cultured autologous GBM tumors (Auto Glioma) or in medium alone as indicated on the X-axis for 48 h. Supernatants were collected and analyzed for IFN γ content by ELISA. Results represent experiments using Auto Glioma for 3 different donors and of gdTCEL of 10 GBM patients against the cell lines. IFN γ secretion in response to GBM cells was compared to secretion in medium only; p values are shown only for significant differences. **D)** Cells of gdTCEL from 10 GBM patients (CPs) and 5 healthy donors (HDs) each containing >50% $\gamma 9\delta 2$ T cells, were incubated with GBM (U251MG, U87MG and T98G), breast cancer (MCF7 and MDA-MB231) and normal breast (H2B) cell lines for 48 h with or without added 2 μ M of Zol. Supernatants were analyzed for IFN γ by ELISA. Bars represent mean and SEM of IFN γ concentration (pg/ml) as indicated on the Y-axis.

volunteers were shown previously to be cytotoxic to progressive gliomas both *in vitro* and in orthotopic model using immunodeficient mice (7). Based on our *in vitro* findings in the cytotoxicity and cytokine assays, a similar effect of GBM patient-derived gdTCEL would be expected *in vivo*. Furthermore, our data reveal three possible ways to increase the anti-tumoral effect of the gdTCEL: concurrent exposure of the tumor to Zol or to an activating anti-butyrpholin 3A1 (BTN3A1, also known as CD277) mAb, or pre-exposure of the tumor to TMZ.

The first two enhancing effects we found using patient- as well as HD-derived gdTCEL are presumably due to the mechanism of $\gamma 9\delta 2$ TCR-mediated interactions with a CD277/IPP complex on the tumor cells, since

CD277 binds IPP either in an extracellular antigen binding cleft, or forming a complex intracellularly, which is recognized by the $\gamma 9\delta 2$ TCR, leading to cell activation and cytokine secretion (11, 19). Monoclonal antibody 20.1, specific to CD277 is thought to stimulate $\gamma 9\delta 2$ T cell activation, by cross-linking of this antigen presenting molecule (21). Accordingly, gdTCEL from GBM patients enhanced their secretion of IFN γ in the presence of CD277+ tumor and mAb 20.1 (Fig 5). Since other GBMDCL, e.g. T98G, that expressed very low CD277 (data not shown), more weakly stimulated gdTCEL IFN γ in the presence of Zol and were less, but still susceptible, to killing in the absence of Zol (Fig. 4) it is likely that alternative antigen presenting molecules e.g. F1-ATPases, could be playing a role

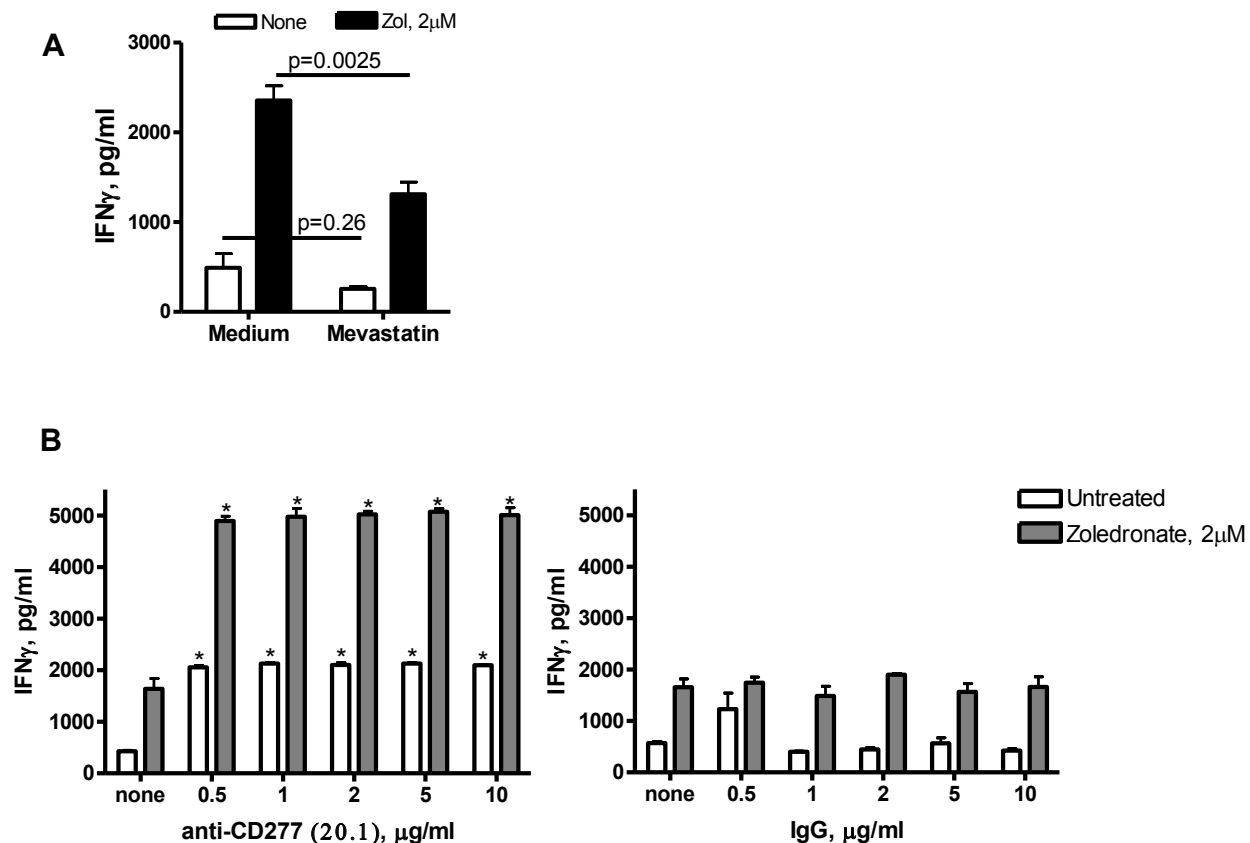


Fig. 5. Response of gdTCEL to GBM involves the mevalonate pathway and is enhanced by BTN3A1-specific antibody. **A)** Cells of gdTCEL from a patient with GBM were cultured with U251MG cells for 48 h at a ratio of 5:1 in the presence or absence of Zol as indicated. Mevastatin (20 μ M) was added for 1 h prior to addition of gdTCEL derived cells. Supernatants were collected and analyzed for IFN γ . Bars represent mean $\pm$ 1 SEM (n=4). P values for statistical comparisons of values in the presence or absence of mevastatin are shown. **B)** Cells from gdTCEL derived from HD were co-cultured for 48 h with U251MG cells, in the presence of increasing concentrations of anti CD277 20.1 antibody or mouse IgG as indicated on the X axis. IFN γ concentration (picogram/ml, pg/ml) in the supernatants was then measured by ELISA. Bars represent mean $\pm$ 1 SEM of 4 replicates from 2 different HDs. *: $p < 0.001$ relative to U251MG cells without antibody.

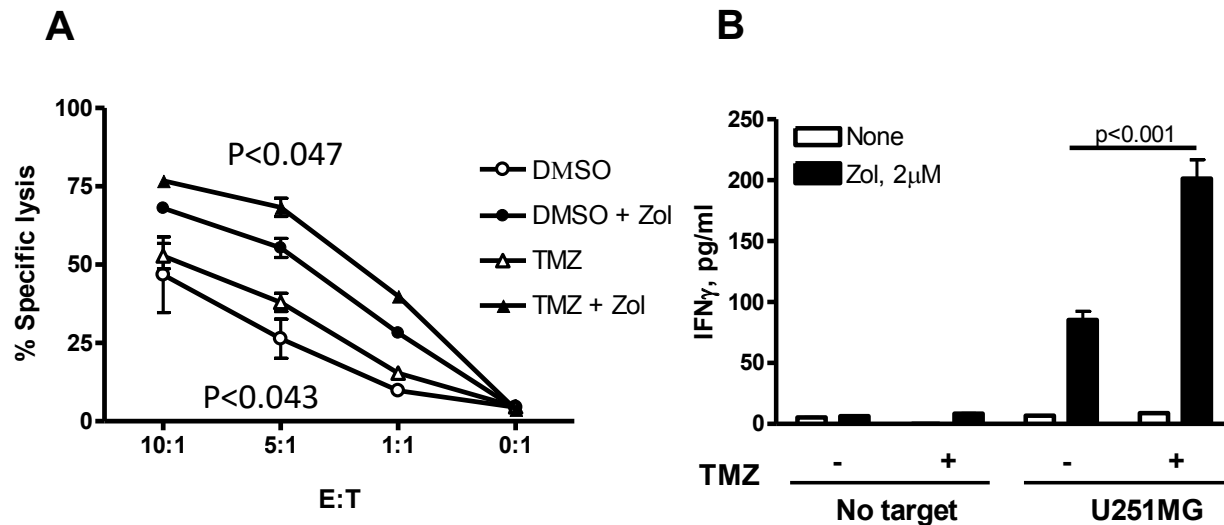


Fig. 6. Temozolomide (TMZ) increases sensitivity of GBM cell lines to $\gamma 9\delta 2T$ cells. **A)** U251MG cells were treated with 20 μM TMZ dissolved in DMSO or DMSO alone, as vehicle control, for 2-4 days. The cells were then harvested, labeled with CFSE and incubated overnight with cells of gdTCEL from healthy donors, at increasing E:T with or without 2 μM of Zol. Specific lysis was measured as described in materials and methods. Bars represent mean of 4 experiments, and *p* values comparing lysis in the presence and absence of TMZ are indicated for E:T ratio 5:1. **B)** IFN γ in the supernatants was measured after 48 hours of culture of cells from the gdTCEL used in A when added at E:T ratio 1:1 to U251MG cells where indicated. The X axis indicates the presence (+) or absence (-) of the target cells (U251MG) to the gdTCEL and presence or absence of zoledronate (Zol) (2 μM) in the cultures. Bars represent mean and 1 SEM of 8 experiments. *P* value comparing results are shown where statistically significant.

but are less efficient in this regard (22). Moreover, the gdTCEL express a polyclonal TCR repertoire (data not shown) and it has been shown that tumor recognition is strongly dependent on the amino acid composition of CDR3 region of $\delta 2$ chain, suggesting that the gdTCEL we derived may recognize different antigens expressed by the GBMTCL (23). Thus, it is likely that the potential use of mAb to CD277 in the clinic to augment therapeutic activity of $\gamma 9\delta 2T$ cells will be restricted to tumors shown to express this surface molecule.

Our data showed, that pre-incubation of target cells with TMZ alone, even in the absence of Zol, enhances both killing and IFN γ anti-tumoral effector functions of the gdTCEL. We speculate that one mechanism may involve increased intracellular ATP that is induced by TMZ (24) which might increase amounts of substrate for formation of ApppI, a substance that is stimulatory for $\gamma 9\delta 2T$ cells (25). In addition, TMZ increases expression of other molecules that are co-stimulatory for NKG2D expressing $\gamma 9\delta 2T$ cell, e.g. MICA, on GBM cells (26). While further research will be required to identify mechanisms of TMZ augmentation of $\gamma 9\delta 2T$ cell-mediated anti-GBM activity, these findings set the stage

for studying the value of combining pretreatment with TMZ with gdTCEL-mediated immunotherapy in GBM patients. Since TMZ may also be directly toxic to T cells, it is likely that a therapeutic protocol of autologous adoptive $\gamma 9\delta 2T$ cell immunotherapy following TMZ preconditioning will be most efficacious. Intra-tumoral administration of TMZ also decreases the number of intra-tumoral immunosuppressive cells, suggesting an additional advantage to this temporal combination (27).

In summary, our results highlight the possibility of clinical use of autologous gdTCEL for control of GBM in patients, and their incorporation with other reagents, notably the already clinically approved zoledronate and temozolomide. Studies to test these concepts in pre-clinical models will be critical to advance this form of immunotherapy for GBM patients.

ACKNOWLEDGEMENTS

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THE ROLE OF NADPH-DERIVED REACTIVE OXYGEN SPECIES PRODUCTION IN THE PATHOGENESIS OF ENDOMETRIOSIS: A NOVEL MECHANISTIC APPROACH

J. NASSIF¹, S.A. ABBASI¹, A. NASSAR¹, A. ABU-MUSA¹ and A.A. EID²

¹Department of Obstetrics and Gynecology, Faculty of Medicine and Medical Center, American University of Beirut, Beirut, Lebanon; ²Department of Anatomy, Cell Biology and Physiology, Faculty of Medicine, American University of Beirut, Beirut, Lebanon

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Endometriosis is defined as endometriotic tissue growing outside the uterine cavity. It is a common gynecological disorder in women of reproductive age and is associated with chronic pelvic pain and infertility. Despite several studies and theories to explain its cause, the exact pathogenesis of endometriosis remains unclear. Retrograde menstruation is the most plausible theory, however, it is not exclusive. The disparity between the actual prevalence of retrograde menstruation and the prevalence of endometriosis suggests that other factors may determine the susceptibility to endometriosis development. Oxidative stress has been associated with endometriosis. This study aimed to explore the role of NADPH oxidase family in the production of reactive oxygen species (ROS) and to determine whether ROS induce the proliferation of endometriotic implants via mammalian target of rapamycin (mTOR) signaling. Anonymous endometriotic tissue samples were collected from women undergoing laparoscopy for endometriosis. The samples were stained with dihydroethidium and fluorescent images of the slides were taken to detect ROS production. After extraction of RNA from the samples and c-DNA generation, quantitative real-time PCR, protein extraction and Western blot were performed to study gene and protein expression of NADPH oxidase 1 (NOX 1), mTOR and fibronectin. The results showed an increase in ROS levels and NOX 1 gene and protein expression in the endometriotic tissues compared to the normal surrounding tissue control. Also, mTOR and fibronectin, gene expression was found to be increased. Up regulation of NOX at gene and protein level leads to increased production of ROS in the endometriotic tissue, which in turn causes proliferation of the ectopic tissue via alteration of the mTOR signaling pathway. Increased fibronectin gene expression points towards tissue injury in endometriosis as compared to the normal surrounding tissue. This manuscript adds a new insight into the pathogenesis of endometriosis and serves as a background for development of new treatments for the disease-associated pain and infertility.

Endometriosis, a common gynecological disorder, is defined as endometriotic tissue growing outside the uterine cavity (1). The endometriotic implants

result in scar tissue and/or adhesions (2). Patients present with a constellation of symptoms which are not related to the extent of the disease but may be due

Key words: endometriosis, reactive oxygen species, NADPH oxidases, mTOR

Mailing address: Dr Assaad A. Eid,
Department of Anatomy,
Cell Biology and Physiology,
Faculty of Medicine, American University of Beirut,
Beirut, Lebanon
Tel.: +961 1 350000
e-mail: ae49@aub.edu.lb

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to the location of implants (3). The most common symptoms associated with endometriosis include chronic pelvic pain and infertility (4). Endometriosis can also be associated with dysmenorrhea (1 to 2 days prior to the onset of menses), dyspareunia, abnormal uterine bleeding, dyschezia, and dysuria (2). The mechanisms leading to all this panoply of injuries is under investigation.

Varying symptomatology amongst patients makes it difficult to assess the definitive prevalence of endometriosis without performing a diagnostic laparoscopy procedure. The overall prevalence for the symptomatic and asymptomatic women is 5-10% (5). The risk factors for endometriosis include: age at menarche, cycle length, dysmenorrhea, lifestyle, family history, PCB, and dioxin exposure (6).

The exact etiology of endometriosis, despite several theories attempting to explain its origin and pathogenesis, remains yet to be elucidated. Some of the hypotheses include: retrograde menstruation, coelomic metaplasia theory, embryonic remnant theory and lymphatic dissemination theory (7).

Retrograde menstruation

Sampson's retrograde menstruation theory (8) is a widely accepted theory to explain the presence and proliferation of endometriotic tissue in the peritoneal cavity. It is supported by high incidence of endometriosis in the peritoneal cavity and its presence in cases with increased endometrial reflux such as Müllerian duct anomalies and abnormal menstruation (5, 9). However, it fails to explain the presence of endometriosis in extra pelvic sites as well as its presence in men (2). Retrograde menstruation occurs in the majority of women (70-90%) of reproductive age, whereas, endometriosis occurs only in 10% of this group (10).

Coelomic metaplasia theory

Meyer's coelomic metaplasia theory, suggests that pathogenic stimuli (hormonal, infectious or environmental) lead to a metaplastic transformation of the coelomic epithelium. The same embryologic origin of both peritoneal mesothelium and endometrial tissue, and the theory's ability to explain the presence of endometrial implants in extra pelvic sites derived from the coelomic epithelium of the developing embryo gives it a

solid basis. Also, the presence of endometriosis in women lacking functional endometrium (Turner's syndrome, uterine agenesis), premenarchal and postmenopausal women (5, 11) further supports this theory. However, drawbacks of this theory include: the uneven distribution of implants in the peritoneal cavity, and the absence of experimental proof that peritoneal cells could be differentiated to functional endometrial cells (8).

Embryonic remnant theory

Embryonic remnant theory suggests that embryonic remnants left behind by the developing Müllerian duct system are transformed into endometrial tissue when subjected to stimuli. This theory remains unproven and is purely hypothetical (11).

Lymphatic dissemination theory

Lymphatic dissemination theory implicates lymphatic and vascular channels or inadvertent iatrogenic dissemination during gynecological surgeries (9). Like the embryonic remnant theory, lymphatic dissemination is also controversial.

Oxidative Stress

Reactive oxygen species

Reactive oxygen species (ROS) are implicated in many physiological and pathological processes. At low concentration, ROS function as secondary signaling molecules that induce different cellular processes (12). In some pathological cases, either due to overproduction or antioxidant depletion, high concentrations of ROS accumulate and react with different cellular components (lipids, nucleic acids, proteins, enzymes, and other small molecules) leading to oxidative stress (13).

NADPH oxidase (NOX) and ROS production

NOX 2 formerly known as NADPH oxidase is a multimeric transmembrane protein with NADPH and FAD binding domains at the C-terminal (Fig. 1). Upon activation, the cytosolic subunits with the Rac protein translocate to the plasma membrane and assemble together with the cytochrome b558. Once assembled, gp91phox (catalytic subunit) uses NADPH as an electron donor in order to reduce oxygen to superoxide anion, thus producing a burst

of ROS that mediate immune response in phagocytic cells (14, 15). To date, 6 isoforms of NOX 2 have been identified in mammalian cells: NOX1, NOX3, NOX4, NOX5, Dual oxidase 1 (Duox1) and Dual oxidase 2 (Duox2) (16-21).

Mammalian target of rapamycin (mTORC1)

mTOR, a serine/threonine kinase, belongs to the phosphatidylinositol kinase-related kinase (PIKK) family which regulates multiple cellular processes induced by different stimuli (22). It forms two distinct complexes mTORC1 and mTORC2, each with different composition and function (23, 24).

Endometriosis and oxidative stress

Several studies have described oxidative stress as a factor contributing to the pathogenesis, start and progression of endometriosis, by measuring oxidative stress biomarkers in the blood, tissue and peritoneal fluid samples of women with endometriosis (25, 26). Carvalho et al. found that 23 out of 36 studied oxidative stress biomarkers were significantly higher in endometriosis patients compared to normal, while 13 markers showed no significant difference. In endometriosis, oxidative stress is thought to be mainly induced by an overproduction of reactive oxygen species (ROS) rather than antioxidant depletion (27).

Although data regarding association between endometriosis and oxidative stress is available, to date no study has looked into the role of NOX-derived ROS in development and pathogenesis of endometriosis in the endometriotic implants and their surrounding normal tissue. This article aims to determine the role of NADPH oxidase family in the production of ROS in endometriotic lesions compared to the surrounding normal tissue taken as control, as well as the role of NADPH-derived ROS to induce the proliferation of the endometriotic implants through mTOR signaling.

MATERIALS AND METHODS

Sample collection

Following an approval from the Institutional Review Board (IRB), consecutive anonymous endometriotic tissue samples to be discarded, from women undergoing laparoscopy for endometriosis were collected. There was no need to receive patient consent for this study since

informed consent is not required for research involving materials (data, documents, records, or specimens) that have been collected for surgery or biopsy and where the purpose of these materials is to be discarded after pathology readings and the identity of the patient(s) is completely unknown. A total of 15 consecutive anonymous deep infiltrating endometriotic implants were collected from endometriosis patients in the follicular phase of the cycle. The collected samples were stored in liquid nitrogen until the experimental phase of the study. When resecting endometriotic implants, it is advisable to pass in normal tissues around the lesions to reduce the recurrence rate (28). Visual identification of surrounding normal tissues is facilitated by the magnification of the laparoscopic view. The excised implants would therefore have surrounding normal disease-free tissue. It was decided to use these normal tissues surrounding the endometriotic implants, instead of eutopic endometrial tissue, as control due to ethical and feasibility issues. The normal tissues surrounding the nodule on the pathology specimen were then isolated and used as normal control. All samples were histologically proven to be either endometriotic or normal.

Detection of reactive oxygen species (ROS) production

Dihydroethidium (DHE) staining was used to measure the intracellular ROS production. DHE specifically reacts with the superoxide anions and undergoes a two-electron oxidation reaction to form a DNA-binding fluorophore ethidium bromide compound which gives out a red fluorescence. The frozen endometriotic implant samples were cut into 5- μ m-thick sections and placed on glass slides, which were then stored overnight at -80°C. The following day, DHE (10 μ mol/l) was applied to each tissue section and the slides were incubated in a light-protected humidified chamber at 37°C for 30 min. Then, fluorescent images of the ethidium-stained tissue were obtained using a laser-scanning Confocal microscope [Zeiss LSM710 confocal microscope (Carl Zeiss)]. Ethidium bromide was excited to 488 nm and fluorescence was detected at 560 nm long-pass filter. Mean fluorescence intensity of the digitized image was then measured with the Zeiss light software (31).

RNA isolation and cDNA synthesis

Total RNA was isolated from the frozen endometriotic and control tissues by homogenization using a Dounce homogenizer in TRI reagent (Sigma), according to the manufacturer's protocol. To check purity of the RNA, the ratio of A260 to A280 was calculated using NanoDrop ND1000 spectrophotometer. One microgram of the total RNA was used to generate cDNA using an Oligo (dT)₁₈ primer, according to the protocol supplied with the trisol

kit (Thermo Scientific RevertAid First Strand cDNA Synthesis Kit).

Quantitative real-time PCR

In order to study gene expression of NOX1, mTOR and fibronectin, quantitative real-time PCR was performed with an iCycler 1Q Real-Time Detection System using the QuantiFast SYBR Green PCR Kit (Qiagen). The set of primers used for qPCR are as follows: NOX1 (forward primer: GCTGTGCTGGTTGGAGCAGGA, reverse primer: CGCACCTGTCTCCCTGCAGATCC), mTOR (forward primer: CCCTGGTGGAGAGCCGGTGT, reverse primer: TCGGAATGCAGCCAAGCGGG), fibronectin, (forward primer: CCCAGAGGTGCCCCAACTCACT, reverse primer: GGTCCACCTCAGGCCGATGCT), GAPDH (forward primer: TGGTGGACCTGACCTGCCGT, reverse primer: CCTTGAGGGGGCCCTCCGAC), and β -actin (forward primer: GCATGGGTCAGAAGGATTCT, reverse primer: TCGTCCCAGTTGGTGACGAT). The PCR reaction was carried out on 1 μ l of the produced cDNA with a total reaction mixture of 25 μ l. The PCR mixture was heat-denatured at 95°C for 10 min, followed by 45 cycles of 9 s at 95°C, 12 s at 61°C and 9 s at 72°C. Fluorescent signals were acquired at the last step of each cycle. A melting curve was calculated at the end of the cycles. The results were normalized to GAPDH or β -actin, as reference genes, and to the normal control results (obtaining $\Delta\Delta C_q$). The fold change was calculated according to the formula: fold change = $2^{-\Delta\Delta C_q}$ (31).

Protein extraction and Western blot

Tissues were homogenized in liquid nitrogen using

mortar and pestle. The homogenate was then mixed with radioimmune precipitation assay buffer (0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, 100 mM EDTA, 50 mM Tris-HCl pH8, 1% NP-40, protease inhibitor cocktail, phosphatase inhibitor cocktail, 1 mM PMSF) and sonicated. The samples were then incubated at 4°C for 2 h and afterwards were centrifuged at a maximum speed of 13200 g. Finally, proteins in the supernatant were measured using the Bradford assay and stored at -20°C. For immunoblotting, 40 μ g of proteins were loaded, separated by a 10% polyacrylamide gel, and transferred to a nitrocellulose membrane. The membranes were blocked with 5% low-fat milk in Tris-buffered saline and then probed with primary antibodies. The primary antibodies used included: rabbit polyclonal anti-Nox1 (1:1000; Santa Cruz Biotechnology), rabbit polyclonal anti-fibronectin antibody (1:1000, Sigma-Aldrich), rabbit polyclonal phospho-Ser 1387 tuberlin (1:500, Cell Signaling), and rabbit polyclonal phospho-Ser 2448 mTOR (1:1000, cell signaling). The primary antibodies were detected using horseradish peroxidase-conjugated IgG (1:1000, Bio-Rad), and the bands were visualized via enhanced chemiluminescence. Densitometric analysis was then performed using Image J software (31).

RESULTS

Endometriosis is associated with an increase in ROS production

DHE staining was used to assess the level of

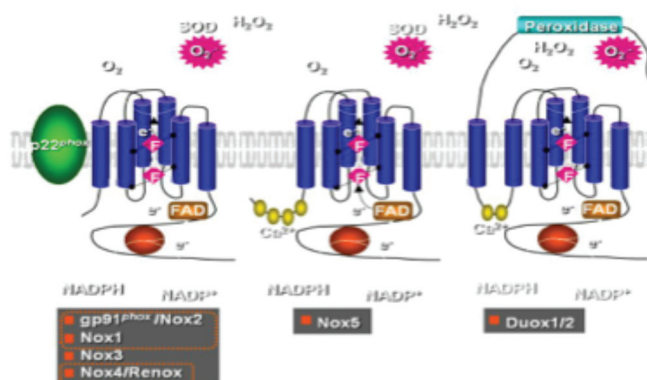


Fig. 1. NADPH oxidase of the Nox/Duox family as source of ROS. Nox NADPH oxidases are electron-transporting membrane enzymes whose primary function is the generation of ROS, linked to the pathobiology of many diseases. The basic catalytic subunit of Nox contains: C-terminal dehydrogenase domain featuring a binding site for NADPH and FAD, and N-terminal domain consisting of six transmembrane alpha-helices that bind two heme groups. Cytosolic NADPH transfers its electron to the FAD, which in turn passes the electrons sequentially to the hemes and ultimately to molecular oxygen on the opposing side of the membrane, to form superoxide anion and thus activating Nox.

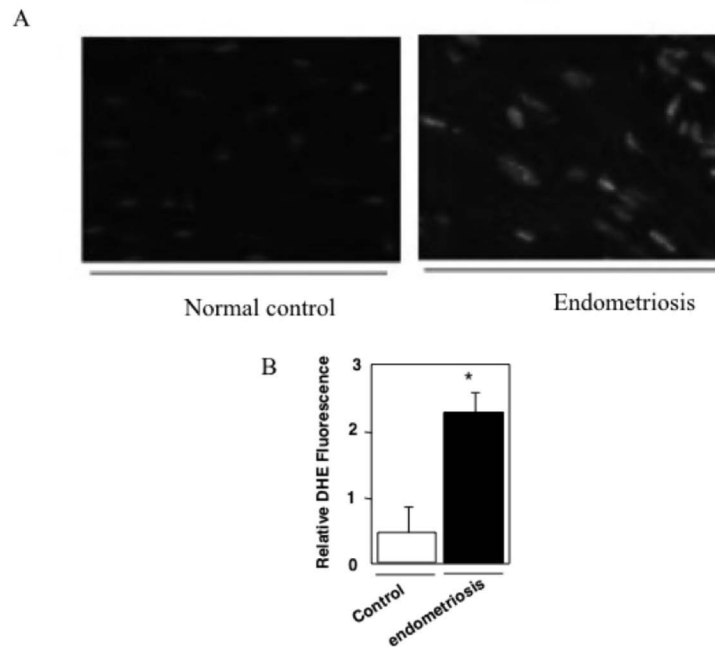


Fig. 2. Endometriosis is associated with an increase in reactive oxygen species production. Reactive oxygen species levels assessed using DHE staining showed an increase in ROS production in endometriotic tissue compared to the normal tissue.

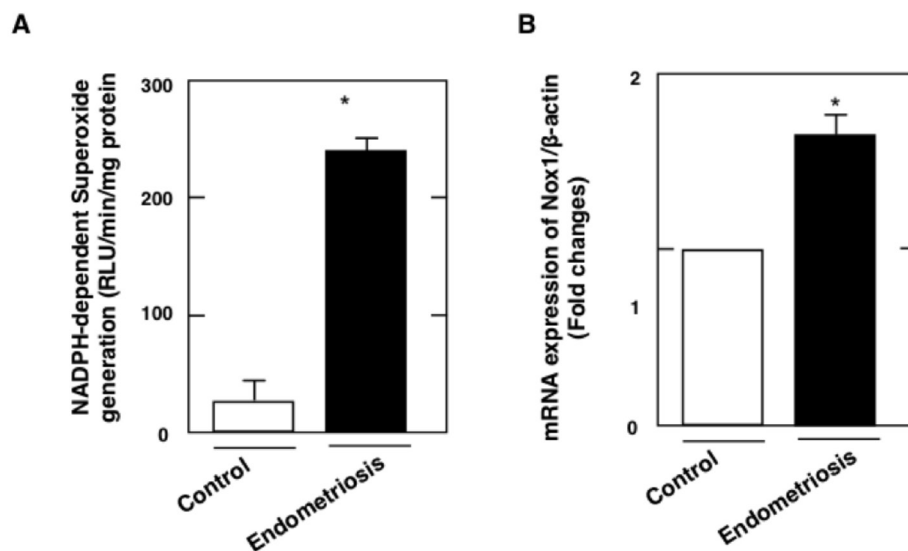


Fig. 3. NADPH dependent superoxide generation (NADPH oxidase activity) and quantitative real-time PCR analysis of Nox 1 in control and endometriotic implants. **A)** NADPH-dependent superoxide generation was increased in endometriotic tissues when compared to normal tissues. **B)** The expression of Nox1 gene, a subunit of the NADPH oxidase family, was up regulated in endometriotic tissues compared to the normal tissues. The results are displayed as fold change, which was calculated relative to the control using the comparative Ct method after normalization against β-actin expression and as the mean ± S.E for 5 different patients.

A

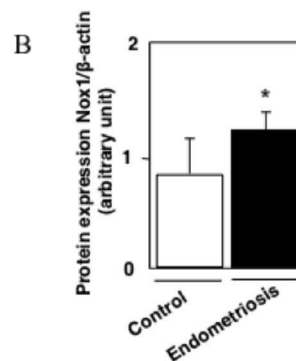
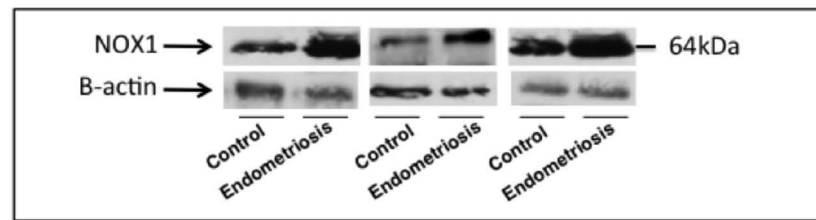


Fig. 4. *NOX1 is one of the contributors of ROS in the endometrial implants. NOX1 protein expression is increased in endometriotic tissues compared to normal tissue (A). Quantitative analysis carried out using Image J software and the results represent the ratio of NOX1/ β -actin as the mean $\pm$ S.E for 3 different patients (B).*

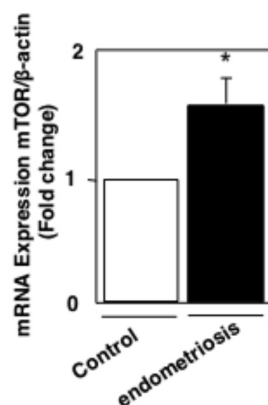


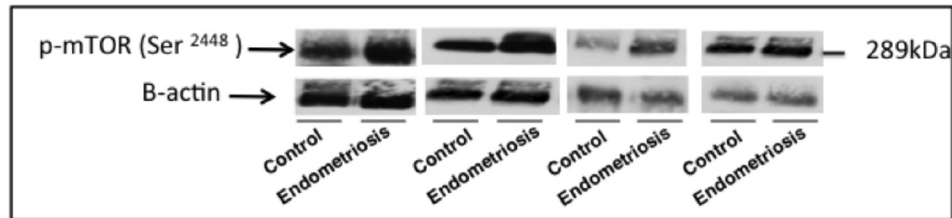
Fig. 5. *Quantitative real-time PCR analysis of cell proliferation related genes. The expression of mTOR was up regulated in the ectopic endometrial tissue as compared to the normal tissue. The results are displayed as fold change, which was calculated relative to the control using the comparative Ct method after normalization against β -actin expression and as the mean $\pm$ S.E for 5 different patients.*

intracellular ROS produced in the endometriotic tissues compared to the adjacent normal tissue (control). DHE specifically interacts with the superoxide anions and is oxidized to produce a red fluorophore “ethidium”, which interacts with double stranded DNA and appears as punctuate nuclear staining. The intensity of DHE fluorescence was found to be enhanced in the endometriotic tissue compared to the control, thus depicting that endometriosis is associated with a significant increase in ROS production (Fig. 2).

Source of ROS in endometriotic lesions

Several factors, including enhanced immune reaction against endometriotic tissue and environmental toxins (e.g., dioxin), have been implicated in increased ROS production but the exact source of ROS remains unknown. NADPH oxidase family has been shown to be an important source of ROS in many tissues (31), however, its role in endometriosis is still unknown. Our results show a significant increase in NADPH-dependent

A



B

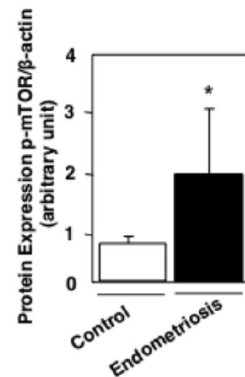
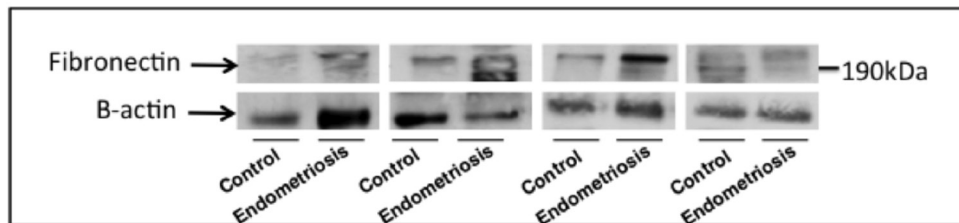
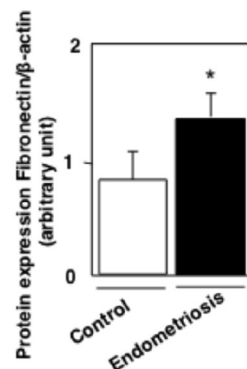


Fig. 6. Western blot analysis of phosphorylated mTOR at Ser²⁴⁴⁸ reveals mTOR activation. MTOR activation is increased in the endometriotic tissues compared to the normal tissue (A). Quantitative analysis was carried out using Image J software and the results represent the ratio of p-mTOR/β-actin as the mean $\pm$ S.E for 4 different patients (B).

A



B



C

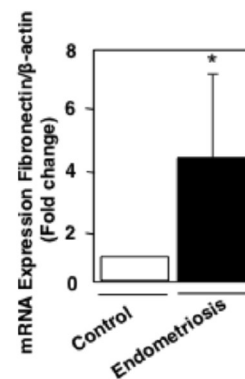


Fig. 7. mTOR activation enhances fibronectin gene and protein expression. Western blot analysis reveals an increase in fibronectin protein expression in ectopic endometrial tissue in respect to normal control tissue (A) and results were quantified using Image J software as the mean of fibronectin/β-actin for $\pm$ S.E for 4 different patients (B). Quantitative real time PCR analysis also showed 4-fold change increase in fibronectin gene expression when normalized to GAPDH, where the results represent the mean $\pm$ S.E for 5 different patients (C).

superoxide generation in endometriotic implants when compared to normal tissues (Fig. 3A). Concomitant with this result, NOX1 mRNA levels were significantly increased in the endometriotic tissue compared to control (Fig. 3B). In addition, the results pointed towards a significant increase in NOX 1 expression at the protein level (Fig. 4).

NADPH-derived ROS trigger a downstream redox signaling, inducing proliferation

NADPH-derived ROS induce proliferation of the endometriotic implants through mTOR signaling. In order to assess the level of mTOR gene expression, quantitative real-time PCR was carried out. It showed that mTOR mRNA expression was slightly significantly increased (Fig. 5). Moreover, Western blot analysis demonstrated an increase in mTOR activation, assessed by the increase in mTOR phosphorylation at its activating site Ser2448 (Fig. 6).

Endometriosis induces fibronectin expression

The expression of fibronectin as a marker of tissue injury in the endometriotic implants compared to the control was studied. Results showed that the expression of fibronectin was significantly increased at both mRNA and protein level in the endometriotic tissue as compared to the control (Fig. 7).

DISCUSSION

The role of NOX-derived ROS has been extensively studied in the pathogenesis of chronic diseases such as Alzheimer's disease, cardiovascular diseases and diabetes, where oxidative stress is a common theme (29). Lambeth uses an antagonistic pleiotropy theory, which states that propagation and expansion of genes has a benefit earlier in life but later leads to harmful effects, to explain this association. These genes, despite their harmful effects, are retained because reproduction occurs before disease manifests and also because natural selection is less effective with age (29). This theory fails to fully rationalize NOX-derived ROS-mediated oxidative stress due to increasing evidence of an association between endometriosis and oxidative stress, with endometriosis being a disease of women in their reproductive years.

Oxidative stress has emerged as an important contributor in the development and progression of endometriosis. In an approach to determine the source of ROS, Ngô et al. demonstrated that the superoxides are mostly produced by the cytosolic enzymes in the stromal endometriotic cells, whereas, in the epithelial endometriotic cells they are produced by mitochondrial complexes (30).

In the present study, the possible role of the NADPH oxidase family in production of ROS at the site of endometriotic implants compared to the surrounding normal tissue was studied. As a first step, the gene and protein expression of NOX1 was explored, since it has been shown to play a role in proliferation, and in signaling pathway that is altered during the development of endometriosis which either regulates or is regulated by the ROS production. Eid et al. (2010) has previously shown that AMP-activated protein kinase (AMPK) regulates ROS production through the NADPH oxidase family (31). AMPK is known to down regulate the expression of NADPH oxidases (30, 32); therefore, it is interesting to explore the pathway of AMPK/ROS production and its signaling cascade during the development of endometriosis.

The results showed an increase in ROS levels and NOX 1 gene and protein expression in the endometriotic implants as compared to the control. In addition, mTOR gene expression was increased in endometriotic tissues, suggesting that NADPH-derived ROS induce proliferation of endometriotic implants by targeting the mTOR pathway. Up regulation and activation of the mTOR pathway suggests that either mTOR is directly activated by an upstream signaling pathway (AMPK pathway) or a larger patient sample is needed to assess the regulation of this pathway.

In addition, an increased expression of fibronectin was found in the endometriotic implants as compared to the control. An increase in fibronectin has been reported in injured tissues (33). Beliard et al. (34) showed an increase in fibronectin expression in the endometriotic implants as compared to the eutopic endometrial tissue. Combining results from the two studies show similarity between eutopic and surrounding tissues in regard to fibronectin expression.

This study is the first, to our knowledge, to identify

the source of an increase in ROS concentrations in endometriosis, as it is an important factor in oxidative stress therapy which can be counteracted by using specific therapeutic inhibitors. Our study demonstrated that an increased NOX1 expression in the endometriotic tissues suggests its role as a major contributor in ROS production. In addition, an increase in mTOR activation and fibronectin expression was observed in endometriotic tissue compared to the surrounding normal tissue, indicating that it might be an important factor in regulation of endometriotic proliferation.

Our data show correlative evidence whereby more work is needed to draw final conclusions, and further studies on the role of NOX derived ROS in endometriosis are needed. The use of primary cell culture with specific inhibitors or silencing of NOX expression using small interfering RNA (siRNA) will help to confirm whether NOXs are the source of ROS. Moreover, it will help to determine whether NADPH-derived ROS are responsible for the up regulation of mTOR expression and activation, as well as for the proliferative phenotype of endometriotic tissue at the ectopic sites.

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PENEHYCLIDINE HYDROCHLORIDE POSTCONDITIONING AMELIORATES CERE-BRAL ISCHEMIA-REPERFUSION INJURY: CRITICAL ROLE OF MITOCHONDRIAL ATP SENSITIVE POTASSIUM CHANNEL

Y. SHU¹, Z. LI² and B. HAN¹

¹*Department of Anesthesiology, The First Affiliated Hospital of Xi'an Medical University, Xi'an, China;*

²*Emergency Department, The First Affiliated Hospital of Xi'an Medical University, Xi'an, China*

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Cerebral ischemia/reperfusion (CI/R) leads to disability and death worldwide. However, limited approaches have been made in developing therapies to decrease the detrimental effects of CI/R. In the present study, we evaluated the effects of penehyclidine hydrochloride (PHC) postconditioning on CI/R injury and elucidated the underlying mechanisms. In CI/R rats, we showed that PHC postconditioning could effectively inhibit I/R-induced brain infarction and edema, and deficit of neurological function. In addition, PHC postconditioning significantly inhibited I/R-induced increase of proinflammatory cytokines and TUNEL-positive cell numbers. Moreover, the opening of mitochondrial permeability transition pore (mPTP) and oxidative stress resulting from I/R were suppressed notably by PHC postconditioning, as evidenced by increased absorbance at 540 nm in Ca²⁺-induced mitochondrial swelling test and increased SOD activity and decreased ROS and MDA level. 5-hydroxydecanoate (5-HD), a specific inhibitor of MitoKATP, was used to evaluate the role of MitoKATP in the neuro-protective effect of PHC postconditioning. The results showed that 5-HD could markedly suppress PHC postconditioning-caused inhibition of brain infarction and edema, deficit of neurological function, inflammation, apoptosis, opening of mPTP and oxidative stress induced by CI/R. The results demonstrated that PHC postconditioning could protect against I/R injury in brain through the inhibition of oxidative stress, mPTP opening, apoptosis and inflammation. Activation of MitoKATP was critical for PHC postconditioning-exhibited neuro-protective effects against I/R injury. These findings may provide a novel foundation for therapeutic strategies targeting cerebral protection against I/R damage.

Cerebral ischemia, also called ischemic stroke, resulting from obstruction of a blood vessel supplying the brain, could cause irreparable cerebral injury which leads to disability and death worldwide. It is reported that acute ischemic stroke causes 10-12% of all deaths in western countries (1). Cerebral ischemia results in disruption of the blood-brain barrier (BBB), and the subsequent vasogenic edema (2-4) and neuronal cell death (5). Reperfusion after ischemia in brain

may further promote the damage to BBB and induce water entry through endothelial cells, resulting in cerebral infarction (6). Cerebral ischemia/reperfusion (CI/R), a major type of ischemia/reperfusion injury, is a complex pathophysiological process. A variety of pathological factors participate in the development of CI/R, including hypoxia, free radical/reactive oxygen species (ROS) burst, mitochondrial dysfunction, intracellular Ca²⁺ overload, leukocyte accumulation,

Key words: penehyclidine hydrochloride, cerebral ischemia/reperfusion, MitoK_{ATP}, mitochondrial permeability transition pore, apoptosis, oxidative stress

Mailing address:

Dr Bin Han, Department of Anesthesiology,
The First Affiliated Hospital
Xi'an Medical University,
Fenghao West Road, Xi'an 710077, China
e-mail: bin_han2001@163.com

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release of inflammatory factors, generation of excitatory amino acid, metabolic disorder and water and electrolyte disturbance etc (7). To date, limited approaches have been made in developing therapies to decrease the detrimental effects of CI/R.

Penethylidine hydrochloride (PHC)-3-(2-hydroxyl-2-cyclopentyl-2-phenyl-ethoxy) quinuclidine is a selective anticholinergic agent, developed by the Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences, People's Republic of China. PHC has become a widely used anesthetic premedication and a treatment for smooth muscle spasm (8-10). A number of studies have shown that PHC protects against lung injury induced by various factors (11-14). Inhibition of inflammation, oxidative stress, stress-sensitive signaling pathways and apoptotic cell death has been reported to be involved in the protective effect of PHC (15, 16). In addition, it has been shown that PHC could attenuate several types of I/R injuries, including renal and gastric I/R (17, 18). More recently, PHC has been shown to attenuate focal cerebral I/R injury in rats, inhibit glutamate release in global brain I/R rats and exhibit potential neuroprotective effect (19, 20). However, the molecular mechanism underlying the effect of PHC on CI/R remains unclear.

The aim of this study was to evaluate the effects of PHC postconditioning on CI/R injury and elucidate the underlying mechanisms. The results showed that PHC postconditioning significantly ameliorated I/R injury in brain and activation of MitoKATP was critical for the neuro-protective effect of PHC postconditioning against I/R injury.

MATERIALS AND METHODS

Chemicals and reagents

PHC injection (1 mg/1 mL) was purchased from List Pharmaceutical (Chengdu, China). β -actin and CytoC antibodies were purchased from Santa Cruz. Bax, Bcl-2 and cleaved caspase 3 antibodies were purchased from CST.

Animal model and treatment

Animal care was in accordance with the guidelines for the Care and Use of Laboratory Animals and the principles presented by Xi'an Medical University. All efforts were

made to reduce suffering and the number of animals used. Ninety adult Wistar rats (200 ~ 250 g) were purchased from the Experimental Animal Centre of Xi'an Medical University. Rats were randomly divided into five groups: Sham (18 rats); CIR group (18 rats); PHC postconditioning group (18 rats); PHC postconditioning + 5-HD group (18 rats); 5-HD group (18 rats).

For the establishment of CI/R animal model, transient middle cerebral artery occlusion (MCAO) of rats was produced by filament occlusion of the right MCA as previously reported (21). Briefly, rats were anesthetized in a facemask filled with isoflurane (3% induction and 1.2% maintenance) in oxygen-enriched air. The experiment was carried out on heating lamps to ensure rectal temperature at $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$. For the performance of unilateral MCAO, a 7-0 nylon monofilament was inserted into the internal carotid artery via an external carotid artery stump and then the filament tip for occlusion was placed at a distance of 8 to 9 mm beyond the internal carotid/pterygopalatine artery bifurcation. After 120 min, MCAO was stopped to allow reperfusion. In the sham group, rats underwent identical surgical procedures and received intravenous (i.v.) injection of isotonic saline without MCA; in the CI/R group, rats received I/R procedures and i.v. injection of isotonic saline 20 min after I/R operation; in the PHC group, rats received I/R procedures and i.v. injection of PHC (0.02 mg/kg) 20 min after I/R operation; in the PHC + 5-HD group, rats received I/R procedures and i.v. injection of PHC (0.02 mg/kg) with 5-HD 20 min after I/R operation; in the 5-HD group, rats received I/R procedures and i.v. injection of 5-HD 20 min after I/R operation. 48 h after reperfusion, rats were anesthetized and samples were collected for further detection.

Evaluation of neurological function

Neurological function was evaluated 48 h after the reperfusion. A modified Bederson score was determined according to the following scoring system: 0, no deficit; 1, forelimb flexion; 2, as for 1, plus decreased resistance to lateral push; 3, unidirectional circling; 4, longitudinal spinning or seizure activity; and 5, no spontaneous motor activity.

Brain infarction measurement

Brain infarct volume was determined using 2, 3, 5-triphenyltetrazolium chloride (TTC) as previous described (21). Forty-eight hours after the reperfusion,

rats were anesthetized with 4% isoflurane and brains were removed and cut coronally at a thickness of 2 mm and incubated in 2% TTC at 37°C for 20 min. After that, brain sections were fixed in 4% paraformaldehyde at 4°C overnight and scanned into a computer. The injury was quantified using Image J software. Infarct volume was expressed as a percentage of the contralateral hemisphere.

Measurement of brain edema

Forty-eight hours after the reperfusion, rats were anesthetized with 4% isoflurane and brains were removed. Wet weight of brain was weighed and then dry weight was measured after drying at 105°C for 24 h. Brain moisture content (%) was calculated as follows: (wet weight – dry weight)/wet weight × 100.

Determination of mPTP opening

The rat brain mitochondria were isolated using a Mitochondria Isolation Assay Kit (Pierce). The entire isolation procedure was carried out at 4°C and mitochondrial pellet was resuspended in an assay buffer (250 mM sucrose, 20 mM MOPS, 10 mM Tris-Base, 100 mM Pi(K), 0.5 mM Mg²⁺, pH 7.0), and the protein content was determined using the BCA protein assay kit. The mitochondria were used in experiments within 4 h of the decapitation of the animal. Ca²⁺-induced mitochondrial swelling could be used as an index of increased mPTP opening. An aliquot of the mitochondria (100 mg) was diluted in 1 ml of assay buffer at 25°C. The swelling was induced by adding of 100 µM CaCl₂ and measured with a spectrophotometer as a decrease in light scattering at 540 nm for 10 min.

Determination of apoptosis

After the experiment, hippocampus CA1 was collected and single-cell suspension was made. Annexin V/PI staining (Roche) was conducted according to the manufacturer's instructions and cell apoptosis was analyzed by flow cytometry. In addition, brain sections were cut and stained with TUNEL Assay kit (Roche) and apoptotic cells were observed under confocal microscope.

Evaluation of oxidative stress

For the evaluation of oxidative stress, brain tissues were homogenized in saline and malonaldehyde (MDA) level and superoxide dismutase (SOD) activity were determined by commercial assay kits according the manufacturer's

protocols. In addition, isolated mitochondria was incubated with H2DCF-DA (10 µM) for 30 min at 37°C. Then ROS level was detected by flow cytometry and results were shown as percentage of Sham group.

Western blot

After the experiment, hippocampus CA1 tissues were isolated and were lysed with ice-cold RIPA buffer (TIANGEN). The lysates were centrifuged at 13,200 rpm for 1 hr at 4°C to produce whole-cell extracts. Protein content was quantified using the BCA method. Protein (50 µg) was separated on a 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane. After blocking with 5% BSA prepared in TBS for 1 h at room temperature, immunoblots were incubated with primary antibodies overnight at 4°C. After washing for four times with TBST buffer, blots were then incubated with horseradish peroxidase-linked IgG secondary antibodies (Pierce, IL, USA) for 30 min at 37°C. Enhanced chemiluminescence was performed by ECL (Pierce, IL, USA).

Quantitative real-time PCR

Total RNA was isolated using a commercial RNA extraction kit (TIANGEN). 500 ng of total RNA from rat brain tissue was reverse-transcribed to cDNA using a commercially available kit (Takara). Quantitative real-time PCR was performed with iCycler Thermal Cycler (Bio-Rad, USA) using 2X SYBR Green master mixes (Bio-Rad, USA). Forty cycles were conducted as follows: 95°C for 30 s, 60°C for 30 s, proceeded by 10 min at 95°C for polymerase activation. Quantification was performed by the delta cycle time method, with rat β-actin used for normalization.

Statistical analysis

Results are presented as mean ± SD. Statistical analysis was performed by GraphPad Software. The statistical significance of differences between groups was analyzed via one-way analysis of variance followed by a Dunnett's t-test for multiple comparisons. A P value <0.05 was considered to be significant.

RESULTS

Effect of PHC postconditioning on CI/R injury in rats

In the present study, rat model of CI/R injury

was established by MCAO, and at 20 min after the reperfusion, PHC was injected intravenously. Forty-eight hours after the operation, brain infarction was determined. Fig. 1A and B show that the I/R operation significantly increased infarction volume in rats. PHC postconditioning notably decreased infarction volume induced by I/R treatment. To study whether opening of MitoKATP was involved in the protective effect of PHC, several rats were injected

with 5-hydroxydecanoate (5-HD), a specific inhibitor of MitoKATP, after the I/R operation. We calculated that in the presence of 5-HD, PHC postconditioning-induced decrease of brain infarction volume was significantly blocked (Fig. 1A and B). 5-HD treatment alone slightly increased brain infarction volume in CI/R-treated rats without statistical significance (Fig. 1A and B). In addition, neurological scores were evaluated and the results showed that I/R operation

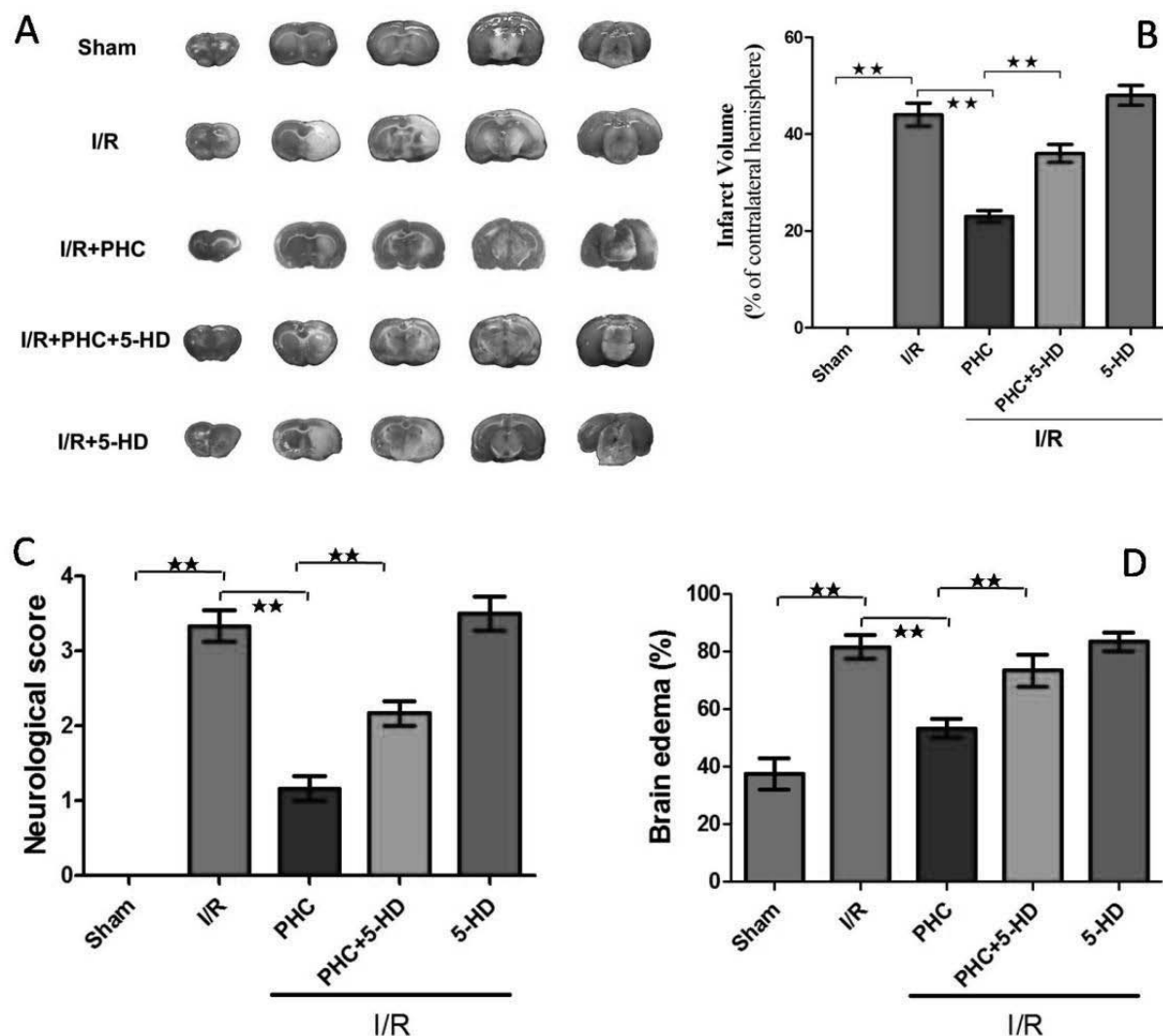


Fig. 1. Effect of PHC on CI/R-induced injury in rats. Rats underwent transient middle cerebral artery occlusion and received intravenous injection of PHC with or without 5-HD. After the experiment, brain infarction was determined by TTC (A) and infarct volume was calculated (B) (8 rats). Neurological score was evaluated by a modified Bederson score (C) (8 rats). Brain edema was also detected by evaluation of water content (D) (8 rats). ** $P < 0.05$ indicates statistical significance between groups.

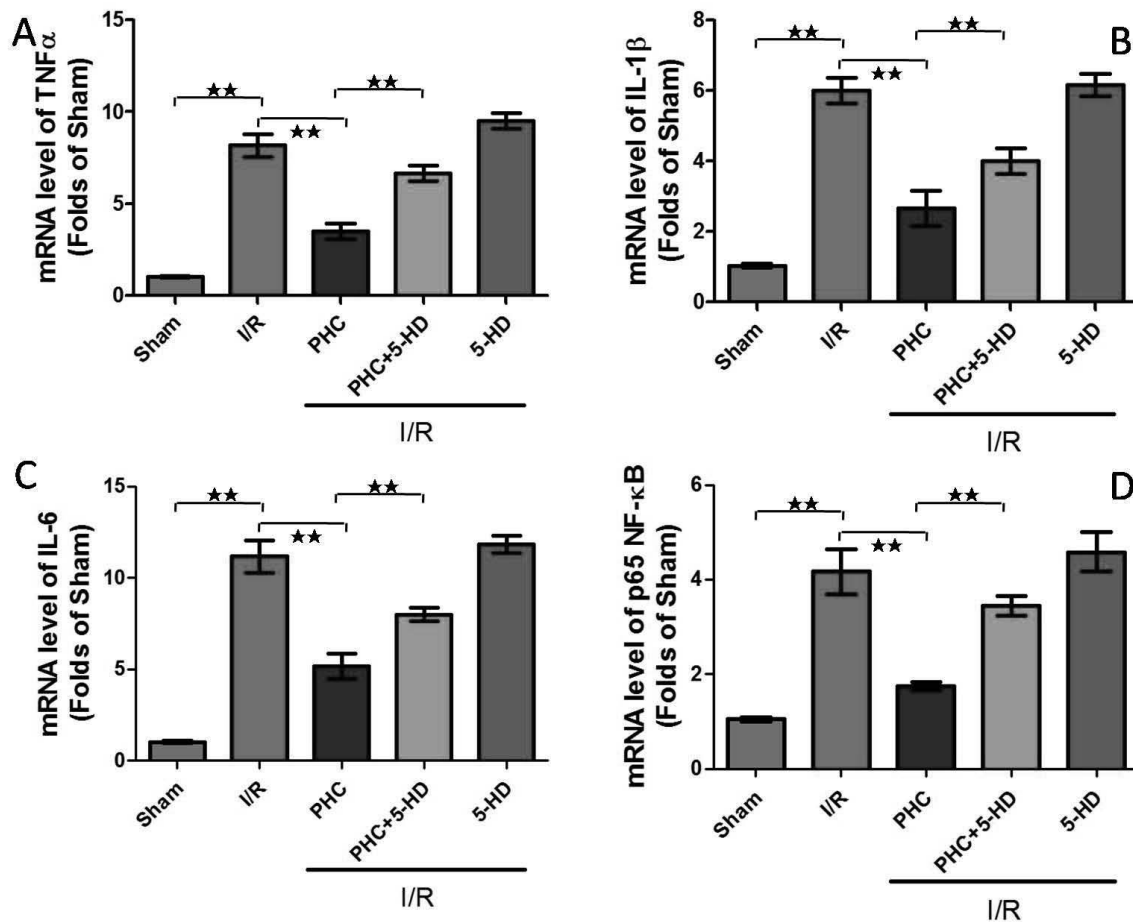


Fig. 2. Effect of PHC on proinflammatory cytokines in rats treated with CI/R. Rats underwent transient middle cerebral artery occlusion and received intravenous injection of PHC with or without 5-HD. After the experiment, mRNA expression of TNF α (A), IL-1 β (B), IL-6 (C) and NF- κ B (D) was determined by Real-time PCR and results were expressed as folds of Sham group (6 rats). ** $P < 0.05$ indicates statistical significance between groups.

induced significant increase of neurological score which was inhibited by PHC postconditioning (Fig. 1C). 5-HD showed notable suppressive effect on PHC-exhibited decrease of neurological score (Fig. 1C). The results of brain edema were consistent with that of infarction volume and neurological score (Fig. 1D). These results demonstrated that PHC postconditioning could protect against CI/R injury and opening of MitoKATP was involved in the protective effect of PHC postconditioning.

PHC postconditioning on proinflammatory cytokines in CI/R rats

Levels of proinflammatory cytokines in brains of CI/R rats were determined by real-time PCR. As reflected in Fig. 2, CI/R resulted in significant increase of

mRNA expression of tumor necrotic factor- α (TNF- α) (Fig. 2A), IL-1 β (Fig. 2B), IL-6 (Fig. 2C) and p65 NF- κ B (Fig. 2D), indicating the severe inflammatory injury. PHC postconditioning remarkably decreased mRNA levels of TNF- α (Fig. 2A), IL-1 β (Fig. 2B), IL-6 (Fig. 2C) and p65 NF- κ B (Fig. 2D) induced by CI/R. Moreover, the inhibitory effect of PHC postconditioning on TNF- α (Fig. 2A), IL-1 β (Fig. 2B), IL-6 (Fig. 2C) and p65 NF- κ B (Fig. 2D) was blocked by 5-HD. The results implicated that PHC postconditioning could inhibit CI/R-induced inflammation which involved the opening of MitoKATP.

PHC postconditioning on apoptosis in CI/R rats

Next, we evaluated the effect of PHC

postconditioning on apoptosis of hippocampus CA1 in CI/R rats. In Fig. 3A, we showed that both Annexin V and PI-positive cells were increased by CI/R treatment. PHC postconditioning could significantly decrease the number of Annexin V-positive/ PI-negative cells, indicating that PHC postconditioning mainly inhibited CI/R-induced apoptotic cell death (Fig. 3A). 5-HD treatment could markedly reversed the effect of PHC on cell Annexin V and PI staining. In addition, apoptosis was determined by TUNEL

staining. As shown in Fig. 3B, we showed that TUNEL-positive cell numbers increased dramatically in CI/R rats. PHC postconditioning significantly decreased TUNEL-positive cell numbers induced by CI/R (Fig. 3B). Consistently, in the I/R + PHC + 5-HD group, TUNEL-positive cell numbers were significantly higher than that of the I/R + PHC group (Fig. 3B). Moreover, apoptotic molecular events were examined by Western blot. As shown in Fig. 3C, protein expression of Bax, cytochrome C

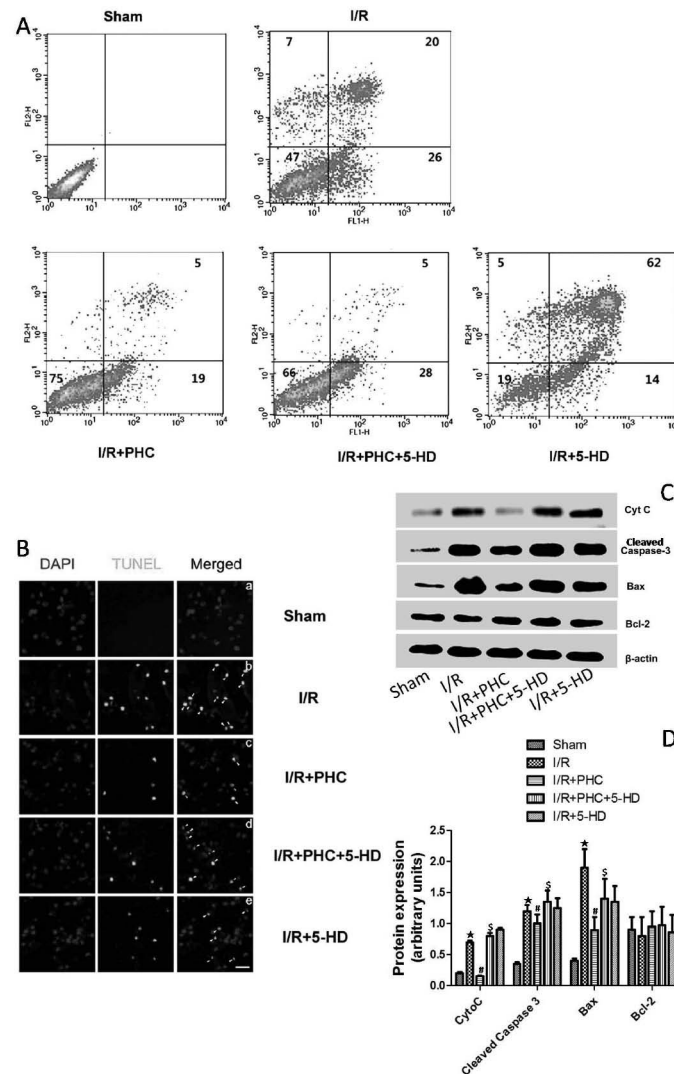


Fig. 3. Effect of PHC on apoptosis in rats treated with CI/R. Rats underwent transient middle cerebral artery occlusion and received intravenous injection of PHC with or without 5-HD. After the experiment, apoptosis was evaluated by Annexin V/PI (A) and TUNEL staining (B) and detected by flow cytometry and confocal microscope, respectively (6 rats). Protein expression of CytoC, cleaved caspase-3, Bax and Bcl-2 was examined by Western blot (C) (6 rats). Representative images and blots are shown. Results of Western blot are shown as mean $\pm$ SD (D) (6 rats). * $P < 0.05$ indicates statistical significance, compared with Sham; # $P < 0.05$ indicates statistical significance, compared with I/R; \$ $P < 0.05$ indicates statistical significance, compared with I/R+PHC.

(CytoC), and cleaved caspase-3 was increased in rats of the I/R group. PHC postconditioning significantly inhibited the increase of Bax, cytochrome C (CytoC), and cleaved caspase-3 expression, compared with that of the I/R group, which was blocked in the presence of 5-HD (Fig. 3C). In the experiment, Bcl-2 protein expression was not significantly influenced by I/R, PHC postconditioning and 5-HD. These results indicate that postconditioning may inhibit I/R operation-induced activation of mitochondrial apoptotic pathway, which involved the opening of MitoKATP.

PHC postconditioning on mPTP opening in CI/R rats

The opening of mitochondrial permeability transition pore (mPTP) is closely related with the initiation of apoptosis (22). In the study, we next investigated the changes of brain mPTP. As shown in Fig. 4, Ca^{2+} -induced mitochondrial swelling was used to evaluate mPTP opening. As the time period prolonged, absorbance at 540 nm in the I/R group decreased more quickly than that of the sham group. The postconditioning of PHC significantly attenuated the decreasing rate induced by I/R treatment. In the presence of 5-HD, absorbance at 540 nm at different

time points was significantly lower than that of the I/R + PHC group (Fig. 4). These results demonstrated that postconditioning of PHC may inhibit I/R-resulted opening of mPTP. Opening of MitoKATP was involved in PHC-induced inhibitory effect on the opening of mPTP.

PHC postconditioning on oxidative stress in CI/R rats

Oxidative stress is a major contributor to the opening of mPTP (23). In the next step, we evaluated oxidative stress in brain tissues of CI/R rats. Isolated mitochondria were incubated with H2DCF-DA to detect ROS generation. As shown in Fig. 5A, ROS level was significantly increased in CI/R rats. In contrast, ROS level in I/R + PHC was notably lower than that of the I/R group. In addition, 5-HD significantly suppressed the inhibitory effect of PHC on ROS level in CI/R rats. Superoxide dismutase (SOD) activity in CI/R rats was markedly decreased and PHC postconditioning significantly inhibited I/R-induced decrease of SOD activity (Fig. 5B). In the presence of 5-HD, PHC-exerted enhancement of SOD activity in I/R rats was notably blocked (Fig. 5B). Consistently, CI/R operation resulted in significant increase of malonaldehyde (MDA) content

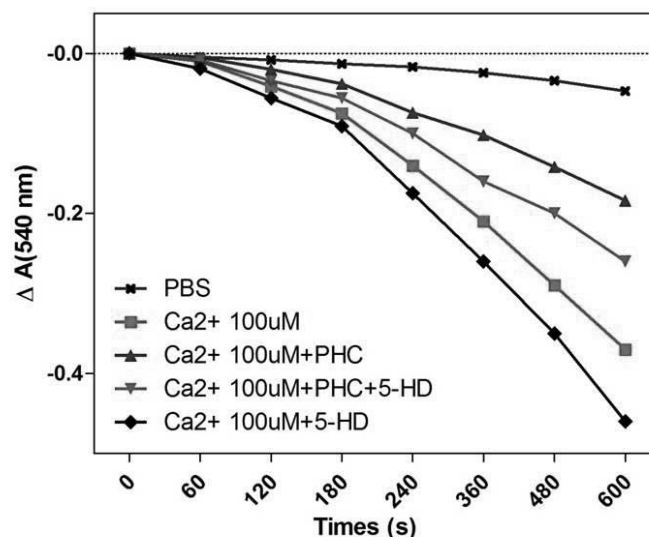


Fig. 4. Effect of PHC on mPTP opening in rats treated with CI/R. Rats underwent transient middle cerebral artery occlusion and received intravenous injection of PHC with or without 5-HD. After the experiment, brain mitochondria were isolated and mPTP opening was determined by Ca^{2+} -induced mitochondrial swelling (6 rats).

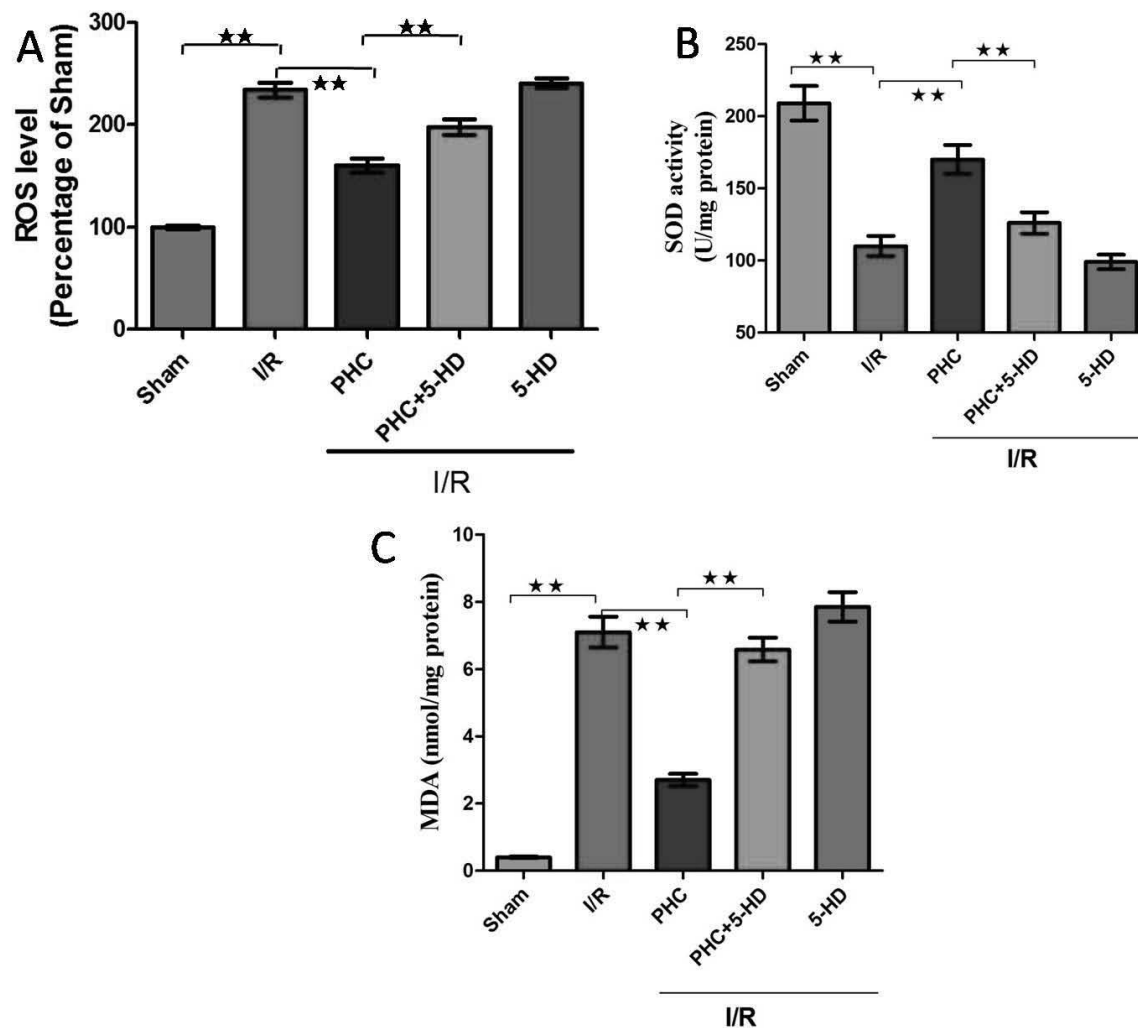


Fig. 5. Effect of PHC on oxidative stress in rats treated with CI/R. Rats underwent transient middle cerebral artery occlusion and received intravenous injection of PHC with or without 5-HD. After the experiment, MDA level (A), SOD activity (B) and ROS level (C) were detected to evaluate oxidative stress (8 rats). ** $P < 0.05$ indicates statistical significance between groups.

which could be suppressed by PHC postconditioning (Fig. 5C). Moreover, the inhibitory effect of PHC postconditioning on MDA level was blocked by 5-HD (Fig. 5C). These results demonstrated that CI/R resulted in significant oxidative injury and PHC postconditioning could exhibit protective effect against I/R-induced oxidative stress through, at least partly, the opening of MitoKATP.

Furthermore, we tried to find the possible molecular pathways that mediated PHC-exerted activation of MitoKATP. We tested adenosine 5'-monophosphate (AMP)-activated protein kinase

(AMPK), phosphatidyl inositol 3-kinase (PI3K)-Akt, and mitogen activated protein kinases (MAPKs) pathways. However, the results showed that PHC had no significant effect on AMPK, PI3K-Akt, and MAPK pathways (data not shown). Previous studies have shown that nitric oxide synthase (NOS) activation and nitric oxide (NO) release are associated with activation of MitoKATP (24). However, our results showed that PHC did not significantly affect NOS expression and NO content (data not shown). Moreover, we tested possible receptors that mediated PHC-exerted activation of MitoKATP. Previous results have shown

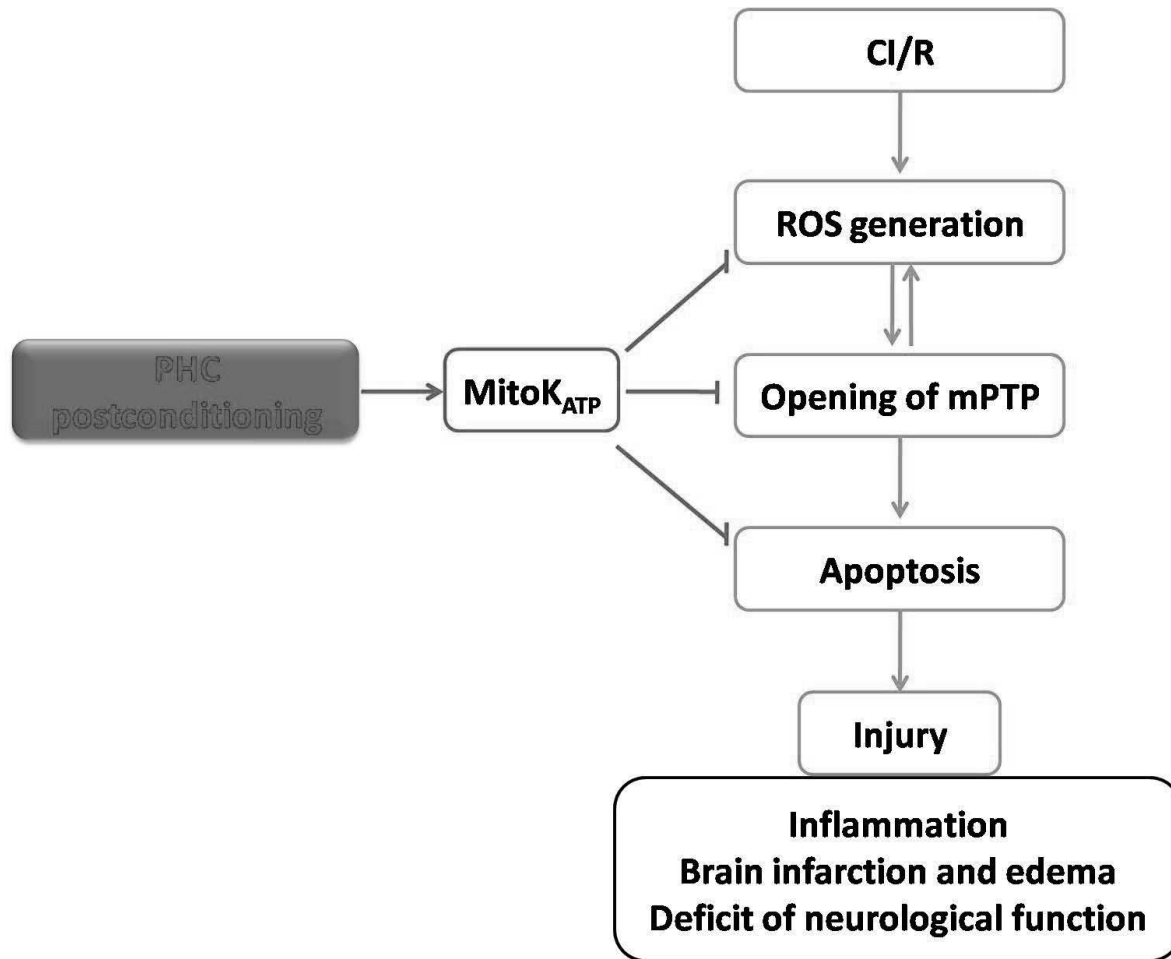


Fig. 6. Mechanism of neuro-protective effect of PHC postconditioning against I/R injury. PHC postconditioning could inhibit I/R-induced inflammation, brain infarction and edema, and deficit of neurological function. The inhibition of apoptosis, opening of mPTP and oxidative stress were involved in the neuro-protective effect of PHC postconditioning against I/R injury. Moreover, activation of MitoKATP was critical for PHC postconditioning-exerted effect on redox status, mPTP opening, apoptosis, inflammation, brain infarction and edema, and deficit of neurological function in CI/R rats.

that inhibition of M1 and M3 receptors were involved in the protective effect of PHC (25). In the present study, we did not observe a significant effect of PHC on M1 and M3 receptors (data not shown). Further studies are needed to clarify the molecular mechanisms that mediate the activation of MitoKATP induced by PHC.

DISCUSSION

Ischemic and pharmacological preconditioning has been shown to benefit the recovery of I/R injury (26, 27). However, compared with ischemia, reperfusion

is more easily to predict. Thus, intervention of postconditioning, through modulation of reperfusion rather than ischemia, has been proposed to protect I/R-induced tissue injury. Several studies have shown the protective effects of postconditioning against I/R injury. For instance, Zhao et al. (28) demonstrated that both ischemic and pharmacological postconditioning could protect against cardiac I/R injury. Ischemic postconditioning has also been shown to reduce CI/R injury (29).

In the present study, we examined the protective effect of PHC postconditioning on CI/R injury in rats

and elucidated the molecular mechanism. We found that PHC postconditioning could effectively inhibit I/R-induced brain infarction and edema, and the deficit of neurological function.

Complex mechanisms are involved in the protective effect of postconditioning against I/R injury, among which opening of the mitochondrial potassium ATP-dependent channel (MitoKATP) has been extensively described (30, 31). It is reported that MitoKATP plays important roles in the protective effects of postconditioning against CI/R injury (32, 33). MitoKATP channel openers protect neuron by mimicking ischemic preconditioning. In the current study, we examined the possible role of MitoKATP in PHC postconditioning-mediated neuro-protective effects. The results showed that 5-HD significantly inhibit the protective effects of PHC postconditioning on I/R-induced brain infarction and edema, and the deficit of neurological function. These results demonstrate that activation of MitoKATP was involved in PHC postconditioning-exhibited neuro-protective effects against I/R injury in brain.

Apoptosis and inflammation contribute to the development of CI/R injury. In the study, we also tested whether PHC postconditioning could markedly inhibit I/R-induced inflammation and apoptosis, which may be involved in the protective effects of PHC postconditioning against CI/R injury. Previous studies have shown that activation of MitoKATP could regulate apoptotic signaling and suppress apoptotic cell death (34-36). Wang et al. (34) showed that nicorandil inhibited hypoxia-induced apoptosis in human pulmonary artery endothelial cells through activation of MitoKATP. Zhang et al. (35) found that MitoKATP opener iptakalim protected against MPP-induced astrocytic apoptosis via mitochondria and mitogen-activated protein kinase signal pathways. Huang et al. (36) reported that MitoKATP opener diazoxide prevented diabetes through inhibiting pancreatic beta-cells from apoptosis via Bcl-2/Bax rate and p38- mitogen-activated protein kinase. In the study, our results showed that inhibition of MitoKATP by 5-HD significantly attenuated the inhibitory effect of PHC postconditioning on I/R-induced molecular apoptotic events and apoptotic cell death. The data suggested that activation of MitoKATP was involved

in the inhibition of I/R-induced apoptosis by PHC postconditioning.

Mitochondrial dysfunction and oxidative stress are closely related with apoptosis. Opening of mPTP initiates the release of various factors, such as CytoC, activating caspase cascade and leading to final apoptotic cell death. Reactive oxygen species (ROS) is mainly generated in mitochondrial respiration chain and in turn, excessive ROS insults mitochondria and influences mPTP, damaging integrity of mitochondria and resulting in mitochondrial dysfunction. Oxidative stress is defined as excessive generation of ROS and/or decreased antioxidant defense which causes oxidative damage and is closely associated with various types of I/R injury. In the current study, we showed that PHC postconditioning could remarkably inhibit I/R-induced opening of mPTP and oxidative stress, indicating that inhibition of mPTP and oxidative stress may be involved in the neuro-protective effect of PHC postconditioning against I/R injury.

MitoKATP was found to inhibit the opening of mPTP and thus suppress mitochondrial dysfunction. Moreover, MitoKATP could regulate redox balance and attenuate the generation of ROS and oxidative damage. Sun et al. (37) showed that diazoxide could ameliorate oxidative mitochondrial DNA damage and deletion after renal ischemic injury. Chai et al. (38) demonstrated that Iptakalim protected PC12 cells against hydrogen peroxide-induced oxidative injury via opening MitoKATP channel. In our study, we found that inhibition of MitoKATP channel by 5-HD markedly suppressed the inhibitory effect of PHC postconditioning on the opening of mPTP and oxidative stress. These results demonstrate that activation of MitoKATP was involved in PHC postconditioning-exerted regulation of mPTP and redox system. Furthermore, we tested the possible receptors and pathways that mediate PHC-induced activation of MitoKATP. However, the results showed that PHC had no significant effect on AMPK, PI3K-Akt, and MAPKs pathways, NOS expression and NO release. In addition, M1 and M3 receptors, which were reported to be inhibited by PHC, were not involved in the activation of MitoKATP induced by PHC. To elucidate the molecular mechanisms

underlying the neuro-protective effect of PHC and PHC-mediated activation of MitoKATP, more attention should be paid to the potential receptors of PHC. The biological effects of PHC were reported to be mediated by β -arrestin and Toll-like receptors (39). β -arrestin 2 pathway protects rat hippocampal CA1 neurons against cerebral I/R-induced injury (40). Toll-like receptors play important roles in the regulation of I/R via modulating inflammation. More studies are needed to test whether these receptors are involved in activation of MitoKATP induced by PHC and to search for other potential receptors.

In conclusion, in the current study, we found that PHC postconditioning could inhibit I/R-induced inflammation, brain infarction and edema, and deficit of neurological function. The inhibition of apoptosis, opening of mPTP and oxidative stress was involved in the neuro-protective effect of PHC postconditioning against I/R injury. Moreover, activation of MitoKATP was critical for PHC postconditioning-exerted effect on redox status, mPTP opening, apoptosis, inflammation, brain infarction and edema, and deficit of neurological function in CI/R rats (Fig.6). The present findings may provide a novel foundation for therapeutic strategies against cerebral I/R injury.

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SILENCING OF MYELOID CELL LEUKEMIA-1 BY SMALL INTERFERING RNA IMPROVES CHEMOSENSITIVITY TO ETOPOSIDE IN U-937 LEUKEMIC CELLS

M. JAFARLOU¹, B. BARADARAN², D. SHANEHBANDI², T.A. SAEDI³, V. JAFARLOU⁴,
P. KARIMI⁵ and F. OTHMAN¹

¹Department of Human Anatomy, Faculty of Medicine and Health sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia; ²Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran; ³Institute of Biosciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia; ⁴Department of Surgery, Imam Reza Hospital, Tabriz University of Medical Sciences, Tabriz, Iran; ⁵Neurosciences Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

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A key issue in the treatment of acute myeloid leukemia (AML) is the development of drug resistance to chemotherapeutic agents. Overexpression of myeloid cell leukemia-1 (Mcl-1), an anti-apoptotic protein, is associated with tumor progression and drug resistance in leukemia and several cancers. The purpose of this study was to investigate the effect of specific Mcl-1 small interference RNA (siRNA) on the proliferation and chemosensitivity of U-937 AML cell to etoposide. The siRNA transfection was conducted using Lipofectamine™ 2000. Quantitative real-time RT-PCR (qRT-PCR) and Western blot analysis were employed to measure the expression levels of mRNA and protein, respectively. To evaluate tumor cell growth after siRNA transfection, Trypan blue exclusion assay was conducted. The cytotoxic effects of siRNA and etoposide were determined using MTT assay on their own and in combination. DNA-histone ELISA and annexin-V/FITC assays were performed to study the apoptosis. Mcl-1 siRNA transfection significantly blocked the expression of Mcl-1 mRNA and protein in a time-dependent manner, leading to a strong growth inhibition and enhanced apoptosis ($P<0.05$). Furthermore, pretreatment with Mcl-1 siRNA, synergistically enhanced the cytotoxic and apoptotic effects of etoposide ($P<0.05$). Our results demonstrated that Mcl-1 plays a fundamental role in the survival and resistance of U-937 cells to etoposide. Therefore, Mcl-1 can be considered an attractive target in gene therapy of AML patients and siRNA-mediated silencing of this gene may be a novel strategy in AML treatment.

Acute myeloid leukemia (AML), also known as acute non-lymphocytic leukemia, is a severe, rapidly progressing disease triggered by blocking of granulocyte and monocyte differentiation and maturation, resulting in accumulation of abnormal myeloid progenitor cells in the blood (1). AML is primarily a disease of the elderly and its prognosis in older patients is much worse than that of the similarly treated younger patients (2). Standard treatment for

AML includes chemotherapy and hematopoietic stem cell transplantation (3), but unfortunately the majority of patients show less sensitivity and high resistance to therapeutics (4). Therefore, alternative therapies are needed to overcome this problem. Recent studies have led to the development of new chemotherapeutic combinations with small interfering RNAs (siRNA) that target and down-regulate gene products which are involved in the

Key words: Mcl-1, siRNA, etoposide, U-937 cells

Mailing address:

Prof. Dr. Fauziah Othman, Department of Human Anatomy,
Faculty of Medicine and Health Sciences,
Universiti Putra Malaysia, 43400,
Serdang, Selangor, Malaysia
Tel.: +60(3)89472330
e-mail: fauziah@upm.edu.my

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formation of neoplasia and subsequent resistance to chemotherapy. Silencing of genes with siRNA is a powerful technology triggered by double-stranded RNA. When it introduces to cells, it cleaves the target mRNA and inhibits its translation to protein (5, 6).

Disorders in apoptotic pathway are among the main reasons for the formation of tumor cells in the majority of malignancies (7). Since most chemotherapeutics exert their anti-cancer effects by targeting apoptosis, simultaneous down-regulation of apoptotic pathway mediators could be a promising strategy for cancer treatment.

Myeloid cell leukemia-1 (Mcl-1) is a tightly regulated member of anti-apoptotic Bcl-2 family of proteins and was first isolated from the ML-1 human myeloid leukemia cells during the process of macrophage and monocyte differentiation (8). Mcl-1 is expressed in different cells and tissues and has a vital role in cell cycle and programmed cell death. Most of the studies on the Mcl-1 and related diseases in the context of cancer suggest that Mcl-1 might be closely associated with the development of tumors and cancer. Mcl-1 is highly expressed in many tumor cells including AML, indicating that Mcl-1 can be a potential marker protein for prediction or prognosis. In addition, up-regulation of Mcl-1 at leukemic relapse can be a possible explanation for its role in survival of leukemic cells after chemotherapy (9, 10).

Due to the fact that any deficiency in Mcl-1 function is sufficient to induce apoptosis in tumor cells, Mcl-1 can be assumed as a unique target for anti-cancer therapies. Therefore, in the current study we investigated the Mcl-1 silencing in sensitizing U-937 leukemic cells to etoposide treatment. In this regard, we examined the effects of Mcl-1 siRNA or etoposide either alone or in combination on promoting apoptosis in U-937 AML cells.

MATERIALS AND METHODS

Cell culture conditions

U-937, the human leukemic monocyte lymphoma cell line, was purchased from Pasteur Institute Cell Culture Collection (Tehran, Iran) and cultured in RPMI-1640

medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 1% antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin) (Sigma-Aldrich), 1% sodium pyruvate and 2 mM of glutamine, at 37°C in a humidified atmosphere containing 5% CO₂. The cells were sub-cultured 24-48 h later with an initial concentration of 5×10^4 cells/ml and used in the logarithmic growth phase in whole experiments.

siRNA transfection

The Mcl-1 siGENOME siRNA (5'-AGAACGAAUUGAUGUGUAA-3') and a negative control (NC) siRNA (Scrambled siGENOME siRNA had no known homology with any human genes) were ordered from Dharmacon (Lafayette, CO, USA). To evaluate the effects of siRNAs on gene silencing, transfections were performed in 6-well cell culture plates for 24, 48 and 72 h. In brief, U-937 cells were plated (1×10^6 cells/well) and allowed to grow overnight. On the day of the transfection, the cells were cultivated in RPMI-1640 medium free of serum and antibiotics. Transfection of siRNA (at a final concentration of 50 nM or 100 nM) was performed using Lipofectamine™ 2000 transfection reagent. According to the manufacturer's recommendations, siRNA and lipofectamine were diluted in Opti-MEM I Reduced Serum Medium separately and incubated for 10 min at room temperature. The diluted solutions were then mixed and incubated for 20 min at room temperature. Subsequently, the mixtures were added to each well containing cells and medium. Moreover, the treated cells with only the transfection reagent were considered a blank control. The cell culture plates were then incubated for 6 h at 37°C in a CO₂ incubator. Subsequently, RPMI-1640 medium containing FBS (final FBS concentration of 15%) was added, with cells being incubated under the above-mentioned conditions. The suppression of Mcl-1 gene expression was then assessed by quantitative real-time RT-PCR (qRT-PCR) and Western blot analysis.

RNA isolation, cDNA synthesis and qRT-PCR

Total RNA was isolated by AccuZol™ Reagent (Bioneer, Daejeon, Korea) using the manufacturer's protocol. Complementary DNA was synthesized using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit. An aliquot of 2 µg of total RNA was reverse-transcribed by Moloney murine leukemia virus

(MMLV) transcriptase using oligo-dT primers according to the manufacturer's instructions (Promega, Madison, WI, USA). qRT-PCR was performed in the Rotor- GeneTM 6000 system (Corbett Life Science, Mortlake, NSW, Australia) using SYBR Premix Ex Taq (Takara Bio, Otsu, Shiga, Japan). The reaction system of real-time PCR was: 3 µl of cDNA template, 1.2 µl of MgCl₂, 1 µM (10 pmol) of each of the primers, 2 µl of reaction buffer, 2 µl of 10 mM dNTP mix, 0.3 µl of *Taq* DNA polymerase and 9.5 µl of nuclease-free distilled water. For Mcl-1, the sense primer sequence was 5'- ACCAGCTCCTACTCCAGCAA-3', and the anti-sense primer sequence was 5'- TAAGGACAAAACGGGACTGG-3'. For β-actin, the sense primer sequence was 5'- TCCCTGGAGAAGAGCTACG-3' and the anti-sense primer sequence was 5'- GTAGTTTCGTGGATGCCACA-3'. Reaction parameters were: 95°C for 3 min followed by 35 cycles consisting of 30 sec at 95°C, 40 sec at a gradient temperature of 54 to 62°C and 30 sec at 72°C. Final extension eventually was performed by heating at 72°C for 5 min. Relative gene expression of Mcl-1 was calculated with the 2^{-(ΔΔCT)} method (11), using β-actin as the reference gene.

Western blot analysis

After washing twice with cold phosphate-buffered saline (PBS), the cells were resuspended in 500 µl cell lysis buffer (1% Triton X-100, 1% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4 and 1 mM EDTA, pH 8) containing protease inhibitor cocktail complete. The Mcl-1 protein and β-actin loading control were separated using 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDSP-AGE). Separated proteins were then transferred from the gel onto a activated polyvinylidene difluoride membranes (PVDF) (40 s rinse in pure methanol) in transfer buffer (0.01% SDS, 25 mM Tris-base, 192 mM Glycine and 10% methanol) for 1 h in 50-60 mA. The membranes were incubated at room temperature for 45 min in blocking buffer (3% fat-free milk and 0.05% Tween-20 in PBS) and then probed overnight at 4°C with primary mouse monoclonal antibodies specific for Mcl-1 (1:500 Abcam, Cambridge, UK) and β-actin (1:2,000 Abcam) diluted in 5% fat-free milk in PBS. Following 5 min washes for three times with PBS containing 0.05% Tween-20, membranes were incubated with corresponding horseradish peroxidase (HRP)-linked goat anti-mouse secondary antibody (1:3,000) diluted in PBS for 2 h at room temperature. Next, the membranes were washed

again and the bound antibodies visualized using enhanced chemiluminescence plus Western blotting detection Kit and X-ray film. Densitometry was performed by ImageJ software (National Institutes of Health, Maryland, USA) and the signal intensity of each protein band was normalized for the respective actin loading control.

Cytotoxicity assay

The effects of Mcl-1 siRNA on sensitivity of U-937 cells to etoposide were tested using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay. U-937 cells (2 × 10⁴ cells/well) were incubated in 96-well plates each containing 200 µl of medium. The cells were divided into eight groups: etoposide, siMcl-1, NC siRNA, siMcl-1 and etoposide, NC siRNA and etoposide, siRNA blank control, etoposide blank control and combination blank control. Transfection of siRNAs was carried out the following day as described previously. The cells of the appropriate groups were treated with different concentrations of etoposide (0.001, 0.2, 0.5, 1, 2 and 4 µM) 6 h after the siRNA transfection. Cells treated with only 1% DMSO or a mixture of 1% DMSO plus Lipofectamine 2000 were considered as etoposide or combination group controls, respectively. After 48 h the cytotoxicities of the treatments were assessed using the MTT cell proliferation kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The absorbances of the solubilized formazan dyes were measured using an ELISA plate reader (Awareness Technology, Palm City, USA) at 570 nm. The survival rate (SR) was measured through the following equation: SR (%) = (A Treatment / A Control) × 100%. The concentration that produced 50% cytotoxicity (IC₅₀) was determined using GraphPad Prism 6.01 software (GraphPad Software Inc., San Diego, CA, USA).

Combination effect analysis

To examine the interaction between Mcl-1 siRNA and etoposide, combination effect analysis was performed, based on the principles described by Chou and Talalay (12). The value of coefficient of drug interaction (CDI) was determined using the following formula: CDI = SAB / (SA × SB), where SA and SB are the survival rates of etoposide and siRNA relative to the corresponding control, SAB is the survival rate of the combination treatment relative to the control. CDI < 1, CDI = 1 and

CDI>1 indicate synergistic, additive and antagonistic effects, respectively.

Cell viability assay

The effect of Mcl-1 siRNA on cell proliferation was assessed by trypan blue exclusion assay. U-937 cells (5×10^4 cells/well) were transfected with specific and NC siRNAs in 24-well cell culture plates and then incubated for 5 days. At different time points after treatments, the cells were harvested and then the cell suspensions were stained with equal volumes of 0.4% trypan blue for 2 min. Subsequently, the number of unstained cells (N, viable cells) was counted using an inverted microscope and a hemocytometer. The percentage of viable cells was quantified using the following equation:

$$\text{Cell viability (\%)} = (\text{N Treatment} / \text{N Control}) \times 100$$

Moreover, the percentage of viable cells for control group was considered 100%.

Cell death detection

The U-937 cells were seeded at a density of 5×10^4 cells/well of 24-well plate and then treated with specific siRNAs, NC siRNA, the IC50 dose of etoposide and the combination of them, as described previously. Forty-eight hours after transfection, cells were harvested and ELISA assay was performed using an ELISA cell death detection kit (Roche Diagnostics GmbH) according to the manufacturer's protocol. In brief, the cell lysates were obtained and centrifuged at 200 g for 10 min. Twenty μ l of the supernatants and 80 μ l of immunoreagent containing antihistone-biotin and anti-DNA-peroxidase were then transferred to each well of a streptavidin-coated plate and the plate was incubated for 2 h at 25°C. The wells were washed with incubation buffer and 100 μ l of 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfon acid (ABTS) solution was added. The reactions were stopped with ABTS stop solution and absorbance was measured using an ELISA plate reader at 405 nm (with a reference wavelength of 490 nm). Results were expressed as the fold increase in the absorbance of treatment groups relative to the blank control group.

Flow cytometry for cell apoptosis detection

U-937 cells were plated in 6-well plates and were divided into the same eight groups as in the MTT assay. The siRNA transfection and etoposide treatments were as before. After 48 h, cells were collected and washed

twice with pre-chilled PBS. The cell concentration was adjusted to $5 \times 10^5 \sim 5 \times 10^6$ cells/well with 100 μ l of pre-chilled binding buffer. Five μ l of Annexin V-FITC and 5 μ l of propidium iodide (PI) were added to each sample and incubated for 10 min in the dark on ice. Finally, 400 μ l of pre-chilled binding buffer was added. The cell apoptosis was determined by flow cytometry. Apoptosis of each group was assayed three times.

Statistical analysis

Data were presented as mean $\pm$ standard deviation (SD). Analysis of variance (ANOVA) followed by Bonferroni's and Sidak's tests was used to determine the significant differences between groups. Values of P less than 0.05 were considered significant. All the statistical analyses were performed using GraphPad Prism 6.01 software.

RESULTS

Mcl-1 mRNA and protein down-regulation in U-937 cells after siRNA transfection

We initially explored the effect of specific siRNA on Mcl-1 expression by qRT-PCR and Western blot analysis at 24, 48 and 72 h after transfection. As shown in Fig. 1, Mcl-1 siRNA transfection significantly reduced the amount of Mcl-1 mRNA. Results of untreated group (blank control) were considered 100% and relative gene expression was calculated in relation to blank control. The levels of Mcl-1 mRNA in the siRNA-treated group at 24, 48 and 72 h post-transfection were about 43.7%, 21.1% and 17.2%, respectively ($P < 0.05$; Fig. 1). There was no significant difference between negative control siRNA group and untreated cells. The Mcl-1 siRNA was also effective in knocking down Mcl-1 protein expression. As shown in Fig. 2, while the β -actin internal control showed equal loading among all groups, the levels of Mcl-1 protein at 24, 48 and 72 h post-transfection were 51.4%, 20.5% and 13.2%, respectively. Meanwhile, NC siRNA had an insignificant effect on Mcl-1 mRNA and protein expression compared with the blank control group.

Mcl-1 siRNA inhibited the proliferation of U-937 cells

Decreased Mcl-1 activity is associated with arrested cell growth; therefore, it was examined

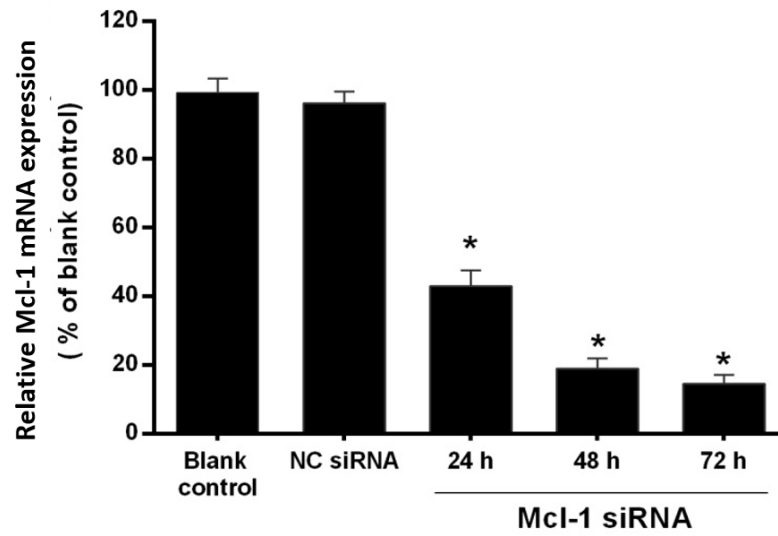


Fig. 1. Silencing of *Mcl-1* mRNA by siRNAs in U-937 cells. Cells were transfected with *Mcl-1* or NC siRNA for 24, 48 and 72 h. Relative *Mcl-1* gene expression was quantified by qRT-PCR using $2^{-\Delta\Delta Ct}$ method as described in Methods. * $P < 0.05$ versus blank control; the data represent mean $\pm$ SD ($n = 4$).

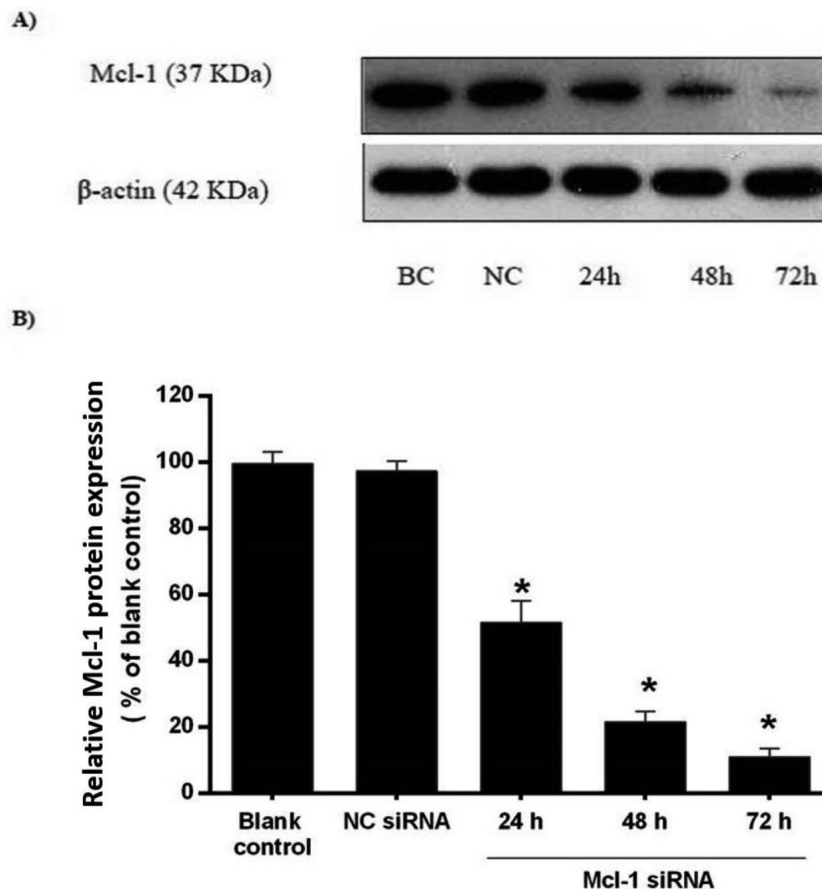


Fig. 2. siRNA suppression *Mcl-1* protein expression in U-937 cells. **A)** Representative Western blot analysis showing the expression of *Mcl-1* protein in U-937 cells at 24, 48 and 72 h after exposure to *Mcl-1* siRNA. **B)** The results of Western blot calculated using densitometry and normalized to the respective β-actin. * $p < 0.05$ vs blank control; the results are expressed as mean $\pm$ SD ($n = 4$).

to determine whether or not siRNA-mediated suppression of Mcl-1 would affect cell proliferation in U-937 cells. The cell viability curve showed that compared with the blank control group, Mcl-1 siRNA significantly decreased the cell viability in a time dependent manner ($P < 0.05$; Fig. 3). Beginning at 24 h after transfection of Mcl-1 siRNA, the cell viability decreased rapidly to 84.68% and dropped to 54.23% on day 5. However, there was no significant difference between the NC siRNA treated group and untreated cells.

Mcl-1 siRNA synergistically increased the sensitivity of U-937 cells to etoposide

To explore whether the down-regulation of Mcl-1 by siRNA would increase the sensitivity of U-937 cells to etoposide, a combination treatment was performed. As shown in Fig. 4, mono-treatment with etoposide enhanced cell toxicity in a dose-dependent manner. The results showed that in comparison with blank control, Mcl-1 siRNA lowered the survival rate to 82.43% ($P < 0.05$). Furthermore, combination therapy further decreased the survival rate in comparison with siRNA or etoposide single treatment. Mcl-1 siRNA transfection significantly decreased the IC_{50} value of etoposide from 2.29 μ M to 1.78 μ M (Fig. 4 and Table I).

To assess whether the effects of etoposide and Mcl-1 siRNA are synergistic, additive or antagonistic, the CDI values as described in the combination effect analysis of methods section were calculated. Results showed that the combination effects of Mcl-1 siRNA and etoposide were synergistic with the CDI values of less than 1 in all concentrations of etoposide (Table II). Meanwhile, NC siRNA had an insignificant effect on the chemosensitivity of the cells compared with etoposide alone ($P > 0.05$; Fig. 4).

Decrease in cell viability caused by the Mcl-1 siRNA was due to increase in apoptosis

To determine whether the decrease in cell viability was due to decrease in cell proliferation or increase in apoptosis, we measured the amount of apoptosis by ELISA cell death kit and Annexin V-FITC and propidium iodide (PI) labeling followed by fluorescence-activated cell sorting (FACS).

Results of ELISA-based cell death detection assay demonstrated that treatment of leukemia cells with Mcl-1 siRNA significantly increased the extent of apoptosis by 6.79-fold, compared with the blank control (Fig. 5). However, treatment with NC siRNA alone or in combination with etoposide displayed no distinct changes in the extent of apoptosis relative to the blank control or etoposide monotreatment, respectively ($P > 0.05$; Fig. 5).

Furthermore, as shown in Fig. 6, flow cytometry analysis demonstrated that after 24-hour incubation the percentage of annexin V+ cells for etoposide and Mcl-1 siRNA were 27.55 and 18.14, respectively. Additionally, when cells were subjected to Mcl-1 siRNA transfection and etoposide treatment, a significant enhancement in the population of annexin V+ cells was observed in comparison with Mcl-1 and etoposide single treatment (Fig. 6 and Table III). Meanwhile, no significant alteration was observed in NC siRNA and NC siRNA plus etoposide compared with the untreated blank group or etoposide alone, respectively ($P > 0.05$; Fig. 5, Fig. 6 and Table III).

DISCUSSION

AML is a malignant disease characterized by an arrest in cell differentiation and uncontrolled proliferation the standard chemotherapeutic treatment of which consists mainly of Cytarabine and Anthracyclines (13), but resistance to chemotherapy is a major obstacle in AML treatment. Patients often relapse after a primary response to cytotoxic agents (14). Therefore, development of new strategies to overcome drug resistance is very important. Combination therapy is widely used for the treatment of lethal diseases such as AIDS and cancer to achieve synergistic therapeutic effect, toxicity reduction and minimized drug resistance (12). Mcl-1 is a member of the Bcl-2 anti-apoptotic family of proteins and is overexpressed in a variety of human malignancies including colon, breast, CNS, lung, renal, melanoma, ovarian, prostate and leukemia. Mcl-1 up-regulation in tumor cells enable them to evade apoptosis, to survive and as a mechanism to develop drug resistance (15). On the other hand, some reports indicate that Mcl-1 down-regulation

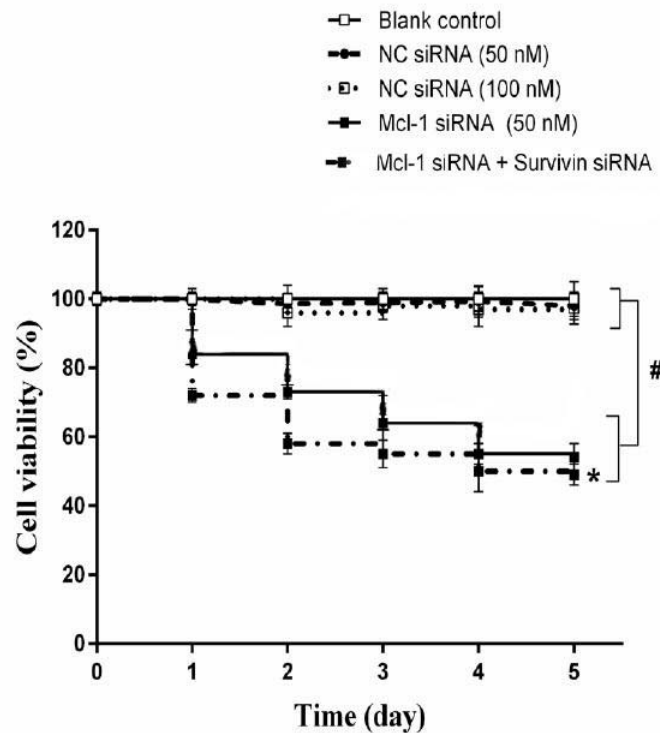


Fig. 3. Effect of Mcl-1 siRNA or NC siRNA transfection on the viability of U-937 cells. Number of viable cells was calculated by trypan blue staining after 24, 48, 72, 96 and 120 h treatment and the viability determined as described in Methods. * $P < 0.05$ versus blank control or NC siRNA; the data represent mean $\pm$ SD ($n = 4$).

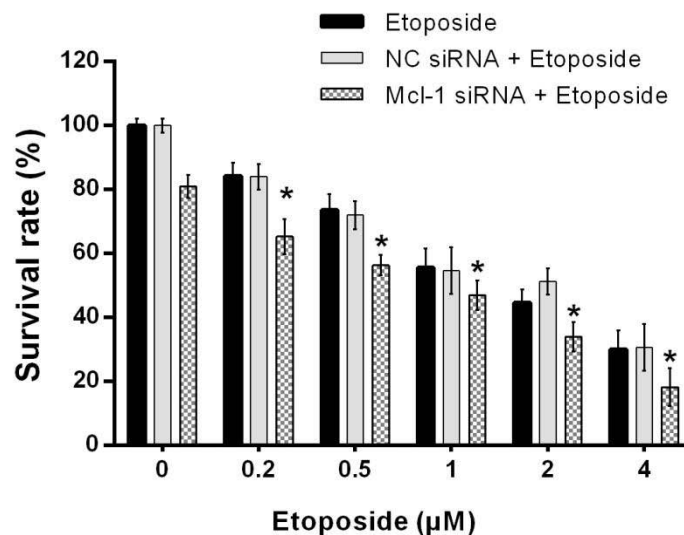


Fig. 4. Effect of Mcl-1 silencing on the chemosensitivity of U-937 Cells. Cells were treated by etoposide, NC siRNA or Mcl-1 siRNA (50 nM) and their combination for 48 h and cytotoxicity was measured by MTT assay. # $P < 0.05$ versus blank control; * $P < 0.05$ versus etoposide or Mcl-1 siRNA alone; The results are expressed as mean $\pm$ SD ($n = 4$).

Table I. Inhibitory concentration (IC_{50}) values of siRNAs and etoposide.

Treatment	IC_{50} (μ M)
Etoposide	2.29
NC siRNA (50nM) + Etoposide	2.23
NC siRNA (100nM) + Etoposide	2.22
Mcl-1 si RNA + Etoposide	1.78

NC: negative control

Table II. The values of coefficient of drug interaction (CDI).

Etoposide (μ M)	0	0.2	0.5	1	2	4
Combination	CDI					
Mcl-1 siRNA	-	0.98	0.93	0.94	0.87	0.78

$CDI < 1$, $CDI = 1$ and $CDI > 1$ indicate synergistic, additive and antagonistic effects, respectively.

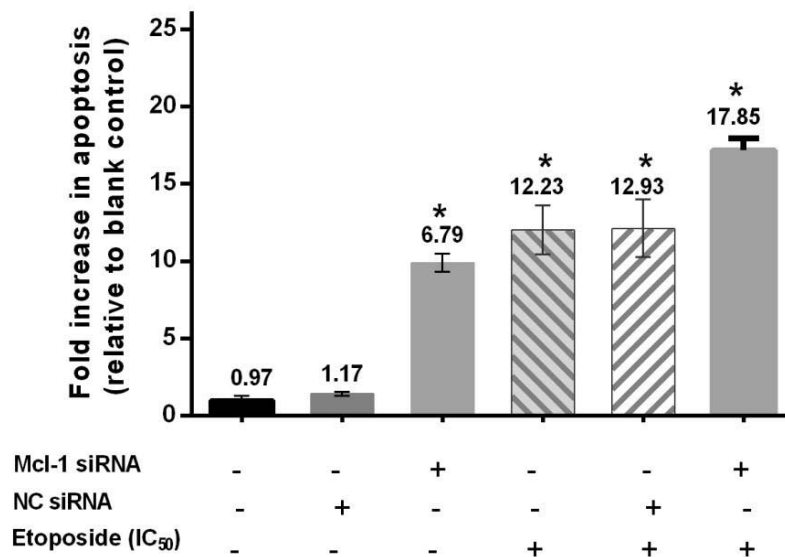


Fig. 5. Effect of Mcl-1 down-regulation on etoposide-induced apoptosis. Cells were treated with siRNAs and etoposide alone or in combination. At 48 h after transfection, apoptosis was measured by ELISA cell death assay as described in Methods. * $p < 0.05$ versus blank control; the results are expressed as mean $\pm$ SD ($n = 4$).

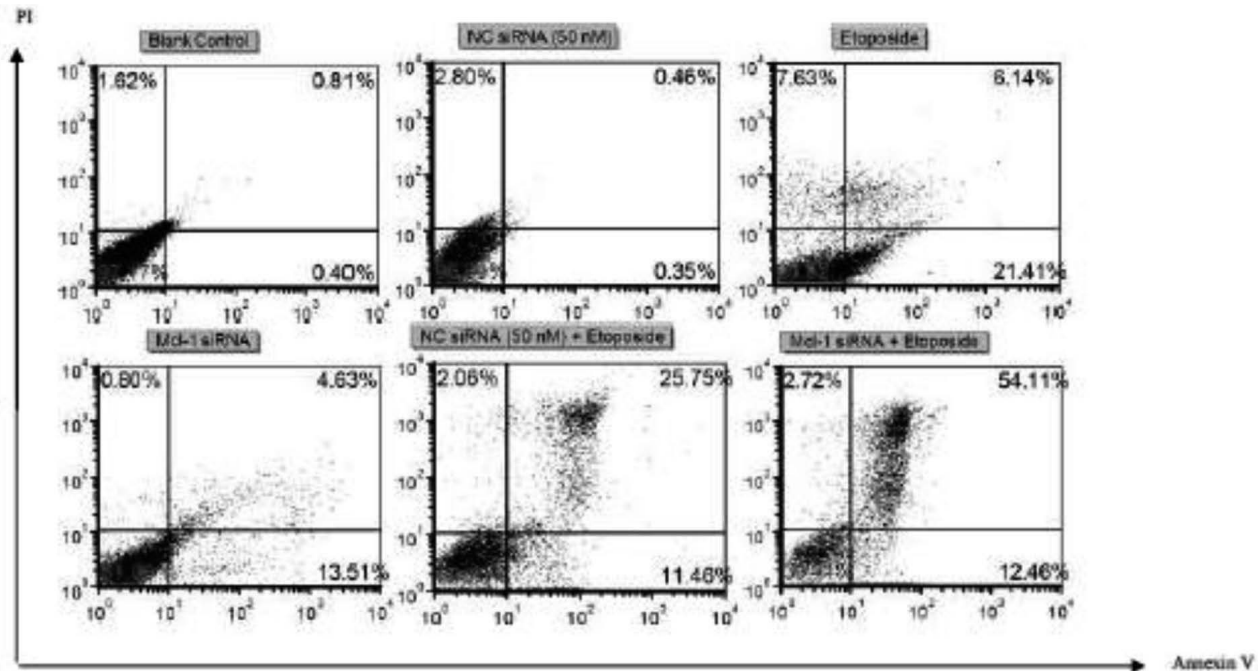


Fig. 6. The effect of Mcl-1 siRNA and etoposide on apoptosis in U-937 cells. After Mcl-1 siRNA transfection (for 48 h) or/and etoposide treatment for 24 h, the cells were stained with AnnexinV-FITC and propidium iodide (PI) followed by flowcytometry; UL: upper left, dead cells. UR: upper right, late-stage apoptotic cells. LL: lower left, viable cells. LR: lower right, early stage apoptotic cells. Data are representative of four experiments.

Table III. Flow cytometric results of U-937 cells treated with Mcl-1 siRNA and etoposide.

Treatment	LL	UL	LR	UR	LR+UR
1 Blank Control	97.17	1.62	0.4	0.81	1.21
2 NC siRNA (50nM)	96.39	2.8	0.35	0.46	0.81
3 Mcl-1 siRNA	81.06	0.8	13.51	4.63	18.14 ^a
4 Etoposide	64.82	7.63	21.41	6.14	27.55 ^a
5 NC siRNA (50 nM) + Etoposide	60.73	2.06	11.46	25.75	37.21 ^a
6 mcl-1 siRNA + Etoposide	30.71	2.72	12.46	54.11	66.57 ^a

UL: upper left. Dead cells. UR: upper right, late-stage apoptotic cells. LL: lower left, viable cells. LR: early stage apoptotic cells. The results are expressed as mean SD (n=3); ^aP<0.05 relative to the blank control.

can sensitize tumor cells to anti-cancer agents (16, 17). The above-mentioned reports indicate that Mcl-1 is related to drug resistance and suppression of Mcl-1 can be a proper target for chemosensitization. Therefore, in the present study to investigate whether

siRNA-mediated silencing of Mcl-1 would affect drug resistance and chemosensitivity of leukemic cells to etoposide, we examined Mcl-1 siRNA in combination with etoposide and alone in U-937 AML cells.

In this study the results of qRT-PCR and Western blot analysis showed that treatment with Mcl-1 siRNA significantly decreased the Mcl-1 mRNA and protein, indicating that siRNA could successfully cleave Mcl-1 mRNA and inhibit its translation to subsequent protein. The results of cell viability assay revealed that Mcl-1 suppression had a significant effect on decreasing U-937 cell growth and proliferation, suggesting that Mcl-1 may have an important role in cell proliferation. To analyze the effect of Mcl-1 siRNA on toxicity of etoposide in AML cells, we measured cell toxicity by MTT assay. Results indicated that combination treatment of Mcl-1 siRNA and etoposide synergistically enhanced cell toxicity. Moreover, IC50 value of etoposide lowered substantially.

In the present study, we measured the amount of apoptosis by two distinct methods to determine whether the decrease in cell viability is because of reduced cell proliferation or increased apoptosis. The results of both assays showed that single treatment with etoposide remarkably increased apoptosis in U-937 cells. Furthermore, simultaneous transfection of Mcl-1 siRNA and etoposide treatment led to a significant increase in sensitivity of leukemic cells to etoposide-induced apoptosis. However, neither NC siRNA nor Lipofectamine 2000 could change the chemosensitivity of etoposide, indicating the important role of Mcl-1 siRNA in chemosensitization. We thus concluded that decreased cell viability in AML cells is due to increased apoptosis.

Cellular apoptosis can be initiated by two main intrinsic and extrinsic pathways. Intrinsic pathway responds to the intra-cellular ligands causing release of cytochrome C and activation of caspase 9. Extrinsic pathway or death receptor pathway is initiated when extra-cellular ligands bind to death receptors in cell surface. Both pathways mutually activate caspase 3, leading to downstream caspase activation and finally resulting in apoptosis (18, 19). Studies have shown that Mcl-1 mainly blocks the apoptosis through intrinsic pathway. This protein is localized to mitochondrial outer membrane and hetero-dimerize with and neutralize pro-apoptotic Bcl-2 family proteins such as Bak, Bax and Bim, leading to inhibition of cytochrome C release

from mitochondria which is needed for apoptosis induction and eventual enhanced cell survival (9, 20, 21). Therefore, down-regulation of Mcl-1 increases etoposide apoptotic effect. Etoposide is an anti-cancer agent which is utilized for the treatment of different malignancies, such as leukemia, and acts by inhibiting enzyme topoisomerase II. This enzyme has a crucial role in DNA replication, and inhibition of this enzyme by etoposide would cause DNA fragmentation (22). DNA damaging is one of the stimulators of apoptotic intrinsic pathway (18), but overexpression of Mcl-1 in tumor cells enables them to develop resistance against chemotherapeutics and, according to the literature, suppression of Mcl-1 expression by siRNA could decrease survival rate in cancer cells (23). Therefore, as the results of our study show, down-regulation of Mcl-1 by siRNA can be a promising strategy to overcome resistance and sensitize leukemic cells to etoposide. However, further investigations are needed to realize the exact mechanism of chemosensitization.

Overall, the results of this study demonstrated that Mcl-1 has a fundamental role in cell survival and sensitivity of U-937 cells to etoposide. Down-regulation of Mcl-1 by specific siRNA and simultaneous exposure to etoposide led to a significant synergistic inhibition of cell proliferation and enhanced apoptosis and anti-tumor effect. Therefore, Mcl-1 can be considered an attractive target for AML treatment and a novel strategy to overcome drug resistance and sensitize leukemic cells to chemotherapeutics.

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EFFECTS OF EPIDURAL BLOCK COMBINED WITH GENERAL ANESTHESIA ON ANTITUMOR CHARACTERISTICS OF T HELPER CELLS IN HEPATOCELLULAR CARCINOMA PATIENTS

XT. WANG¹, M. LV² and HY. GUO³

¹Second affiliated hospital of Shandong University of Traditional Chinese Medicine, Shandong, China; ²Qianfoshan hospital, Shandong University, Shandong, China; ³Second affiliated hospital of Shandong University of Traditional Chinese Medicine, China

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The first two authors contributed equally to this article

This study discusses the changes of T helper cells (Th cells) of patients who received different anesthesia methods in liver cancer resection. We selected 122 patients who were diagnosed with hepatocellular carcinoma and underwent liver cancer resection and divided them into a general anesthesia combined with epidural anesthesia group (group A) and general anesthesia group (group B). Peripheral blood was collected to detect Th cells on the day of surgery, and on the second and seventh days after surgery. Th1 and Th2 cell frequency and mRNA expression of interferon- γ (IFN- γ) of all patients significantly rose on the second day but recovered to the previous level on the seventh day. Th1/Th2 increased remarkably on the seventh day compared to the second day. Compared to the day of surgery, Th17, regulatory T (Treg) cells as well as mRNA expression of interleukin-17 (IL-17) and FoxP3 had no obvious changes on the second day, but dramatically declined on the seventh day. Compared to group B, Th1 cell frequency and Th1/Th2 in group A had a slight increase on the second day, and a remarkable increase on the seventh day; but Th2, Th17 and Treg cell frequency in group A slightly decreased on the second day and remarkably decreased on the seventh day. mRNA of IFN- γ , cytokine levels and IFN- γ /IL-4 of group A were all higher than group B on the seventh day, while mRNA of IL-17, concentration of IL-17 as well as concentration of transforming growth factor- β 1 (TGF- β 1) in group A were much lower than group B. These findings suggest that improving antitumor activity of Th cells can benefit patients who receive liver cancer resection.

Liver cancer, one of the most frequently seen malignant tumors (1-2), has the third highest incidence and mortality among all tumors, and has the highest incidence and mortality among males (3). T helper (Th) cells are a type of immune cells to which substantial importance has been given recently. Undifferentiated CD4+Th cells can differentiate into Th1, Th2, Th17 and Treg cells under different cytokine environments. Th17 cells, differently from Th1 and Th2, have been proved to play an important role in autoimmune

disease and infection. Correlation of tumor with Th1 and Th2 is greater and more definite than with Th17. It has been reported that patients who develop liver cancer, gastric carcinoma, prostatic cancer, colon cancer, lung cancer and ovarian cancer have a higher proportion of Th17, which may be in a negative correlation with prognosis, indicating that Th17 may promote occurrence and development of tumor.

Th1 resists tumor through producing interferon- γ (IFN- γ). In contrast, Th2, Th17 and Treg cells

Key words: liver cancer resection, T helper cells, anesthesia, cell frequency, general anesthesia combined with epidural anesthesia

Mailing address:

Dr Xuetao Wang,
Second affiliated hospital of Shandong University
of Traditional Chinese Medicine,
No.1, Jingba Road, Jinan, Shandong
250001, China
e-mail: wxtdsci@163.com

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are beneficial for tumor growth. The number and distribution of Th cell subsets are critical issues for tumor patients. However, the balance between Th cell subsets is more important, as it determines the tendency of tumor immunity as well as prognosis of patients. Appropriate management in the perioperative period, such as the correct choice of anesthesia method can help patients regain balanced Th cell subset and further maintain the balance of anti-tumor reaction. General anesthesia and general anesthesia in combination with epidural block are two frequently used anesthesia methods. Epidural block is confirmed as being able to prevent or eliminate excessive stress reaction induced by surgery. It can prevent harmful stimulation from reaching the central nervous system (4). Previously, the choice of anesthesia was correlated to stress and immune function during the perioperative period, but in recent years it has been reported to be also correlated to long-term prognosis, recurrence and metastasis of tumor. General anesthesia in combination with local anesthesia can reduce metastasis and recurrence of tumor after surgery (5-6). Although its mechanism has not yet been defined, the balance of Th cell subset is hypothesized to be one of the important factors, therefore the selection of the method of anaesthesia for tumor patients has been gradually given more importance.

MATERIALS AND METHODS

One hundred and forty patients who were diagnosed with liver cancer and received liver cancer resection in the hepatic surgery department of the First People's Hospital in Jining, Shandong between October 2013 and May 2014 were enrolled in this study. They were aged from 19 to 65 years and had category I or II American Society of Anesthesiologists physical status. All the patients had normal leukocyte and lymphocyte counts, were confirmed with no distant metastasis by imaging diagnosis, were able to receive radical resection and had not undergone any previous anti-tumor treatment. The subjects were divided into a general anesthesia combined with epidural anesthesia group (group A), and a general anesthesia group (group B). Those who were diagnosed as not having hepatocellular carcinoma (HCC) pathologically or received transfusion therapy during or after surgery were excluded. A total of

122 patients remained, 62 from group A and 60 from group B. Of these 122 patients, 28 received right hepatectomy, 22 left hepatectomy and 72 segmental hepatectomy. Gender, age, clinical pathological factors, hemorrhage, duration of anesthesia and Visual Analog Scale (VAS) Score in the two groups were found with no statistically significant difference. All the patients signed an informed consent to participate, and the study was approved by the ethics committee of the First People's Hospital in Jining, Shandong.

Anesthesia method

All patients did not eat or take medication 8 h prior to surgery. Patients in group A were given an epidural puncture in the space between T8 and T9. Then an epidural catheter was inserted for 3.3 cm and 8 ml lidocaine (2%) was injected. If no spinal anesthesia was found and epidural block was confirmed to be successful 5 min later, patients were immediately given 8 ml bupivacaine (0.5%), followed by 4 ml injection through the epidural catheter every 50 min.

Induction and maintenance of general anaesthesia: after 5-min preoxygenation, all patients were intravenously injected with induction drugs including 5 µg/kg fentanyl, 2.0 mg/kg propofol and 0.9 mg/kg rocuronium bromide. Tracheal intubation was performed 90 s later. Anaesthesia was maintained with sevoflurane (2.25%).

Postoperative analgesia: both group A and group B were given patient controlled epidural analgesia (PCEA) using a portable infusion pump (Auto Med, Ace medical AM3400, Korea) after surgery, but 0.125 % bupivacaine + morphine (30 µg/mL) for group A and morphine (0.4 mg/mL) for group B. Postoperative analgesia lasted for 48 h. When analgesia was over, VAS was used to evaluate pain suffering by the patients.

Blood samples

Ten ml venous blood samples were taken from the patients in a semi-supine position in the morning of the day of surgery (d0), and on the second (d2) and the seventh (d7) days. The venous blood collected was added with 0.2 ml heparin. Peripheral blood mononuclear cells were then isolated using Ficoll density gradient method, followed by detection with flow cytometry and real-time Polymerase Chain Reaction (PCR). Plasma obtained by centrifuge was also detected.

Flow cytometry

Cells used for Th1 and Th17 detection were added into a 24-well plate. Then 50 ng/ml polymethyl acrylate (PMA), 709 ng/ml (1 μ mol/L) ionomer (IO) and 10 μ g/ml biochemical fulvic acid (BFA) were added to stimulate cells. Four hours later, cells were removed and washed by 100 μ l phosphate buffer saline (PBS). Cells used for Treg detection were added directly. Afterwards, the two groups of cells were both added with 5% bull serum albumin and then preserved at 4°C for 10 min. Next, they were centrifuged for 5 min and resuspended by adding 70 μ l staining buffer. Antibody was added as per instruction, as shown in Table I, and then cells were kept in the dark for 30 min. Next, cells were washed by 100 μ l staining buffer and then preserved at 4°C for 5 min. Treg group and PE isotype IgG2a group were added with 100 μ l newly prepared Foxp3 fixation/permeabilization buffer and then preserved in the dark. Four hours later, another 100 μ l permeabilization buffer was added and then cells were washed by PBS for 5 min. Th1 and Th17 and PE isotype IgG1 group were added with 100 μ l fixation solution, shaken and then kept in the dark at room temperature. Twenty min later, 100 μ l permeabilization buffer was added, followed by PBS washing. All cells were resuspended with 70 μ l permeabilization buffer, followed by addition of fluorescent antibody (Table II) and then kept in dark at 4°C. All cell groups were added with 100 μ l permeabilization buffer and then washed by PBS once. Afterwards, all cells were

fixed with 100 μ l paraformaldehyde (1%). Centrifuge was performed 10 min later and cells were resuspended with 425 μ l staining buffer. Finally, they were preserved at 2-8°C. Detection was carried out within 24 h.

Quantitative real-time polymerase chain reaction (qRT-PCR)

MRNA extraction. First, medium was removed from cells. Cells were washed by 2 ml PBS twice before being transferred into a culture bottle. One ml Trizol reagent was added and maintained at 22°C for 5 min to isolate ribonucleoprotein complex. Afterwards, the mixture was added with 0.2 ml chloroform and shaken for 15 s. After being placed at 22°C for 2.5 min, cells were centrifuged at 12,000. Fifteen min later, supernate was removed and added with 0.5 ml isopropanol. Ten min later, cells were centrifuged at 12,000 g at 5°C for 10 min. Supernate obtained was abandoned. Pre-cooled ethyl alcohol (75%) containing diethylpyrocarbonate (DEPC) was used to wash RNA sediment. Then it was centrifuged at 7,000 g at 5°C for 5 min. RNA was redissolved in DEPC after being dried at room temperature or in a vacuum environment (1 μ l RNA: 99 DEPC). Then concentration and purity of RNA were measured.

For reverse transcription, all the operations were carried out on ice. Firstly, RNA sample and relevant reagents were vibrated and centrifuged after unfreezing. Reagents (PrimeScript™ RT reagent Kit) were added into a thin-

Table I. Comparison of baseline data of patients in two groups.

Cells	Fluorescent antibody added
Treg	FITC-CD4, APC-CD25
TH1&TH17&TH2	FITC-CD4
Blank	

(mean $\pm$ SD)

Table II. Addition of second antibody.

Cells	Fluorescent antibody added	Incubating time
Treg	PE-Foxp3	Overnight
Th1&Th17	APC-IL17, PE-IFN γ	20 min
Th2	APC-IL4	20 min

wall PCR tube and incubated at 37°C for 15 min, followed by 5-s enzyme inactivation under 85°C according to the manufacturer's instructions. cDNA were preserved at 4°C. The volume could be amplified accordingly. Ten μ l reaction systems were available for 500 ng total RNA at the maximum volume.

Fluorescence quantitative PCR primers and probe were designed as follows:

IFN- γ : F: 5'-TGCAGGACCCATATGTAAAAGA-3'
 R: 5'-TCAAAATGCCTAAGAAAAG-3'
 IL-4: F: 5'-TACAGCCACCATGAGAAGGACA-3'
 R: 5'-GCCAGGCCCCAGAGGTT-3'
 IL-17: F: 5'-CGCTGATGGGAACGTGGACTAC-3'
 R: 5'-GGTGGACAATCGGGGTGACA-3'
 FoxP3: F: 5'-CGCTGATGGGAACGTGGACTAC-3'
 R: 5'-GGTGGACAATCGGGGTGACA-3'
 β -actin: F: 5'-CAACTGGGACGACATGGAGAAAAT-3'
 R: 5'-CCAGAGGCGTACAGGGATAGC AC-3'

Enzyme-linked immunosorbent assay (ELISA)

Levels of IFN- γ , interleukin-4 (IL-4), IL-17, IL-10 and transforming growth factor- β 1 (TGF- β 1) in plasma were detected using ELISA with R and D systems. Coefficient of difference within sample and between samples was less than 5% and 10%, respectively. Sampling was carried out three times.

Statistical analysis

SPSS19.0 statistics software was used to process data. Measurement data was expressed as mean $\pm$ standard error (SE). Comparison between two samples was carried out using bilateral t-test, while categorical variables were compared using Fisher's exact test. Difference was considered to be statistically significant if $p < 0.05$.

RESULTS

General data of cases

There were no differences in age, gender and pathological factors between the two groups. Dosage of fentanyl, morphine and sevoflurane in the two groups had statistically significant difference (Table III).

Th1, Th2, Th17 and Treg cell frequency

Th1 (CD4+IFN- γ +CD4+T cells) and Th2 cell

frequency (CD4+IL-4+CD4+T cells) were much higher at d2 compared to d0 ($p < 0.001$, $p < 0.001$, respectively), but recovered partially at d7 ($p = 0.015$, $p < 0.001$). Th1/Th2 had no difference at d0 and d2, but significantly increased at d7 ($P < 0.001$). In contrast, Th17 (CD4+IL-17+CD4+T cells) and Treg (CD4+CD25+FoxP3+CD4+T cells) cell frequency had no changes at d2 compared to d0, but significantly decreased at d7 ($p < 0.01$, $p = 0.021$, respectively) (Table IV and Fig. 1).

Th1, Th2, Th17 and Treg in the two groups had no differences at d0. At d2, only Th2 cell frequency in group A was lower than group B ($P = 0.028$), while Th1, Th17 and Treg cell frequency and Th1/Th2 had no differences between the two groups. At d7, Th1 cell frequency in group A was much higher than group B ($p < 0.01$), while Th2 cell frequency in the two groups had no differences ($p = 0.231$). Thus Th1/Th2 in group A was much higher than group B ($p < 0.01$); in contrast, Th17 and Treg cell frequency in group A were remarkably lower than group B ($p = 0.047$, $p = 0.003$, respectively) (Table V and Fig. 2).

mRNA expressions in IFN- γ , IL-4, IL-17 and FoxP3 in peripheral blood mononuclear cells

IFN- γ , IL-4 and IL-17 are major cytokines secreted by Th1, Th2 and Th17. FoxP3 is a major transcription factor of Treg cells. Therefore, changes were studied of Th cells at RNA level by detecting mRNA expression of IFN- γ , IL-4, IL-17 and FoxP3 in peripheral blood mononuclear cells.

MRNA expression of IFN- γ significantly increased at d2 ($p < 0.01$), but returned to the previous level at d7 ($p = 0.042$). MRNA expression of IL-4, IL-17 and FoxP3 had no significant difference at d0 and d2, but mRNA expression of IL-17 and FoxP3 remarkably decreased at d7 compared to d2 ($p = 0.032$ and $p < 0.01$, respectively) (Fig. 3).

Similar to Th cell frequency, mRNA expression of IFN- γ , IL-4, IL-17 and FoxP3 in the two groups had no significant difference. At d2, mRNA expression of IL-4 in group A was much lower than group B ($p < 0.01$). At d7, mRNA of IFN- γ and IFN- γ /IL-4 in group A was much higher than group B ($p = 0.029$ and $p = 0.003$, respectively); mRNA of

IL-17 in group A was remarkably lower than group B ($p = 0.042$); mRNA of FoxP3 in group A was lower than group B, but there was no statistically significant difference ($p = 0.019$). Variation of mRNA expression of representative cell factors of Th cells was consistent with variation of Th cell frequency detected by flow cytometry. Table VI demonstrates comparison of mRNA of IFN- γ , IL-

4, IL-17 and FoxP3 and Th1/Th2 between group A and group B.

Concentration of IFN- γ , IL-4, IL-17, IL-10 and TGF- β 1 in plasma

IFN- γ , IL-4 and IL-17 are cytokines produced by Th1, Th2 and Th17, while IL-10 and TGF- β 1 are cytokines produced by Treg cells. A significant

Table III. General data of cases.

	Group A (n=62)	Group B (n=60)	P value
Age(year)	49.2 $\pm$ 1.3	44.8 $\pm$ 1.6	0.423
Male (n)	53	45	0.218
Male (n)	9	15	
With liver cirrhosis (n)	43	45	0.620
Without liver cirrhosis (n)	19	15	
Child-Pugh grade A (n)	57	56	0.976
Child-Pugh grade B (n)	5	4	
Tumor differentiation level I-II (n)	48	42	0.472
Tumor differentiation level III-IV (n)	14	18	
With one tumor (n)	46	50	0.517
With multiple tumors (n)	16	10	
With invaded blood vessels (n)	12	16	0.553
Without invaded blood vessels (n)	50	44	
Duration of anesthesia $\leq$ 3h (n)	36	42	0.419
Duration of anesthesia $>$ 3h (n)	26	18	
VAS score	3.46 $\pm$ 0.48	3.81 $\pm$ 0.32	0.879
Dosage of fentanyl (μ g)	0.30 $\pm$ 0.01	0.50 $\pm$ 0.01	<0.001
Dosage of morphine (mg)	2.28 $\pm$ 0.06	95.21 $\pm$ 1.09	<0.001
Dosage of sevoflurane (ml)	20.89 $\pm$ 0.68	37.20 $\pm$ 1.46	<0.001

Note: *t*-test

increase was found in the concentration of IFN- γ , IL-4, IL-17 at d2 compared to d0 ($p < 0.01$, $p < 0.01$, $p = 0.011$ and $p = 0.021$, respectively). Afterwards, the concentration of IFN- γ , IL-4 and IL-17 gradually recovered to the previous level at d7 ($p = 0.008$, $p < 0.001$, respectively), but no significant decrease was observed in the concentration of IL-4, IL-10 and TGF- β 1 at d7 (Fig. 4).

There was no difference in concentration of all plasma cytokines between two groups at d0. IFN- γ and IL-4 concentration in Group A was remarkably

lower than group B at d2 ($p = 0.018$ and $p = 0.013$, respectively), while IFN- γ /IL-4 in Group A was significantly higher than group B at d7 ($p < 0.001$). However, the concentration of IL-17 and TGF- β 1 in Group A was significantly lower than group B ($p = 0.009$ and $p = 0.031$, respectively). The variations were highly consistent with the variation of mRNA expression detected by flow cytometry. Table VII demonstrates comparison of concentration of IFN- γ , IL-4, IL-17, IL-10 and TGF- β 1 between Group A and Group B.

Table IV. *Th1, Th2, Th17 and Treg cell frequency.*

	d0 (%)	d2 (%)	d7 (%)	<i>P</i> (d0 v d2)	<i>P</i> (d0 v d7)	<i>P</i> (d2 v d7)
Th1	7.25 $\pm$ 1.04	18.83 $\pm$ 1.96	13.42 $\pm$ 0.80	<0.001	<0.001	0.015
Th2	1.13 $\pm$ 0.06	3.31 $\pm$ 0.30	0.92 $\pm$ 0.11	<0.001	0.18	<0.001
Th17	2.57 $\pm$ 0.24	2.98 $\pm$ 0.25	1.24 $\pm$ 0.11	0.268	<0.001	<0.001
Treg	6.04 $\pm$ 0.25	6.94 $\pm$ 0.36	5.43 $\pm$ 0.52	0.160	0.327	0.021
Th1/Th2	8.11 $\pm$ 1.25	8.50 $\pm$ 1.13	14.87 $\pm$ 1.26	0.823	<0.001	<0.001

Note: t-test

Table V. *Comparison of Th1, Th2, Th17 and Treg cell frequency and Th1/Th2 between group A and group B.*

	Variable	Group A (%)	Group B (%)	<i>P</i> value
Th1	d0	7.44 $\pm$ 1.55	7.38 $\pm$ 1.34	0.870
	d2	14.67 $\pm$ 1.95	20.42 $\pm$ 2.66	0.098
	d7	16.56 $\pm$ 0.95	10.36 $\pm$ 0.85	<0.001
Th2	d0	1.08 $\pm$ 0.14	1.14 $\pm$ 0.22	0.745
	d2	2.65 $\pm$ 0.30	3.97 $\pm$ 0.50	0.027
	d7	0.74 $\pm$ 0.06	1.14 $\pm$ 0.15	0.233
Th17	d0	2.80 $\pm$ 0.25	2.65 $\pm$ 0.35	0.424
	d2	2.55 $\pm$ 0.29	3.02 $\pm$ 0.35	0.374
	d7	1.07 $\pm$ 0.10	1.45 $\pm$ 0.15	0.047
Treg	d0	5.44 $\pm$ 0.25	6.63 $\pm$ 0.45	0.372
	d2	7.52 $\pm$ 0.70	6.20 $\pm$ 0.80	0.194
	d7	4.50 $\pm$ 0.55	6.19 $\pm$ 0.80	0.003
Th1/Th2 ratio	d0	8.65 $\pm$ 1.76	7.62 $\pm$ 1.32	0.688
	d2	9.67 $\pm$ 1.95	7.40 $\pm$ 1.26	0.254
	d7	14.64 $\pm$ 0.56	6.03 $\pm$ 0.83	<0.001

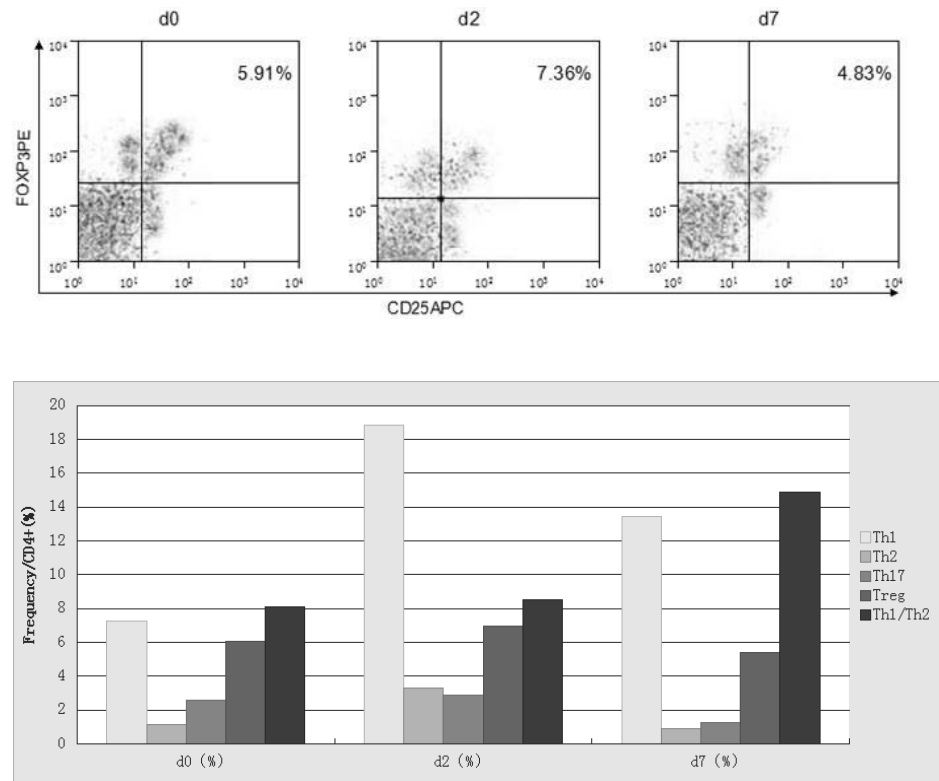


Fig. 1. Typical flow cytometry scatter plot of Treg ($p < 0.05$) and analysis of Th cell subset ($p < 0.05$).

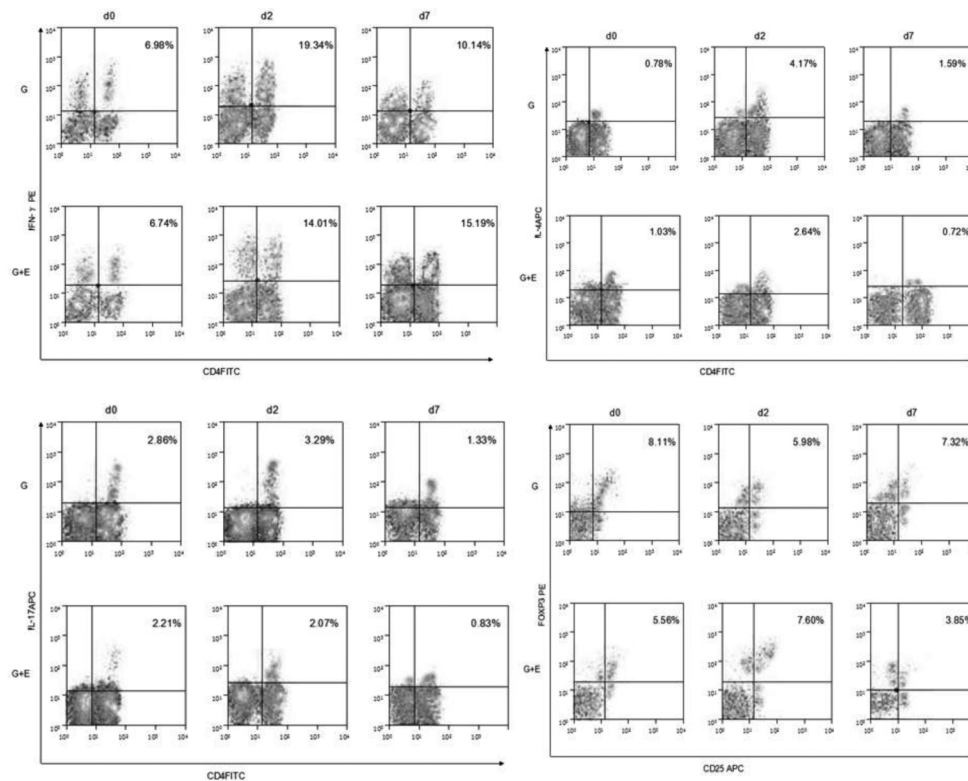


Fig. 2. Flow cytometry scatter plot of Th cell subset (Th1 cell frequency in group A was higher than group B at d7; Th2 cell frequency in group A was lower than group B at d2; Th17 and Treg cell frequency in group A was lower than group B at d7).

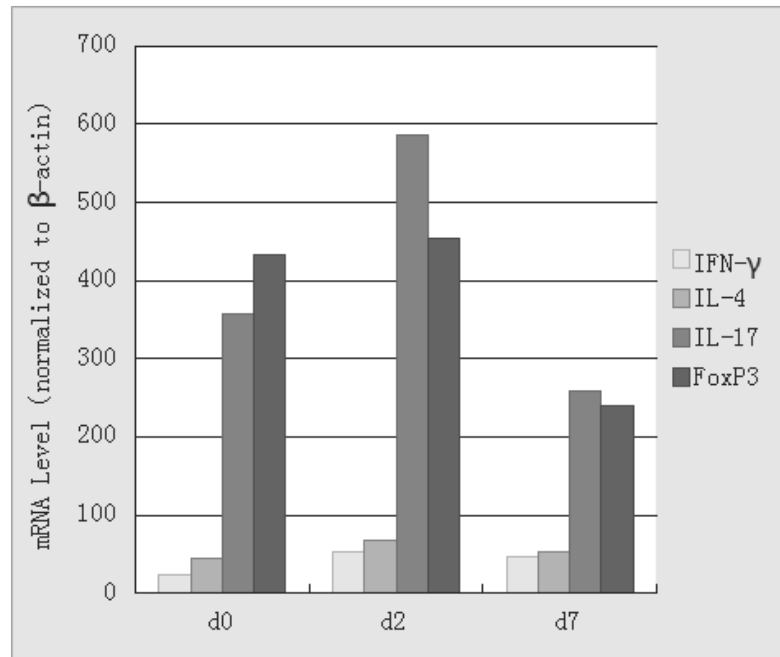


Fig. 3. mRNA expression of IFN- γ , IL-4, IL-17 and FoxP3 at d0, d2 and d7 ($p < 0.05$).

Table VI. Comparison of mRNA of IFN- γ , IL-4, IL-17 and FoxP3 and Th1/Th2 between Group A and group B.

	Variables	Group A ($\times 10^5$)	Group B ($\times 10^5$)	<i>P</i> value
IFN- γ	d0	21.85 $\pm$ 2.55	17.38 $\pm$ 1.34	0.570
	d2	50.97 $\pm$ 1.45	60.42 $\pm$ 2.66	0.098
	d7	41.56 $\pm$ 1.15	29.36 $\pm$ 0.85	0.029
IL-4	d0	41.48 $\pm$ 28.64	59.14 $\pm$ 15.22	0.061
	d2	66.65 $\pm$ 18.30	113.97 $\pm$ 19.50	<0.01
	d7	30.74 $\pm$ 14.06	58.14 $\pm$ 5.15	0.224
IL-17	d0	352.80 $\pm$ 55.25	386.65 $\pm$ 60.35	0.768
	d2	406.55 $\pm$ 60.29	677.02 $\pm$ 133.35	0.171
	d7	221.07 $\pm$ 18.10	429.45 $\pm$ 10.15	0.043
FoxP3	d0	431.44 $\pm$ 55.25	386.63 $\pm$ 42.45	0.408
	d2	467.52 $\pm$ 89.70	432.20 $\pm$ 44.80	0.338
	d7	248.50 $\pm$ 58.55	361.19 $\pm$ 46.80	0.109
Th1/Th2 ratio	d0	0.65 $\pm$ 0.76	0.62 $\pm$ 0.32	0.883
	d2	0.67 $\pm$ 0.15	0.40 $\pm$ 0.26	0.198
	d7	1.24 $\pm$ 0.06	0.04 $\pm$ 0.03	0.003

Note: *t*-test

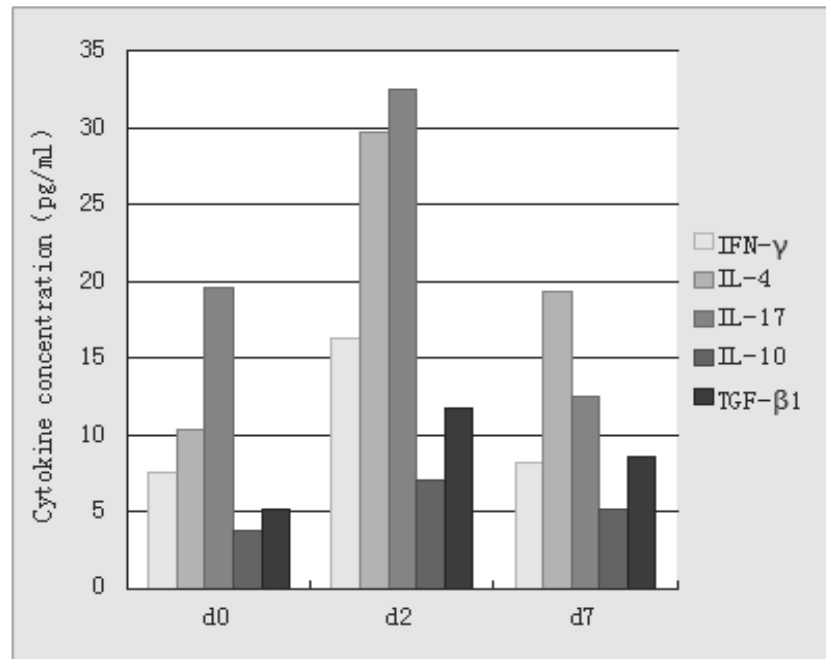


Fig. 4. concentration of IFN- γ , IL-4, IL-17, IL-10 and TGF- β 1 at d0, d2 and d7 ($p < 0.01$).

Table VII. Comparison of Concentration of IFN- γ , IL-4, IL-17, IL-10 and TGF- β 1 between Group A and Group G.

	Variable	Group A(pg/ml)	Group G (pg/ml)	P value
IFN- γ	d0	7.85 $\pm$ 0.55	6.38 $\pm$ 0.34	0.670
	d2	11.97 $\pm$ 0.45	19.42 $\pm$ 1.66	0.018
	d7	8.56 $\pm$ 0.15	5.36 $\pm$ 0.5	0.129
IL-4	d0	10.48 $\pm$ 1.64	10.14 $\pm$ 1.22	0.861
	d2	21.65 $\pm$ 3.30	36.97 $\pm$ 6.50	0.013
	d7	13.74 $\pm$ 1.06	25.14 $\pm$ 2.15	0.065
IL-17	d0	20.80 $\pm$ 5.25	18.65 $\pm$ 3.35	0.304
	d2	27.55 $\pm$ 10.29	40.02 $\pm$ 10.35	0.218
	d7	9.07 $\pm$ 1.10	16.45 $\pm$ 2.15	0.060
IL-10	d0	3.44 $\pm$ 0.25	3.63 $\pm$ 0.45	0.508
	d2	7.52 $\pm$ 0.70	5.20 $\pm$ 0.80	0.238
	d7	4.50 $\pm$ 0.55	5.19 $\pm$ 0.80	0.009
TGF- β 1	d0	6565 $\pm$ 1667	5312 $\pm$ 1102	0.583
	d2	15680 $\pm$ 4254	8375 $\pm$ 1395	0.198
	d7	7555 $\pm$ 1555	10691 $\pm$ 2571	0.303
INF- γ /IL-4	d0	0.74 $\pm$ 0.10	0.65 $\pm$ 0.13	0.618
	d2	0.67 $\pm$ 0.09	0.56 $\pm$ 0.05	0.309
	d7	1.10 $\pm$ 0.14	0.35 $\pm$ 0.08	< 0.001

DISCUSSION

Influence of hepatectomy on the balance of Th cell subsets

Since the operative wound has significant influence on the immune function (7-8), we hypothesized that it would also have significant impact on Th1/Th2 ratio and levels of Th17 and Treg cells. It was found that on the second day after hepatectomy, all Th cells showed a tendency to increase, while on the seventh day after surgery, all Th cells recovered to previous levels. The results revealed that the operative wound could increase the quantity of Th cells but this stimulation did not last for long. There was no significant variation in Th1/Th2 on the second day after surgery, but on the seventh day of surgery, Th1 dominated, and at the same time, there was a remarkable decrease in the cell frequency of Treg and Th17 compared with before surgery. All these results indicated that the removal of tumor relieved the inhibition of the immune system, thus enabling auto-immunization to develop in a direction which was beneficial to the prognosis of patients. Therefore, variation of Th cell after hepatectomy played an important role in the anti-tumor cells mechanism. Some Authors (14-16) held that removing primary tumor with surgery could reverse the immunosuppression of tumor. For other Authors, the increase of Th cells after surgery might be caused by surgical stress, and inflammation reached the peak on the second day after surgery but recovered to the previous state on the seventh day. Variation of Th cell frequency was the same as above.

Influence of anesthetic techniques on Th1/Th2, Th17 and Treg cells

Anesthetic relates to immunosuppression during the perioperative period, because it directly inhibits cells in the body and humoral immunity. Hence, anesthetic and anesthetic methods should be carefully selected for each patient. In the present study, Th1/Th2 in group A significantly increased compared to group B and there were remarkable decreases in Th17 and Treg cell frequency in group A, which showed the advantage of general anesthesia in combination with epidural anesthesia. Moreover, Th1, Th2 and Th17 cell frequency in group A was lower than

group B on the second day of surgery, which also verified that general anesthesia in combination with epidural anesthesia had a better effect in reducing or eliminating the stress reaction related to surgery. Differences between these two anesthetic methods may cause difference of Th cell subsets. Firstly, local anesthesia greatly eliminates the stress response caused by surgery. Secondly, opioid medicines inhibit cellular and humoral immunity (9). In group A, drugs were administrated through an epidural space while in group B they were given intravenously. Animal experiments (10, 11) have confirmed that morphine system administration can inhibit the proliferation of lymphocytes. Moreover, epidural injection of opioid medicines and local anesthetics carries out a better protective effect on the proliferation of lymphocyte and cytokine production than venous injection. Thirdly, intravenous anesthetics and inhalation anesthetics correlate to levels of catecholamine and corticosteroids in plasma (12). Glucocorticoid and catecholamine have significant impact on immunoregulation, including reducing the production of Th1/Th2 cytokines and increasing the mRNA expression of FoxP3. Thus, it is not surprising that general anesthesia has a significant inhibitory effect on immunoreaction during the perioperative period compared to epidural anesthesia in combination with general anesthesia. Apart from surgery and anesthesia, other factors may also influence Th cells, including hepatitis virus infection, liver cirrhosis, blood transfusion and pain level (13). The results of flow cytometry, cytokine expression and concentration detection are all quite consistent, showing the repeatability and reliability of this research (14-16).

Clinical prognosis

Epidural anesthesia in combination with general anesthesia has proved to be a better choice for patients with developing tumor. Among patients who received epidural anesthesia in combination with general anesthesia, Th1 dominated and meanwhile Th17 and Treg cells declined obviously. Thus we believe that epidural anesthesia in combination with general anesthesia is more beneficial for clinical prognosis of liver cancer patients.

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A NOVEL QUANTITATIVE METHOD OF PTEN EXPRESSION ASSESSMENT IN TUMOR TISSUE

D. WANICZEK¹, M. SNIETURA², A. KOPEC², D. SCIEGLINSKA³, W. PIGLOWSKI^{2,3},
Z. LORENC¹, M. MUC-WIERZGON⁴ and E. NOWAKOWSKA-ZAJDEL⁵

¹Department of Propaedeutics Surgery, Chair of General, Colorectal and Polytrauma Surgery, School of Health Sciences in Katowice, Medical University of Silesia, Katowice, Poland; ²Tumor Pathology Department, Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice Branch, Poland; ³Center for Translational Research and Molecular Biology of Cancer, Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice Branch, Poland; ⁴Department of Internal Medicine, School of Public Health in Bytom, Medical University of Silesia, Katowice, Poland; ⁵Department of Nutrition-Related Diseases Prevention, School of Public Health in Bytom, Medical University of Silesia, Katowice, Poland

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Phosphatase and Tensin Homolog deleted on chromosome 10 (PTEN) gene is one of the most important tumor suppressor genes which is involved in the regulation of many signaling cascades (AKT/PKB and MAPK). Subtle changes in its activity lead to cancer susceptibility or aggressive tumor behaviour. Despite the diversity of mechanisms leading to PTEN inactivation, it is frequently associated with a decreased or complete loss of protein expression. About 20% decrease in PTEN expression could lead to the development of cancer. There have been no objective, quantitative methods of PTEN expression assessment that allow to measure the subtle variations of the protein concentration in a tissue-contextual manner. A new quantitative algorithm of immunostaining evaluation based on combination of color deconvolution and relative chromogen signal intensity was used in the study. The proposed algorithm was implemented in the popular ImageJ image analysis software and positively verified in cancer cell lines and tissue models as well as in the tissue samples of colorectal cancer (CRC) patients. The proposed quantitative method of PTEN expression assessment creates an alternative to currently available subjective methods and forms the basis for inter-case and inter-tissue comparisons. Using the algorithm it would be possible to identify three groups of patients with advanced colorectal cancer which could significantly differ in the overall survival. The research should be continued.

The tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) is a dual-specific phosphatase that acts as a negative regulator of the PI3K-AKT-mTOR pathway, thus controlling a variety of processes related to cell survival, proliferation and growth. Along with known

cytoplasmic functions, PTEN exhibits a plethora of nuclear activities that include the regulation of genomic stability, cell cycle progression, differentiation, and gene expression (1-3). These factors form the basis of an increased interest in PTEN and other elements of the AKT pathway as potential

Key words: PTEN expression assessment, a new quantitative method, colorectal tumor tissue

Mailing address:

Dr Ewa Nowakowska-Zajdel,
Department of Nutrition-Related Diseases Prevention,
School of Public Health in Bytom,
Medical University of Silesia,
41-902 Bytom, ul. Piekarska 18, Katowice, Poland
Tel.: +48 32 39 76 541 - Fax: +48 32 39 76 532
e-mail: ewanz@onet.eu

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prognostic and predictive markers in various types of cancers, of which head and neck, breast, prostate and lung cancers are the best examples.

PTEN is one of the most mutated and deleted tumor suppressors in human cancers, especially in advanced stages but, importantly, it is also found partially downregulated in tumors in the absence of genetic loss or mutation (4, 5). It has recently become evident that *PTEN* expression and its activity is a subject of subtle regulatory processes that can be disrupted in any stage of carcinogenesis. The latest studies on genetically modified mice with hypomorphic *PTEN* alleles demonstrating a full range of *PTEN* expression from 80% to complete loss of the protein showed that as small a change as a 20% loss of *PTEN* activity markedly increases cancer susceptibility in healthy individuals. The idea that subtle changes in the expression of gene can have profound effects on tumorigenesis, called quasi-insufficiency, was thus proposed (6, 7). Moreover, the spectrum of tumors developed by *PTEN*-hypomorphic animals is distinct from that observed in heterozygous mice. These data suggest that there is tissue-specific sensitivity to the reduction of *PTEN* levels, with some tissues requiring a more profound downregulation in *PTEN* for cancer progression to occur (like prostate cancer), while other tissues are more sensitive to small changes, such as mammary glands (6,7).

Another important paradigm defined by research on *PTEN* functioning, obligate haploinsufficiency, can radically influence tumor biology. According to obligate haploinsufficiency, the complete loss of *PTEN*, rather than continuing to promote cancer, may be less tumorigenic than a heterozygous loss of genes due to a fail-safe cellular senescence mechanism triggered by functional P53 (8).

All of the known data show that there is an urgent need for an objective, quantitative method of *PTEN* expression assessment in cells that takes into account the tissue context. In this work, we propose an algorithm of *PTEN* immunoreactivity quantification in standard immunohistochemistry slides based on the color deconvolution method (9) in combination with a subsequent calculation of the mean signal strength of the chromogen. The functioning of the

algorithm was verified in cultured cell lines with known *PTEN* expression levels as well as in the squamous epithelium playing the role of a tissue model.

MATERIALS AND METHODS

To visualize *PTEN* expression in neoplastic cell lines, squamous epithelium, as well as the Western blotting procedure of the monoclonal rabbit, anti-human *PTEN* antibody clone 138G6 (Cell Signaling Technology, Denver), raised against the last 100 C-terminal aminoacids of the protein, and the monoclonal mouse anti-human *PTEN*, clone 6H2.1 (Dako, Denmark), raised against the full-length recombinant protein, were used.

Cell blocks with known PTEN expression

In order to demonstrate the different levels of *PTEN* expression, formalin-fixed, paraffin-embedded cell blocks of neoplastic cell lines with a known *PTEN* status and expression were prepared. Short characteristics of the cell lines used are presented in Table I (10-12). The procedure of cell block preparation was as follows: the cells were cultured in an optimal growth medium supplemented with 10% fetal bovine serum and 40 µg/ml gentamycin sulfate at standard cell culture conditions (5% CO₂, 37°C) in Nunclon Δ EasY Flask 175 cm² (Nunc) to approx. 80% confluence. Next, cells were harvested by trypsinization, washed in isosmotic PBS and prefixed (60 min) in a 10% neutral buffered formalin supplemented with the phosphatase inhibitor cocktail PhosSTOP (Roche). Cells were counted (TC10TM Automatic Cell Counter, Bio-Rad), aliquoted (8 x 10⁶ cells), harvested by centrifugation (2000 x g, 5 min), re-suspended in 100 µl PBS, mixed with 1% agarose (dissolved in PBS by boiling, then brought to 50°C), and transferred to a 500 µl PCR tube (capped, inverted, and placed on ice until solidification). An agarose cylinder containing immobilized cells was fixed (24 h) in a 10% neutral buffered formalin supplemented with phosphatase inhibitors, washed in tap water for 60 min, dehydrated and paraffin-embedded to simulate a routine tissue processing protocol.

Western blotting of cell line lysates

To prepare the total protein extracts, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.5),

Table I. *Characteristics of cell lines with known PTEN status used in the study.*

Cell line	Species	Origin	PTEN expression	Loss of PTEN expression mechanism
MCF-7	human	breast cancer	moderate	normal expression
A-431	human	epidermoid carcinoma of the vulva	moderate	normal expression
U-2 OS	human	osteosarcoma	weak	loss of one allele
NCI-H1299	human	non-small cell lung cancer	weak/negative	promoter methylation
PC-3	human	prostate adenocarcinoma	negative	gene deletion

150 mM NaCl, 0.1% Nonidet P-40, 50 mM NaF, 1 mM DTT, 1 mM PMSF, and the protease inhibitor mixture Complete™ (Roche). After 20-min incubation on ice, the lysates were centrifuged at 14,000 x g for 10 min at 4°C and supernatants were aliquoted and stored at -80°C. The total protein content was determined using a Protein Assay Kit (Bio-Rad). Then, a 4×sample buffer (200 mM Tris-HCl 6.8, 20% β-mercaptoethanol, 8% sodium dodecyl sulfate, 40% glycerol, and 0.4% bromophenol blue) was added and the lysates were incubated at 98°C for 5 min. Protein samples (40 µg of the total protein extract) were separated in 10% polyacrylamide gels and transferred to the nitrocellulose membrane Protran BA 85 (Whatman) using a Mini-PROTEAN II electrophoresis system (Bio-Rad) according to the manufacturer's instructions. After blocking for 60 min with 5% skimmed milk in TTBS (250 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH7.5), the blots were incubated overnight with a primary antibody diluted in a blocking solution with gentle agitation at 4°C. The following antibodies were used for the Western blot analysis: monoclonal rabbit anti PTEN (138G6, Cell Signaling Technology) was used at a 1:4000 dilution, monoclonal mouse anti-PTEN (6H2.1, Dako, Denmark) at a 1:250 dilution, and monoclonal mouse anti-pan-actin (MAB1501R C4 clone, Chemicon; Millipore) at

a 1:1000 dilution. The primary antibodies were detected by polyclonal horseradish peroxidase conjugated secondary goat anti mouse/rabbit IgG H+L (#31430 or #32460, Pierce; Thermo Scientific) and visualized using SuperSignal® West Pico Chemiluminescent Substrate Kits (Pierce; Thermo Scientific) according to the manufacturer's instructions on X-Omat Blue film (Kodak).

Tissue model of stratified PTEN expression

Immunostaining was performed according to standard guidelines: three sections of cell blocks, and squamous epithelium of 4-µm thickness were cut, mounted on slides, deparaffinized, and rehydrated in xylene and in a graded series of ethanol. Epitope retrieval was performed for 30 min at 98°C in TRIS buffer/ EDTA 10 mmol/l, pH 9.0. Endogenous peroxidase activity was inactivated by a 3% solution of hydrogen peroxide for 5 min. Next, primary antibodies were applied to the slides in three increasing dilutions (1:50; 1:100; 1:200) in a background reducing antibody diluent, (Dako, Denmark) and incubated for 30 min at room temperature. Finally, a mixture of polymer conjugated with horseradish peroxidase and affinity-purified goat anti-rabbit/anti-mouse Fab' antibody fragments and DAB chromogen was used to

visualize the reaction (EnVision+Dako, Denmark). After counterstaining in Mayer's hematoxylin solution (Sigma-Aldrich, St. Louis, MO, USA), the specimens were coverslipped with a DPX mounting medium (Sigma-Aldrich, St. Louis, MO, USA).

PTEN expression in colorectal cancer tissues

Sixty patients with histopathologically confirmed colorectal carcinoma (CRC) after major surgical treatment were enrolled in the study. Clinical data, disease-free survival and overall survival were recorded for all patients. A comprehensive characteristic of the study group is presented in Table II. Expression of PTEN in formalin-fixed, paraffin-embedded tumor samples taken from the patients during major surgery was assessed according to the procedure described above. Anti-human PTEN antibody clone 138G6 (Cell Signaling Technology, Denver) diluted 1:100 was used to demonstrate expression and location of PTEN protein in tumor sections. Sections of squamous cell epithelium played a role of positive and negative control while basal cells of this epithelium served

as reference during calculation of relative chromogen intensity in neoplastic tumor tissue, as described below.

Digital image capture

All microphotographs were obtained using an AxioCam MRc5 five megapixel, color, Peltier-cooled digital camera coupled to an AxioImager microscope equipped with a 63x ECPlanNeofluar objective N.A.= 0.95 (Carl Zeiss, Germany). Control of the microscope, the exposure parameters, as well as the image acquisition in a loss-less tiff format, were performed with AxioVision 4.8 software (Carl Zeiss, Germany). For every immunohistochemical slide 5 representative microphotographs of cells (containing about 35 cells each) or squamous epithelium were taken.

Quantification of the chromogen

All image analyses were performed by means of ImageJ version 1.47t - a public domain, Java-based image processing software with the color deconvolution plug-in version 1.3 installed (13). This plug-in implemented

Table II. Clinico-pathological characteristics of the CRC patients enrolled in the study group. No statistically significant differences between groups (recurrent and no evidence of disease) were observed.

Feature	Study group	Recurrent disease	No evidence of disease
Number of cases	60	20	40
Median of age	67.7 (45-84)	70.5 (45-83)	65.0 (40-84)
Gender	F: 32 (53.3%) M: 28 (46.7%)	F: 12 (60.0%) M: 8 (40.0%)	F: 20 (50.0%) M: 20 (50.0%)
Tumor location	Right bowel: 22 (36.7%) Left bowel: 38 (63.3%)	Right bowel: 6 (30.0%) Left bowel: 14 (70.0%)	Right bowel: 16 (40.0%) Left bowel: 24 (60.0%)
Staging	T3: 60 M0: 60 (100%) N0: 32 (53.3%) N1: 20 (33.3%) N2: 8 (13.3%)	T3: 20 M0: 20 (100%) N0: 8 (40.0%) N1: 7 (35.0%) N2: 5 (25.0%) p=0.051	T3: 40 M0: 40 (100%) N0: 24 (60.0%) N1: 13 (32.5%) N2: 3 (7.5%)
Adjuvant therapy	Administered: 37 (61.7%) Not administered: 23 (38.3%)	Administered: 13 (65.0%) Not administered: 7 (35.0%)	Administered: 24 (60.0%) Not administered: 16 (40.0%)
Tumor grading	G1: 4 (6.7%), G2: 45 (75%), G3: 11 (18.3%)	G1: 1 (5.0%), G2: 14 (70.0%), G3: 5 (25.0%)	G1: 3 (7.5%), G2: 31 (77.5%), G3: 6 (15.0%)

the method described by Ruifrok et al. (9) and is based on un-mixing an RGB image produced by subtractive mixing (inks, histological dyes) into separate channels corresponding to up to three determined colors. A detail graphical representation of the implemented algorithm is shown on Fig. 1. The principle of the proposed method consists of calculating chromogen concentration in target cells in relation to its concentration in the reference cells exhibiting a strong, case-to-case uniform color reaction. The resulting relative chromogen concentration was additionally corrected for residual background staining present in negative reference cells. In the present analysis, the cellular and tissue model were studied and the MCF-7 cells, as well as the basal cells of squamous epithelium of buccal mucosa, were suggested as positive references, respectively. The PC3 cell line, as well as superficial cells of the squamous epithelium, served as negative references in the cellular and tissue models, respectively. Three reference slides were cut and processed together with every diagnostic batch, ensuring the same conditions for the immunostaining reaction. One slide was stained with hematoxylin only and another two were immunostained for PTEN, one of them without hematoxylin counterstaining. The single-stained slides were used for the generation of color mask matrices in the color deconvolution algorithm, as described elsewhere (13).

Statistical analysis

The statistical analysis was performed using Statistica 9.0 (Statsoft). Differences between groups were evaluated by ANOVA. All values presented are expressed as means with standard deviations. Differences with p-values equal or less than 0.05 were considered to be statistically significant. Comparison and analysis of overall survival and disease-free survival in groups of patients with colorectal cancer were performed using Log-rank test and Kaplan-Meier estimation of survival curves.

RESULTS

The results of immunostaining for PTEN protein with the two antibodies used are shown in Fig. 2. The MCF-7 cells in both cases demonstrated strong, uniform chromogenic reactions, which were clearly visible due to the abundant cytoplasm of these cells. The PC3 cells revealed no evident reaction with

the 138G6 antibody, in contrast to the broad range of intensities seen in individual cells with 6H2.1 antibody. Taking into account the biallelic loss of the *PTEN* gene in the PC3 cell line, this result may suggest the non-specific staining confirmed in Western blotting analyses of cell lysates (Fig. 3). The expression of PTEN in A-431 cells was slightly lower than in MCF-7 cells, while in the U-2 OS line carrying monoallelic loss of PTEN the expression was distinctly lower. NIH-1299 cells with *PTEN* promoter hypermethylation were characterized by very weak reaction.

The mean relative chromogen concentrations calculated in MCF-7 cells according to the proposed algorithm are presented in Fig. 4A and are in good agreement with microscopic findings. It is also evident that the relative chromogen intensity does not significantly depend on the primary antibody dilution in the examined range of 1:50-1:200 (Fig. 4B).

The results of immunostaining of the non-keratinizing squamous epithelium for the PTEN protein, as well as the staining separation by means of color deconvolution, are shown in Fig. 5. It is worth noting that, due to the stratified expression of the PTEN protein in the squamous cell epithelium, which reveals a full range of immunostaining from the strong one in basal cells to the lack of staining in the superficial layer, this tissue could be used simultaneously as a positive and negative reference. As shown in Figs. 5D and 5E, the changes of the chromogen concentration in subsequent epithelium layers were not linear and could be best approximated by a logarithmic curve demonstrating relatively high variations in the intermediate layers. However, the DAB concentration in opposite external cellular layers indicated low inter-case variability, as shown by the low value of the standard deviation.

Finally, we tested the proposed algorithm of relative PTEN expression assessment in the cohort of patients with locally advanced colorectal cancer. We could identify 3 groups differing in overall survival and similar tendency in disease-free survival as shown by the Log-rank test ($p=0.071$). As shown in Fig. 6, overall survival was the longest in patients with expression of PTEN greater than 80% of basal cells in batch control. Mean survival in this group

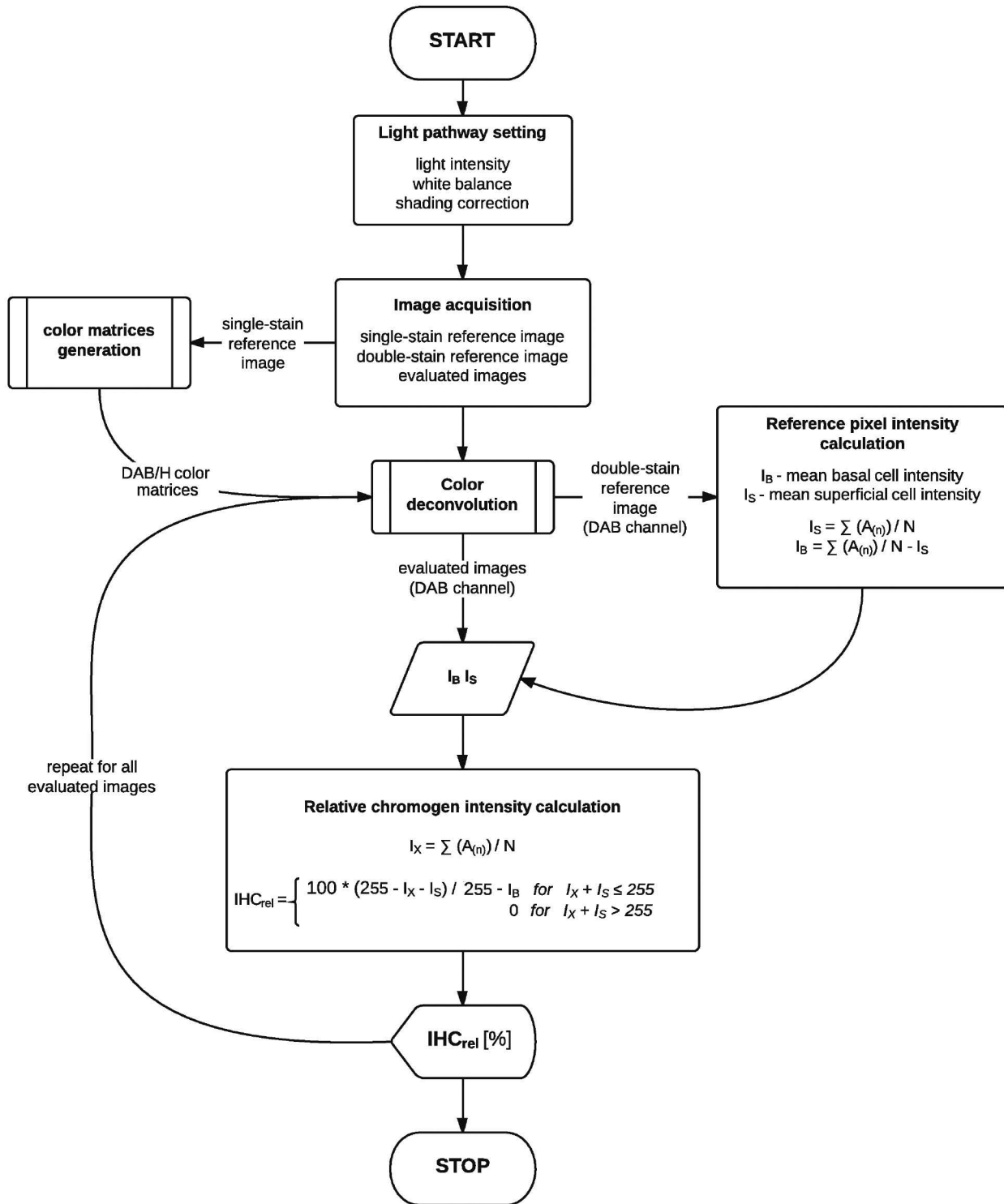


Fig. 1. Proposed algorithm of the relative chromogen intensity calculation. I_S – mean residual background staining calculated in the superficial cells of squamous epithelium or PC3 cells; I_B – mean DAB intensity calculated in the basal cells of reference epithelium or MCF-7 cells corrected for residual background staining (I_S); A_n – pixel value of the selected region of interest; N – number of pixels in the given region of interest; I_X – mean DAB intensity in the target cells or tissue; IHC_{rel} – relative chromogen intensity calculated in the target cells.

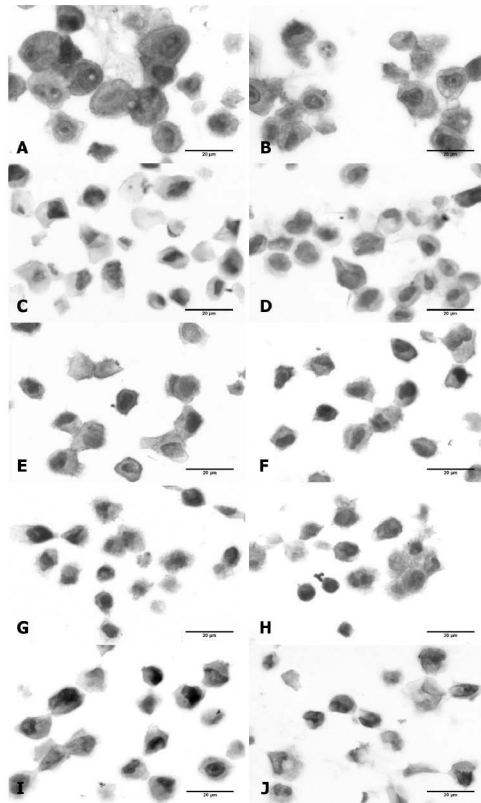


Fig. 2. Immunostaining for PTEN protein in cell lines. Variety of PTEN expression profiles in the investigated cell lines. Left column (A,C,E,G and I) - immunostaining with 138G6 antibody (Cell Signaling Technology), right column (B,D,F,H and J) - immunostaining with 6H2.1 antibody (Dako, Denmark). **A,B**) Strong PTEN expression in MCF-7 cells; **C,D**) lack of staining in PC3 cells (note range of positive staining in PC3 cells demonstrated by the 6H2.1 antibody); **E-J**) intermediate PTEN expression in A-431 (E,F), U-2 OS (G,H) and NIH-1299 (I,J) cell lines.

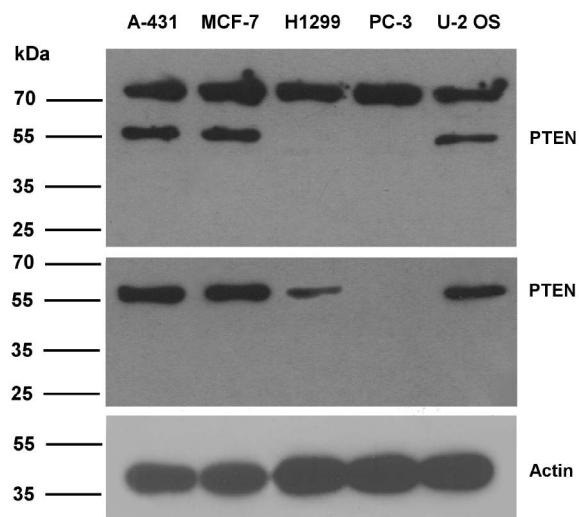


Fig. 3. Results of Western blotting analysis of cell lysates with 6H2.1 (topmost); 138G6 (middle) and control AC-74 anti- β -actin (undermost) antibodies. Cell lines from left to right: A-431, MCF-7, NIH-1299, PC-3, U-2 OS.

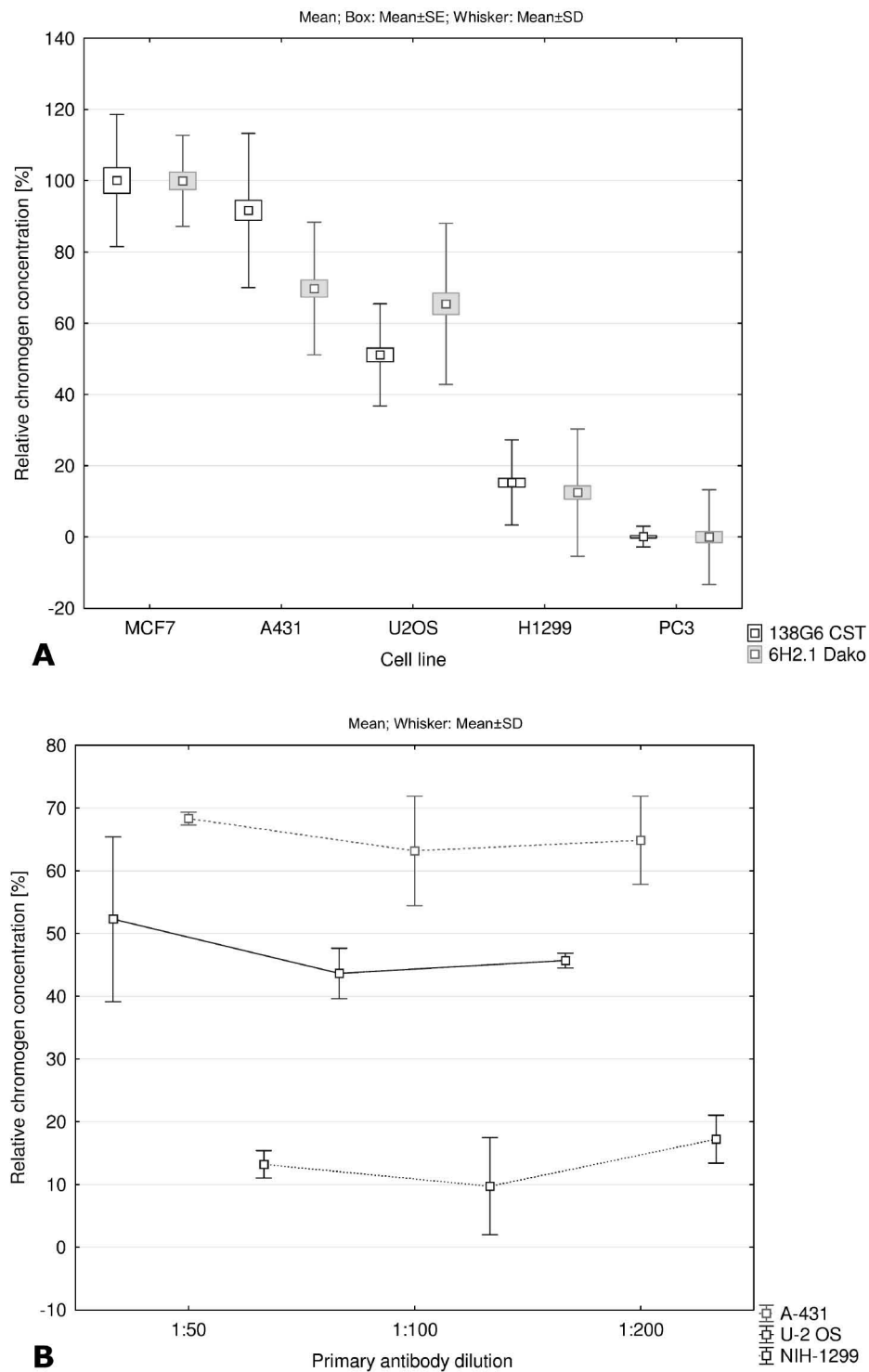


Fig. 4. Relative chromogen concentration in particular cell lines. Intensity of PTEN immunostaining in particular cell lines calculated in relation to MCF-7 cells (**A**); the influence of the primary antibody dilution on the measured chromogen intensity (mean of 138G6 and 6H2.1 antibodies) (**B**). No statistically significant differences between antibodies and antibody dilutions were observed. Each data point shown, comprises approximately 175 individual cells.

was 1,210 days (SD=528 days). Slightly shorter survival was observed in individuals with only partial loss of PTEN with relative staining intensity in range between 20% and 80%. Mean survival in this group was 1,190 days (SD=397 days). Finally, the shortest OS of 878 days (SD=519 days) was associated with nearly complete loss of PTEN expression in tumor tissue characterized by less than 20% of PTEN staining intensity.

DISCUSSION

PTEN is one of the most important tumor suppressor genes involved in the regulation of many signaling cascades including AKT/PKB and MAPK. Subtle changes in its cellular activity, which in many cases result from decreased expression, lead to cancer susceptibility or aggressive tumor behavior in late stages of carcinogenesis. Recently, Pandolfi et al. (6-8) demonstrated that a decrease in PTEN expression by as little as 20% leads to significant changes in cell proliferative activity. Furthermore, the biological effects of these changes are non-linearly dependent on the actual PTEN protein concentration, in particular the tissue context. The conclusion of this basic biological research is that there is an urgent need for objective, quantitative methods of PTEN expression assessment that allow one to measure and compare these subtle variations of the protein concentration in a tissue-contextual manner.

Until now, there have been no standardized procedures of PTEN immunostaining assessment. Three methods are currently widely used. The first one is based on an almost arbitrary discrimination of the immunohistochemical reactivity into one of three intensity categories: high/medium/low (14). Another method relies on a semi-quantitative immunoreactivity graduation by considering the percentage and intensity of the staining of the tumor cells (15). The last procedure, proposed by Perren et al. (16), has a relative nature and is based on comparing target immunostaining with the neighboring histological structures chosen as a local positive reference. All of the methods discussed above suffer from a lack of objectivity

because the interpretation of the staining depends on the experience and alertness of the interpreter and/or the absolute selection of a positive reference. This situation creates problems with inter-case, inter-observer, and inter-laboratory variations and makes comparisons extremely difficult, leading to ambiguous or contradictory results (14, 17).

It would seem evident that in order to achieve a quantifiable result with an IHC stain, thereby converting it to a quantifiable immunoassay, the total staining process must itself first be standardized (18, 19). In this context, reproducibility can be achieved only if rigorous quantifiable reference standards are available and are used to calibrate the system (20). In the present study, we proposed an algorithm of quantitative PTEN concentration assessment in routine immunohistochemistry slides based on the color deconvolution method (9) in combination with the subsequent calculation of a mean signal strength of the chromogen (chromogen concentration) in relation to the well-defined expression standard processed in tandem with target slides.

This standard plays a double role in the analysis sequence. First, single-stained slides are treated as a source for the calculation of the color deconvolution matrices necessary for DAB signal extraction from composite DAB/haematoxylin images. As indicated by Ruifrok et al. (9), this pair of chromogens can be successfully unmixed using simple RGB imagery if conversion to optical density is performed during color deconvolution. However, it may be necessary to measure the spectrum of the chromogens in the actual sample, after all the staining procedures have been performed, since the spectra can be affected by the presence of other dyes and reagents (21).

The second role that the standard plays is as a common reference of chromogen intensities in the context of the factors influencing the particular immunostaining process. In the current study, we proposed that the normal squamous epithelium of buccal mucosa or cell block matrices containing MCF-7 and PC-3 cells lines can play the role of a common reference. Due to simultaneous PTEN-positive and PTEN-negative cell lines present in the matrix, as well as the stratified expression of the PTEN protein in the squamous cell epithelium,

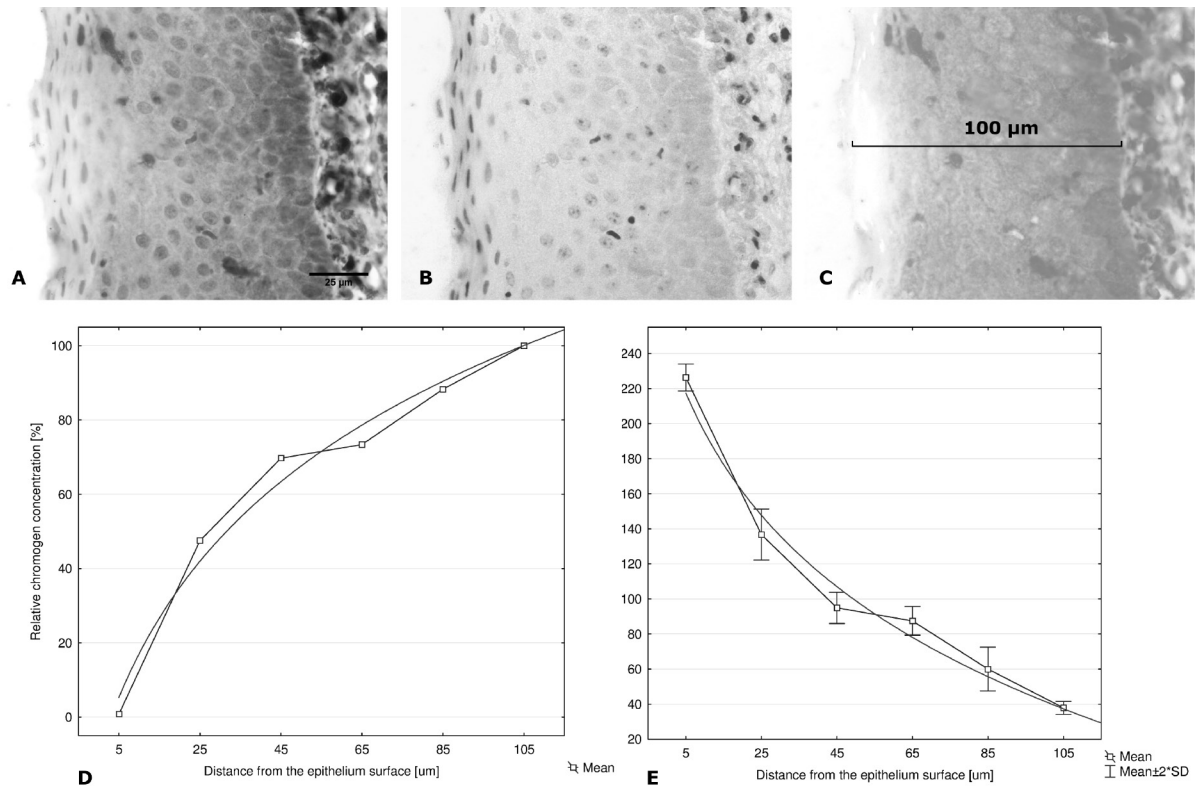


Fig. 5. A stratified expression of the PTEN protein in the squamous cell epithelium. A full range of immunostaining, from the strong in basal cells to a lack of staining in the superficial layer, is seen (A). The results of color deconvolution of double-stained image (A) show hematoxylin staining (B) and DAB chromogen localization (C). Mean profile of absolute pixel intensities (E) and DAB relative concentration calculated according to the proposed algorithm (D) related to the distance from surface of the epithelium (number of measurements $n=25$ for each data point). Note very low values of the standard deviation of DAB concentration in superficial and basal cell layers what makes them a good candidates for common negative and positive references.

which revealed a full range of immunostaining from the strong in basal cells to a lack of staining in the superficial layer, these standards could be used both as a positive and a negative reference in cell lines or tissue immunohistochemical studies, respectively. This approach allows one to calculate the target DAB intensity in relation to the MCF-7/basal cells and subsequently make corrections for residual background staining, if present in the PC3/superficial cells. It is important to note that the algorithm under consideration does not quantify the PTEN protein in cells or tissues. It provides only a coarse approximation of the expression due to the non-stoichiometric nature of antigen-antibody reactions and fact that DAB does not

fulfill Lambert-Beer's law. There is only a linear relationship between the amount of antigen and the staining intensity at low levels of the latter (21). Despite this disadvantage, the proposed method forms the basis for standardization and comparisons in PTEN research, particularly since all the image analyses are possible using the open-domain ImageJ software and the free color deconvolution plug-in; the method is free and widely available.

In order to proof usefulness of the proposed method in clinical practice we tried to assess PTEN expression profiles in a relatively homogenous group of patients with locally advanced colorectal cancer. As we demonstrated elsewhere (22) expression of PTEN may be an early event in

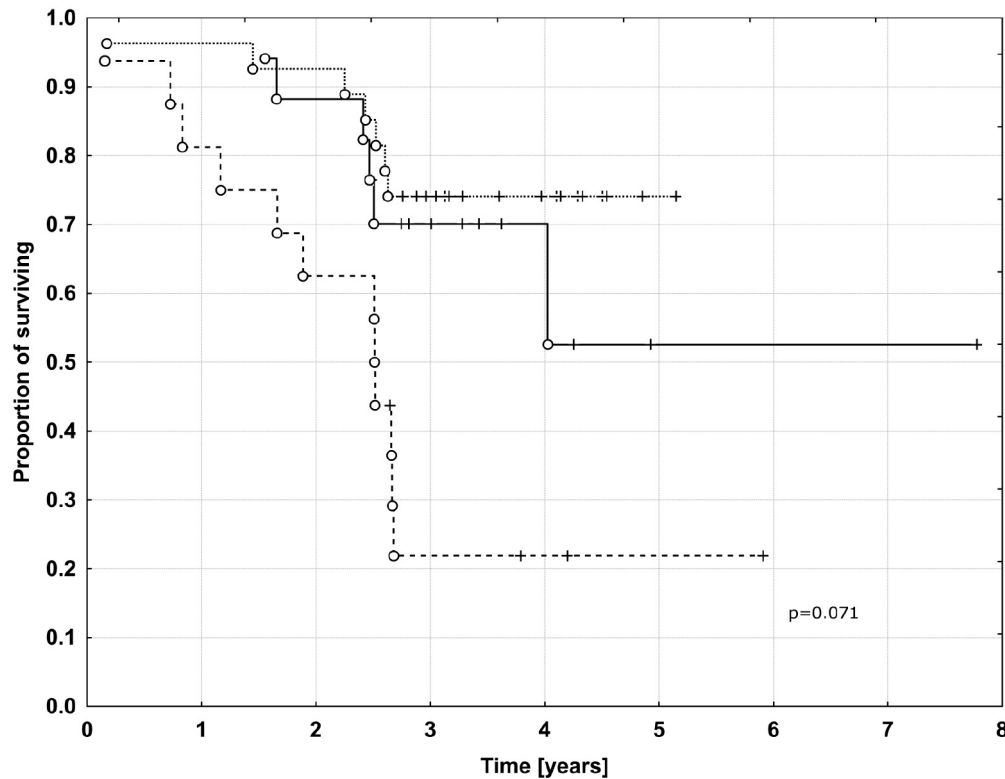


Fig. 6. Kaplan-Meier curves of the overall survival in the CRC patients with different relative expression of PTEN. The expression was calculated in relation to PTEN staining intensity in basal cells of squamous cell epithelium playing role of the batch control. Dotted line, solid line, dashed line - survival of patients with expression of respectively: over 80% of basal cells staining, 20%-80% of basal cells staining, less than 20% of basal cells staining intensity. Circles and crosses: incomplete and complete observations respectively. Statistical Log-rank test significance: $p=0.071$. All lines with known PTEN status used in the study.

colorectal carcinogenesis and can be already observed in adenomatous polyps of the large intestine with increasing frequency in more advanced stages. Other authors showed, as we did, correlation between loss of PTEN expression and poor prognosis (23-25) but they also difficulties in explicit assessment of PTEN expression and in making comparisons between cancer tissues (26). Using our relative quantification algorithm allowed us to distinguish more subtle changes in PTEN expression and demonstrated that worsening of prognosis may occur in cases with only slightly lowered PTEN expression. This finding remains in good concordance with Pandolfi's concept of quasi-insufficiency (7).

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INFLUENCE OF CONTINUOUS INTERVENTION ON GROWTH AND METASTASIS OF HUMAN CERVICAL CANCER CELLS AND EXPRESSION OF RNAmiR-574-5p

DL. MA¹, JY. LI¹, YE. LIU², CM. LIU³, J. LI⁴, GZ. LIN⁵ and J. YAN⁶

¹Department of Oncology, Yishui Central Hospital of Linyi, Linyi, China; ²Department of Infectious Disease, Yishui Central Hospital of Linyi, Linyi, China; ³Department of Oncology, Binzhou Medical College Affiliated Hospital, Binzhou, Shandong, China; ⁴Department of Clinical support, Dongming People's Hospital, Shandong, China; ⁵Department of Health Materials Management, Dongming People's Hospital, Shandong, China; ⁶Medical Social Work section, Dongming People's Hospital, Shandong, China

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The first two authors contributed equally to this work

This study was carried out to acquire solid evidence that some common treatments could affect micro ribonucleic acids (miRNAs) by revealing the regulatory effect of genes, so as to provide a reference for further exploration of the prevention and treatment of cervical cancer. Nude mouse tumorigenicity assay was used to study the effect of inhibiting miR-574-5p on development and tumorigenic ability of Henrietta Lacks (HeLa) tumor. Cell wound scratch assay, flow cytometry and real-time quantitative polymerase chain reaction (RT-qPCR) were adopted to study the effects of anoxia and temperature, etc., on expression of miR-574-5p and QKI in HeLa as well as on the clone and migration ability of cells, to provide prevention and treatment of cervical cancer with new ideas and evidence. The results demonstrated that cervical cancer tissues had a significantly increased miR-574-5p expression compared with para-carcinoma tissues; conversely, GomaFu, overall QKI (pan-QKI) and QKI-5 messenger ribonucleic acid (mRNA) and protein expression all decreased. Part of the common nursing methods had a certain influence on miR-574-5p expression, HeLa reproduction and metastasis, and even cell cycle. For example, ultraviolet (UV) irradiation was effective in decreasing miR-574-5p expression of HeLa and inhibiting cell migration; severe hypoxia significantly decreased the survival rate of HeLa, leading to the increase of programmed death percentage and cell ratio in G2/M phase as well as the decrease of cell ratio in G1 phase. Incubation at different temperatures also affected miR-574-5p expression and cell proliferation. Thus, it can be known that miR-574-5p, GomaFu and QKI expression in cervical cancer tissues and para-carcinoma tissues are significantly up-regulated or down-regulated. Some treatments, such as UV irradiation, hypoxia, incubation temperatures, etc., can affect miR-574-5p expression and HeLa proliferation as well as metastases in different degrees. These findings provide a reference and basis for further study.

Quaking (QKI) signaling protein is a type of protein that aggregates signal transduction function and RNA activation function (1). In recent years, studies

on QKI gene protein (2) indicated that QKI protein might be closely related to microRNAs (miRNAs) and the development of tumors. miRNA belongs to

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Mailing address:

Dr Changmin Liu,
Department of Oncology,
Binzhou Medical College Affiliated Hospital,
The Yellow River two Road No 661,
Binzhou, 256603, Shandong, China
e-mail: liuchangmingll@163.com

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non-coding RNA and studies have found that, on the one hand, miRNA could exist as cancer genes (3-4); on the other hand, increasing evidence in recent years showed that miRNA could play an important role as cancer suppressor genes in inhibiting cancer (5).

Recently, miR-574-5p-related researches discovered that miR-574-5p could be used as biological diagnosis markers of non-small cell lung cancer (6); moreover, miRNA could combine with the miRNA response element (MRE) of 3'-UTR of target mRNA to induce degradation of target mRNA, inhibition of protein translation or change of cellular localization, to further regulate the expression of target genes (7). Approximately 400 kinds of potential binding sites of miRNA can be found on 3'-UTR of QKI gene, including multiple binding sites of miR-574-5p, suggesting that QKI may be affected by miR-574-5p-mediated post-transcriptional control of gene silencing. However, whether miR-574-5p and QKI have similar effects on development of other tumors remains unknown. Research in recent years verified that long non-coding RNA (lncRNA) had important functions in regulation of gene expression and signal transduction and quite a few lncRNAs could affect the expression of target genes through interacting with mRNA or miRNAs (8).

Gomafu, also known as myocardial infarction associated transcript (MIAT), is a recently discovered lncRNA. Preliminary bioinformatic analysis shows that miR-574-5p may have a certain correlation with Gomafu. On the other hand, miR-574-5p, QKI protein and Gomafu are usually expressed in the same tissue (such as nervous tissue) and make an important contribution to the same disease (such as schizophrenia). In order to discover the interaction among QKI, Gomafu and miR-574-5p, this study aims to provide information on prevention and treatment and nursing intervention means of cervical cancer for future studies through revealing the effect of gene regulation and obtaining evidence that some common clinical nursing intervention methods can affect miRNAs.

MATERIALS AND METHODS

Experimental materials

Human cervical cancer HeLa cells were obtained

from the national key laboratory of China, *Escherichia coli* DH5 α in competent state was used for plasmid transformation, and cervical cancer tissue samples were also collected from the laboratory.

PFlag-CMV2 carrier (Cat#:E7398), phsa-miR-574 and phsa-QKI-5 overexpression plasmids were pre-constructed.

Interfering viruses included LV-anti-miR-control (concentration: 1×10^9 TU/ml), LV-anti-miR-574-5p (concentration: 5.28×10^8 TU/ml), lncRNA Gomafu siRNAs (MIAT-homo-2602, MIAT-homo-3211, MIAT-homo-520) and negative control.

Analysis of cell cycle

Cells in logarithmic phase which were in good growth state were digested using pancreatic enzymes and then inoculated into a culture plate; the concentration in each well was 4×10^5 /ml. After cells were cultured overnight, growth and adherence status was observed. Corresponding experiment was performed if cells were in good status. The cells were then digested using 10% pancreatic enzymes at corresponding time points and collected and were centrifuged at 800 g for 6 min at room temperature and then supernatant was removed. 1 ml of phosphate buffer saline (PBS) was added for resuspension of cells, and were centrifuged at 800 g for 6 min at room temperature, and supernatant was removed. Next, 300 μ l of PBS was added to resuspend cells. Afterwards, the cells were slowly added to pre-cooled absolute ethyl alcohol (700 μ l) dropwise to fix cells overnight at 4°C without light, and the final concentration of ethyl alcohol was 70%. Cells obtained from the last step were centrifuged at 800 g for 10 min at room temperature and washed by PBS 2-3 times after supernatant was removed. Cells were resuspended in PBS (500 μ l) containing 100 μ g/ml RNaseA, then, wrapped in aluminum foil and incubated in the dark for 30 min at 37°C. Polyimide (PI) in 2 mg/ml was added to the solution obtained to a final concentration of 50 μ g/ml. And then, it was wrapped in aluminum foil and incubated in the dark for 30 min at 37°C. Lastly, samples were loaded and the number of cells in each cell cycle was measured; ratio after x-coordinate and y-coordinate in flow cytometry were set as PI fluorescence readings and the number of cells, respectively.

Analysis of cell survival rate

Cells in logarithmic phase and good status were digested using pancreatic enzymes, then, inoculated into

a culture plate at the concentration of 4×10^5 /ml per well and cultured overnight. The growth and adherence of cells were then observed. A corresponding experiment was performed if the cells were in a good status. Culture medium and PBS used for washing cells was collected at corresponding time points. Then cells were collected in the same tubes after being digested with 10% pancreatic enzymes and centrifuged at 800 g for 6 min at room temperature and then supernatant was removed. 1 ml of PBS was added in cells obtained before and cells were separated for resuspension. After that, cells were centrifuged at 800 g for 10 min at room temperature, and supernatant was removed, then 500 μ l of PBS containing 5 μ g/ml PI was added to gently separate cells for full mixing. Tubes were wrapped by aluminum foil and put on ice away from light for 10 min of incubation; then the numbers of surviving cells, and necrosis and apoptosis cells were counted after x-coordinate and y-coordinate in flow cytometry were set as FSC-A and PI-A, respectively.

Cell clonality detection

After the digestion of cells in logarithmic phase, the number of cells was counted using blood counting chamber to make sure that each well of the culture plate was inoculated with 100-500 cells. Cell growth was observed every day and Dulbecco's modified eagle medium (DMEM) was replaced every 2-3 days. The clone culture lasted for 5-10 days. Culture was terminated at the expected incubation time point. Culture medium was removed using a micro-suction pump and cells were washed gently by PBS once or twice. The cells were fixed using 100% methyl alcohol for 20 min at 4°C, after which they were washed by PBS once or twice and stained using 0.2% crystal violet staining fluid for 30 min at room temperature. After staining, the cells were washed by PBS for three times and then dried at room temperature and photographed.

Detection of migration ability of cells

Cells were observed to ensure they were in good growth status; then cells in logarithmic phase were used and digested using pancreatin and inoculated in a culture plate in 4×10^5 /ml. Cells were cultured overnight and then cell growth and adherence status was observed; corresponding experiments could be carried out if cell adherence was in good status. At corresponding time points of the

experiment, an appropriate amount of 10% pancreatin was used to digest cells; then cells were centrifuged in 800 g for 6 min at room temperature. An appropriate amount of culture medium without serum or double antibody was added to obtain single-cell suspension; blood counting chamber was used to count cells and make their concentration be 1×10^6 /ml. The Transwell upper chamber of each well was added with 200 μ l of DMEM, while the lower chamber was added with 500 μ l of DMEM; the whole culture plate was incubated at 37°C for 2 h and then received hydration. After hydration, the culture medium in upper and lower chambers of Transwell was removed gently using a micro-vacuum pump without damaging the membrane in Transwell. The Transwell upper chamber of each well was added with 150 μ l of the single-cell suspension prepared previously; lower chambers were added with 200 μ l of DMEM containing 10% fetal bovine serum (FBS); cells were cultured at 37°C for 24 h. After 24 h of incubation, Transwell chambers were taken out and washed gently by PBS for three times; cells on the upper layer of Transwell membrane were wiped off gently and cells on the lower layer of membrane were fixed using 100% methyl alcohol for 20 min at 4 °C. After the fixation, cells were washed by PBS for 2-3 times and stained for 10 min using 0.2% crystal violet staining solution. Crystal violet staining solution was removed and cells were washed by PBS for 3-5 times and dried at room temperature. Under an inverted microscope, 4-6 fields of view were selected randomly and the number of cells was counted and cells were photographed.

Statistical analysis

All data obtained in this experiment were analyzed using GraphPad Prism5.00 288 software and expressed as mean $\pm$ SD. Groups were analyzed by student's *t*-test or one-way analysis of variance. There was a statistical significance if $p < 0.05$, a significant difference in statistics if $p < 0.01$ and an extremely significant difference in statistics if $p < 0.001$ (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; NS: not significant).

RESULTS

Expression of miR-574-5p, pan-QKI, QKI-5 and Gmafu in cervical cancer tissues

It was seen from real-time quantitative polymerase

chain reaction (qPCR) detection that compared with para-carcinoma tissues, overall QKI (pan-QKI) messenger ribonucleic acid (mRNA) expression decreased sharply (19 cases) in 24 cervical cancer tissue samples, accounting for 79.17%. On the contrary, miR-574-5p expression was markedly up-regulated in cancer tissues (18/24, 75.0%) compared with para-carcinoma tissues. Change of pan-QKI mRNA expression was negatively related to overall change of miR-574-5p, which was similar to colorectal cancer results. QKI-5 mRNA and Gomaflu expression were also studied, although QKI-5 mRNA was unlikely to be the direct target gene of miR-574-5p and QKI-5 mRNA had an obviously decreased expression, just like pan-QKI in most of the tumor samples. Similarly, Gomaflu expression decreased significantly in cervical cancer tissues.

Apart from Real time qPCR detection, Western blot was used to further detect pan-QKI and QKI-5 expression in clinical samples of cervical cancer and para-carcinoma tissues. The results showed that pan-QKI of 19 (79.17%) samples decreased and QKI-5 of 16 (66.67%) samples decreased. In general, declining tendency of QKI-5 and pan-QKI expression in cancer tissues was basically the same.

Correlation between Gomaflu expression and miR-574-5p and QKI-5 expression in cervical cancer tissues

In correlation analysis, a certain negative correlation between pan-QKI and mRNA expression and miR-574-5p expression was found. However, the p value had no remarkable statistical significance which might be due to the lack of samples. Similarly, QKI-5 and mRNA expression in cervical cancer tissues and miR-574-5p also showed negative correlation.

To comprehend the correlation between lncRNA Gomaflu and miR-574-5p, a similar correlation analysis was performed. Gomaflu expression was negatively correlated with miR-574-5p in cervical cancer tissues to some extent, which was basically consistent with expectations. The value of Pearson r was -0.1137 and p value was 0.6618.

In the cervical cancer tissue samples above, QKI-5 and QKI-5 mRNA expression had positive

correlation with Gomaflu, respectively, through analysis ($r=0.6138$ and 0.6942 ; $p < 0.01$) as shown in Fig. 2A, 2B.

Influence of miR-574-5p on pan-QKI, QKI-5 mRNAs and protein expression in HeLa

Overexpressed miR-574-5p was transfected in HeLa with polyethyleneimine (PEI). Twenty-four hours later, cells were collected to verify the effect of miR-574-5p overexpression, results indicated that transfected miR-574-5p was 2.6 times more likely to overexpress than pFlag-CMV2 in the control group (Fig. 3A).

Pan-QKI and QKI-5 mRNAs expression decreased by 25% ($p < 0.01$, Fig. 3B). On the contrary, Gomaflu expression in HeLa increased significantly (60%, $p < 0.01$) resulted from miR-574-5p overexpression.

Western blot was performed on total HeLa cell protein and the results revealed that pan-QKI and QKI-5 protein in HeLa decreased sharply with the overexpression of miR-574-5p, and falling range was in accordance with mRNA change degree (Fig. 3C).

Protein expression of HeLa cells was also analyzed. Results of Western blot indicated that after inhibition of miR-574-5p, protein expression of pan-QKI and QKI-5 of HeLa cells also increased significantly, and the increasing extent was basically the same as that of mRNA in the previous experiment (Fig. 4).

Significant influence of miR-574-5p on HeLa cell migration

Cell wound scratch assay was carried out to explore the influence of inhibiting miR-574-5p on HeLa cell growth and migration. It was found that HeLa cell had a significantly decreased wound healing ability in comparison with the control group after 18- or 24-h scratch (Fig. 5), which demonstrated that miR-574-5p inhibition was effective in notably decreasing migration ability of HeLa cell.

Significant influence of miR-574-5p and Gomaflu on invasion and migration ability of HeLa cells

After 96-h slow virus inhibition of miR-574-5p on HeLa cells, virus-infected cells were digested and collected. Then invasion and migration ability of HeLa cells were detected according to operating

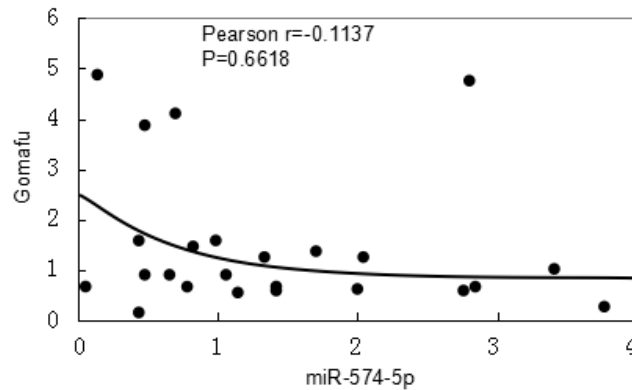


Fig. 1. Relationship between *Goma fu* and *miR-574-5p* expression in cervical cancer tissues.

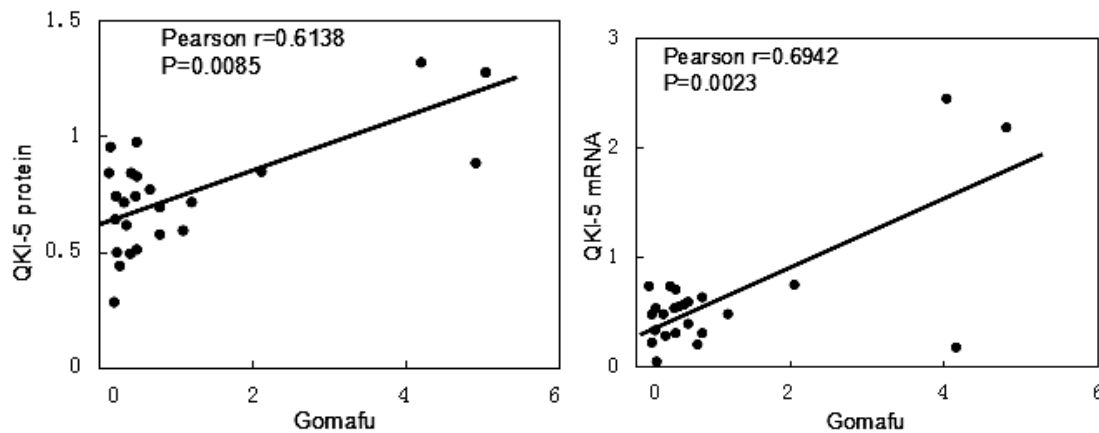


Fig. 2. Correlation between *QKI-5* and *Goma fu* expression in cervical cancer tissues.

procedures of Transwell. Results verified that after virus inhibition of *miR-574-5p*, number of HeLa cells passing through filter membranes and matrigel decreased significantly (Figs. 6 and 7). After statistical analysis, *p* values in both figures were less than 0.05 and had statistical differences compared with the control group. Transwell experiment verified that inhibiting *miR-574-5p* could inhibit invasion and migration ability of cervical cancers.

Influence of UV irradiation on miR-574-5p expression of HeLa cell

Cells (40 cm) were irradiated for one minute with UV (30 W and 70u W/cm²) after petri dish cover was removed. After irradiation, cells were incubated

conventionally at 37°C. After 24 h, total RNA was extracted to conduct *miR-574-5p* expression and cell wound scratch assay. One minute UV irradiation was found to be capable of dramatically reducing *miR-574-5p* expression (Fig. 8).

Influence of hypoxia intervention on miR-574-5p and HeLa cell proliferation and migration

CoCl₂ strong reductant was used to prepare cell hypoxia model in different degrees which was designed to study the possible influence of oxygen deficit on HeLa cell in detail. Firstly, 50 μM of CoCl₂ culture solution was made to construct a mild hypoxia condition for cell growth. Cells were collected after being treated for 0 h, 1 h and 2 h.

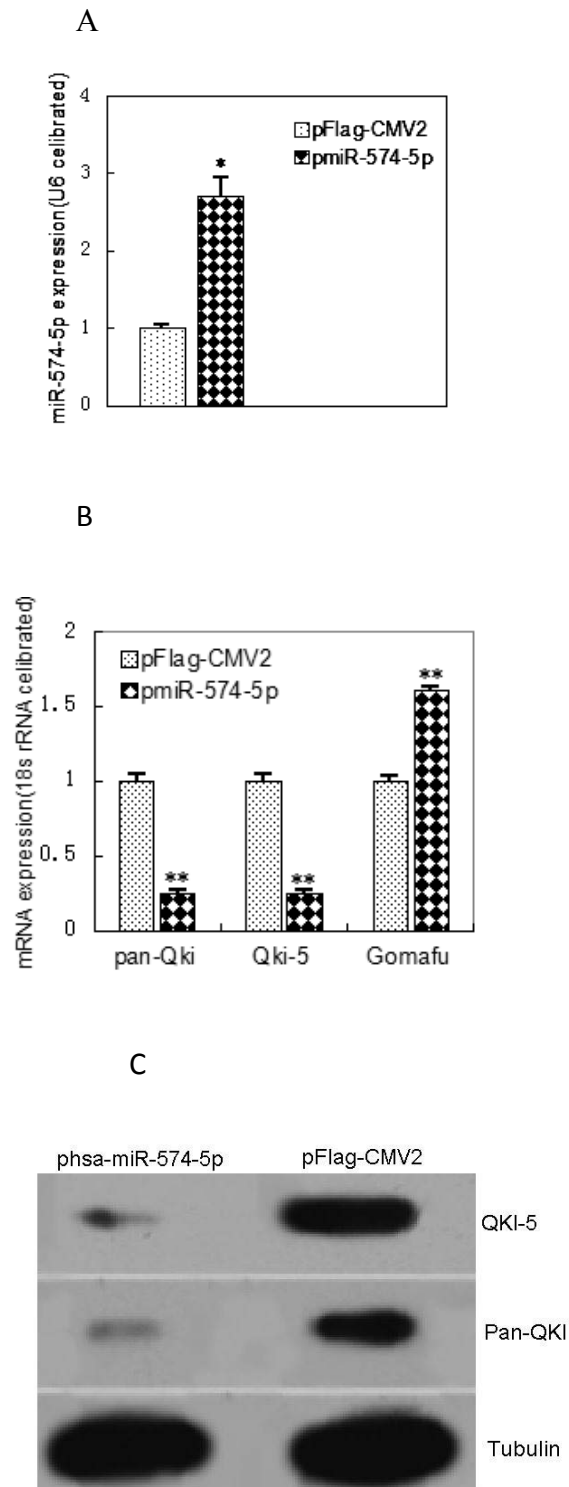


Fig. 3. *A) Effect of miR-574-5p overexpression. B) Significant decrease of pan-QKI and QKI-5 mRNA expression and increased Gomaflu resulted from miR-574-5p overexpression. C) Significant decrease of pan-QKI and QKI-5 protein expression resulted from miR-574-5p overexpression (Tubulin: internal reference).*

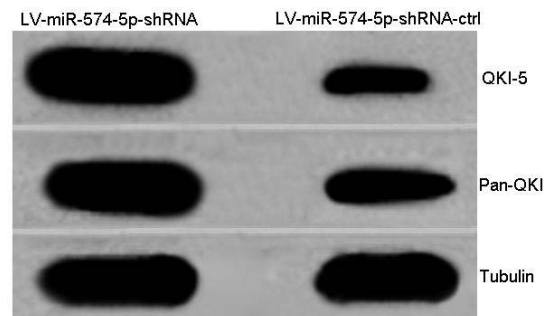


Fig. 4. Significant increase of pan-QKI and QKI-5 expression after inhibition of miR-574-5p.

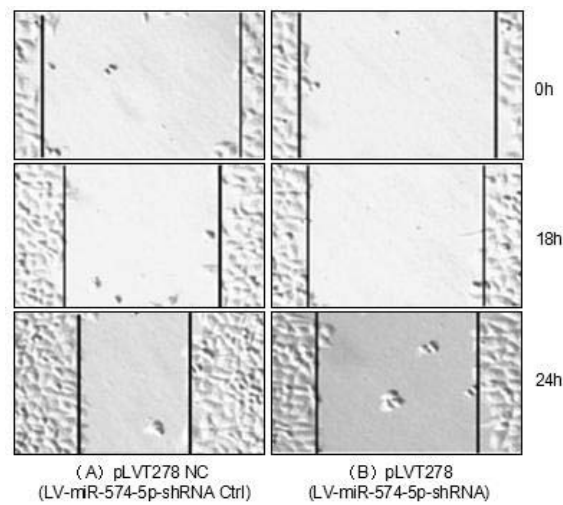


Fig. 5. Significant inhibitory effect of miR-574-5p on HeLa cell migration.

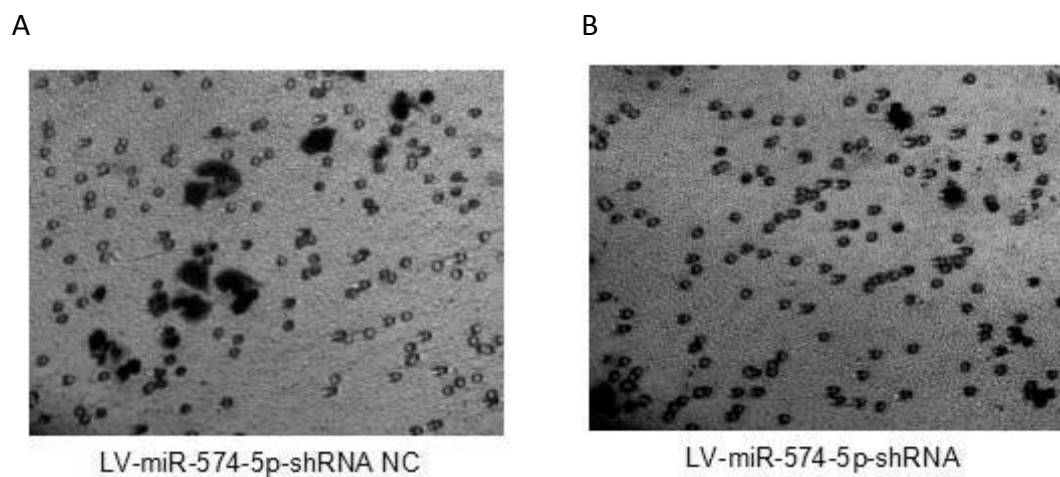


Fig. 6. Inhibition effect of inhibiting miR-574-5p on invasion ability of HeLa cells.

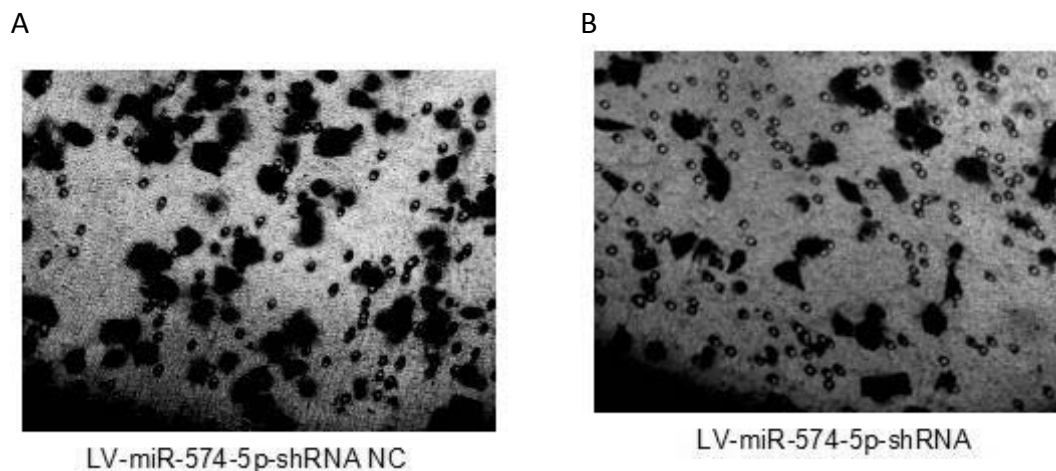


Fig. 7. Inhibition effect of inhibiting miR-574-5p on migration ability of HeLa cells.

Then, total RNA was extracted to detect HeLa cell by real-time qPCR. miR-574-5p expression in HeLa cell declined obviously after one-hour mild hypoxia treatment ($p < 0.001$), and miR-574-5p expression increased by 30% after HeLa cell was stimulated immediately for 2 h. miR-574-5p expression had a significant difference after 1 and 2 h processing ($p < 0.001$). Compared with miR-574-5p expression treated with 0 h, decreasing range of miR-574-5p had no statistical significance, although it was lowly expressed after 2-h mild hypoxia processing (Fig. 9A, 9B).

Research findings showed that both moderate and severe hypoxia stimulation could decrease miR-574-5p expression sharply. Severe hypoxia decreased miR-574-5p expression slightly, accounting for 23.8% compared with the control group, and miR-574-5p expression decreased by 50% by moderate hypoxia. Cells were likely to adapt to hypoxia stimulation immediately after reaching a low point, and miR-574-5p expression was improved gradually. Changes of miR-574-5p expression resulting from hypoxia stimulation in different degrees were in the same trend (Figs. 10 and 11).

Influence of cold and heat intervention on miR-574-5p expression and cell proliferation and metastasis

Besides oxygen delivery, cold and heat therapy is

also the most commonly used nursing intervention in practice. It could be seen that incubation temperature (38°C) was able to enhance the clone formation ability of HeLa cell notably in comparison with room temperature, and the normally expressed miR-574-5p group had a consistent changing trend with the inhibiting group (Fig. 12).

Obviously, temperature affected HeLa cell migration, and had a more apparent influence on miR-574-5p overexpressed HeLa. To further explore molecular biological mechanisms, HeLa cells in the same growth state were plated and incubated at 33°C, 35°C, 37°C and 38°C, respectively. Twenty-four hours later, cells were collected to perform routine total RNA extraction and reverse transcription and analyze miR-574-5p and pan-QKI mRNA expression at different incubation temperatures.

Compared with room temperature, miR-574-5p expression in HeLa cell incubated at 38°C had statistically significant increase ($p < 0.01$). However, a significant decrease occurred in miR-574-5p expression as incubation temperature declined (35°C and 33°C), in which, comparing 33°C with room temperature, miR-574-5p decreased by over 50% ($p < 0.001$, Fig. 13A). Conversely, pan-QKI mRNA expression in HeLa cell incubated at different temperatures showed an opposite tendency. In comparison with room temperature, pan-QKI mRNA

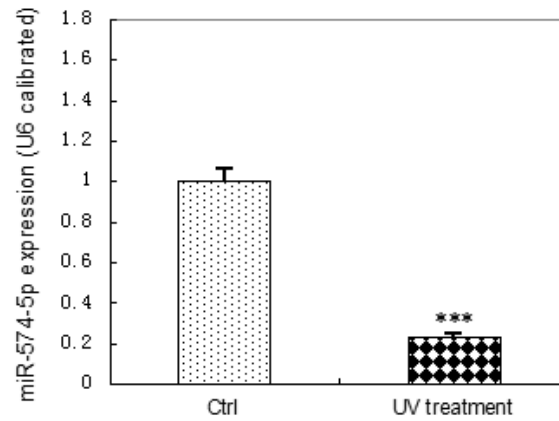


Fig. 8. Significant decrease of miR-574-5p expression in HeLa cell induced by UV irradiation intervention.

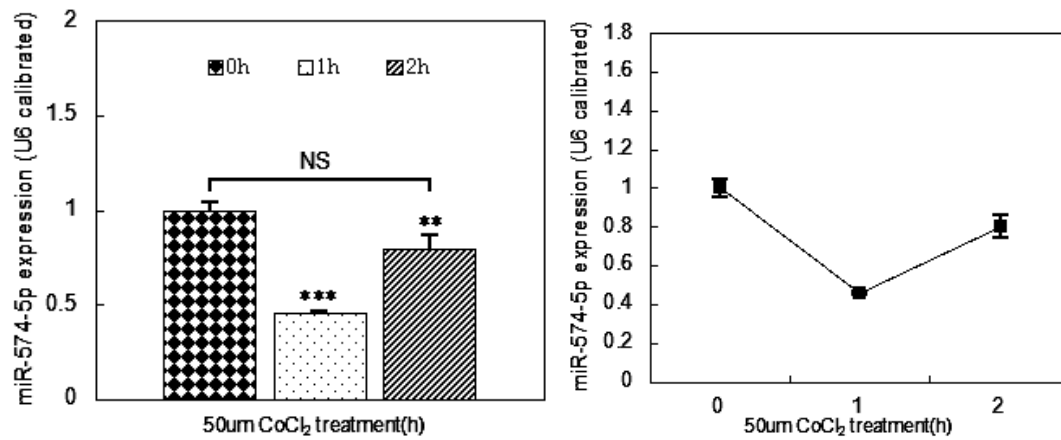


Fig. 9. Significant instant change of miR-574-5p expression resulted from mild hypoxia (** $p < 0.01$, *** $p < 0.001$).

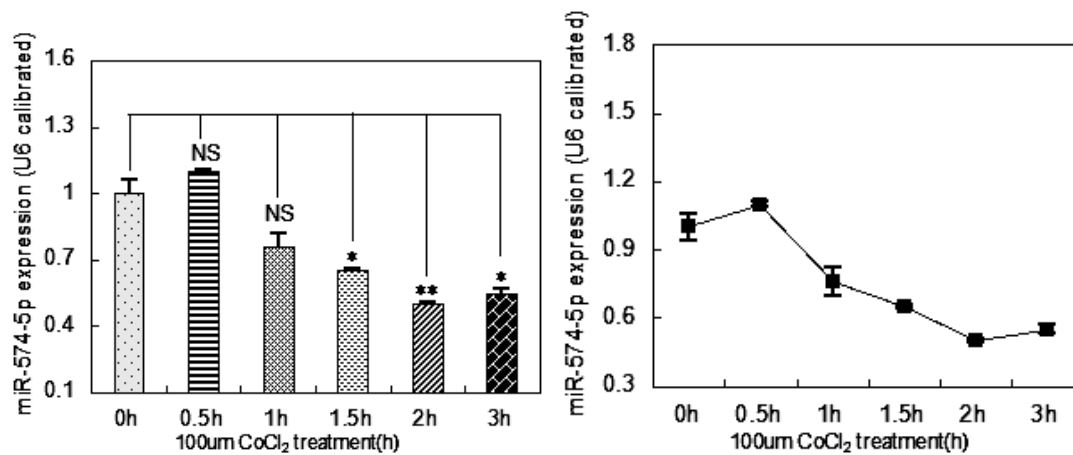


Fig. 10. Changes of miR-574-5p expression resulted from moderate hypoxia (compared with 0 h, * $p < 0.05$, ** $p < 0.01$; NS: not significant).

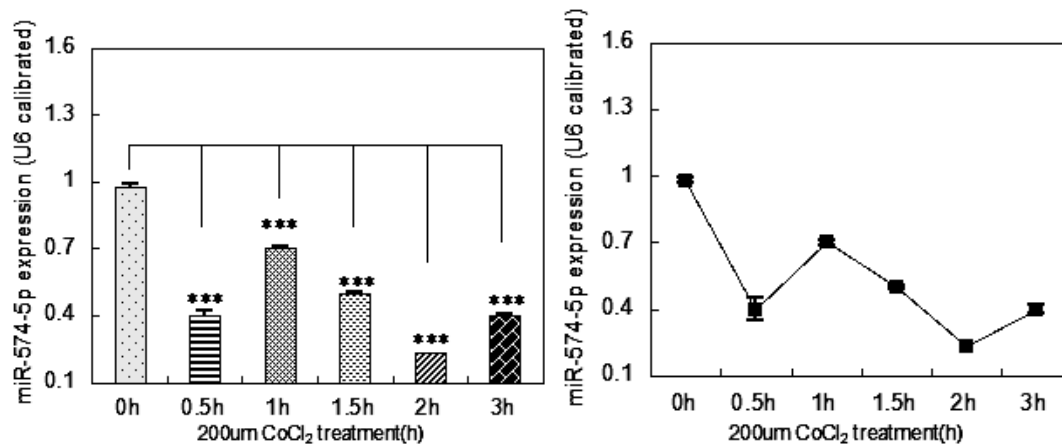


Fig. 11. Changes of miR-574-5p expression resulted from severe hypoxia (compared with 0 h, ***P<0.001).

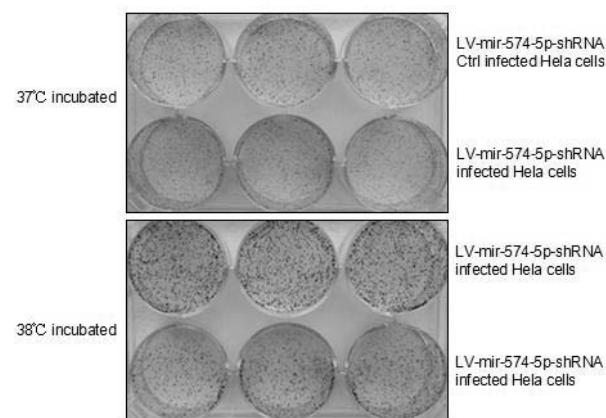


Fig. 12. Significantly enhanced clone formation ability of HeLa at 38°C.

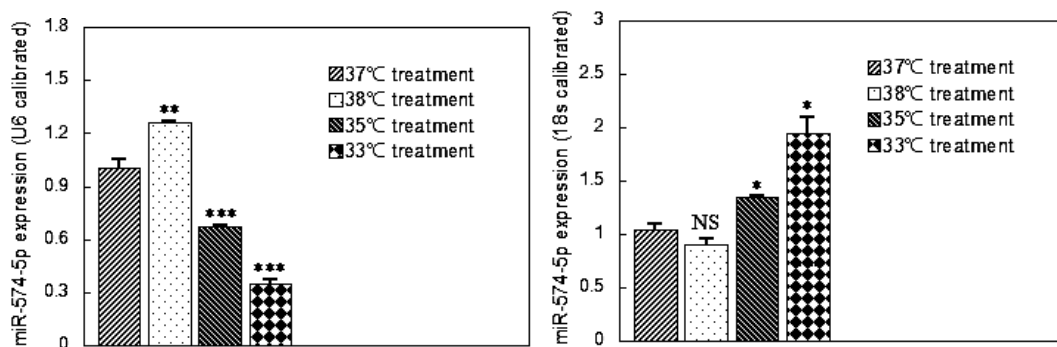


Fig. 13. Significant influences of different temperatures on expression of miR-574-5p (A) and pan-QKI (B) in HeLa cell.

expression (38°C) had a slight decrease without statistical significance, while pan-QKI mRNA expression (35°C and 33°C) was in an upward trend with a statistical significance, and increased sharply as temperature declined (Fig. 13B).

The influence of temperature variation on pan-QKI mRNA and miR-574-5p expression conformed to previous research expectations. Factors above were likely to be changed under the stimulation of temperature, thereby changing cell proliferation and migration ability. Of course, temperature stimulation could also totally have an effect on migration and proliferation of HeLa cell by other ways. Limited by time, mechanisms of influence of cold and heat stimulation on HeLa cell growth and proliferation as well as deeper reasons could not be explored in this study.

DISCUSSION

Ultraviolet radiation and sterilization, commonly used methods in practice, are designed to destroy DNA or RNA molecular structure in microbiological cells by taking advantage of ultraviolet irradiation at the appropriate wavelength, to cause growing cell death and/or regenerative cell death and achieve sterilization. UV irradiation has been applied to treat some diseases, such as alopecia areata, leucoderma, psoriasis and other dermatoses at the beginning. Successively UV irradiation was used in the treatment of herpes zoster as well as fast absorption of subcutaneous extravasated blood, and even blood irradiation and oxygenation. The field of application expanded from skin diseases to cardiovascular diseases (9-11). UV irradiation is capable of inducing light, aging and destroying DNA or RNA molecular structures. One report (12) claims that UV irradiation changes human immune factors, involving IL-12, IL-18 and IL-23. It is reported that narrow UV irradiation is able to cause T cell apoptosis when treating psoriasis (13), which is basically identical with the research results acquired from this experiment. In this study, UV irradiation was observed to be capable of affecting cell proliferation and decreasing miR-574-5p expression significantly, which might be one of the reasons influencing tumor cell proliferation and resulting in necrocytosis.

Oxygen delivery therapy, a frequently used basic

treatment, improves human arterial oxygen tension and arterial oxygen saturation to correct hypoxia resulting from various factors, promote tissue metabolism and maintain vital movement. Hypoxia, a kind of pathological process or result, may lead to abnormal changes in metabolism, functions and structures when tissue is short of oxygen supply or breaks down. Research findings showed that the occurrence and development of tumor has a relationship with tumor micro-environment in which changes of temperature and oxygen concentration are critical factors. Low oxygen environment increasing tumor resistance to radiation and chemotherapy is a significant factor resulting in tumor recurrence, metastasis and difficult treatment (14).

It was found from this study that mild, moderate and severe hypoxia stimulation differently affected miR-574-5p expression. MiR-574-5p expression was decreased sharply by severe hypoxia stimulation, which was similar to change of miR-210 expression results from hypoxia found by other Authors (15-16).

Cold and heat therapy is frequently applied in clinical nursing. According to reports published by the Proceedings of the National Academy of Sciences of the United States of America (PNAS) in October 2013, raising temperature could change the growth and proliferation of cancer cells in mice, thereby impacting the occurrence and development of tumor. Under a higher temperature than room temperature, such as, 30–31°C, cancer in mice was easier to control. Scholars (17-18) also found that cancer cells in mice grew easily in a colder environment. The study inspired this research greatly, and research findings laid an important foundation for carrying out research on cold and heat intervention in cervical cancer cell.

This study verified the influence of treatment on miR-574-5p expression and HeLa cell proliferation and migration, to provide a basis for the prevention and of cervical cancer in clinic.

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EXPRESSION OF CARTILAGE ANTITUMOR COMPONENT RanBP9 IN OSTEOSARCOMA

LL. ZHU¹, CH. WANG¹, HP. YANG¹ and WH. SHU²

¹*Department of Orthopaedics, the People's Hospital of Liaocheng City, Shandong, China;*

²*Medical Basic Teaching and Research Office, School of Nursing, Binzhou Polytechnic, Shandong, China*

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This study was carried out to investigate the expression changes of RanBP9 in tissue specimens and osteosarcoma cell strains and preliminarily explore its mechanism in osteosarcoma, so as to provide a theoretical foundation for follow-up experiments. The expression of RanBP9 in human osteosarcoma tissue specimens was detected by immunohistochemistry and the expression of RanBP9 messenger ribose nucleic acid (mRNA) in osteosarcoma cell strains was detected in real-time with polymerase chain reaction (PCR), and finally the expression of RanBP9 protein in osteosarcoma cell strains was detected by immunofluorescent staining and Western blot. Results demonstrated that RanBP9 was widely expressed in tissues, but also highly expressed in cells; moreover, the expression of RanBP9 was mainly concentrated in cytoplasm and nucleus, and partial expression was found in cell membrane. Thus, it can be concluded that RanBP9 is positively expressed in bone tumor tissues and cell strains.

Osteosarcoma, one of the most common malignant bone tumors (1), is derived from mesenchymal cell line and grows rapidly. Cells in weight-bearing bones of the lower limbs can mutate under the effect of external factors, which may be related to the formation of osteosarcoma. In recent years, study of tumor mechanism has become a hotspot and researchers have realized that the occurrence of tumor is closely associated with gene expression (2). Research on osteosarcoma gene expression has also made great advances and research findings show that osteosarcoma gene expression can not only determine the occurrence and development of osteosarcoma, but is also closely associated with the treatment outcome of osteosarcoma and affects patient prognosis (3). Many scholars in China and overseas have their own views on this aspect.

For example, González-Fernández R et al. (4)

indicated that the effect of Id1 gene on regulating the occurrence and development of osteosarcoma provided a new idea for the research on osteosarcoma pathogenesis, and Id gene was likely to be a new target for the treatment of osteosarcoma. In 2012, Couto et al. (5) discovered that MiRNA-145 could inhibit the formation and growth of tumors in tumor-bearing nude mice *in vivo*. In 2014, Wang et al. (6) found that TRPS1 was able to inhibit the expression of TGF α -1 in embryonic kidney tissues in mice as well as TGF β signal transduction pathways. Paupert et al. (7) investigated the effect of SATB1 on biological behaviors of human osteosarcoma cells and explored its molecular mechanism in 2014, which provided a basis for the treatment of osteosarcoma in clinic. This study was to perform an in-depth discussion on the expression of cartilage antitumor component - RanBP9 in osteosarcoma.

Key words: RanBP9, osteosarcoma, protein expression, cell culture

Mailing address:

Dr Linlin Zhu,
No. 67, Dongchang West Road,
Department of Orthopaedics,
The People's Hospital of Liaocheng City,
Liaocheng City, Shandong,
252000, China
e-mail: linlinzhull@163.com

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MATERIALS AND METHODS

Reagents

DMEM/RPMI1640 complete medium was mixed with NaHCO₃ (4.5 g) and then dissolved in 1000 mL of ultrapure water. PH value was adjusted to 7.5 filtered, thus Dulbecco's modified eagle medium (DMEM)/RPMI1640 basal medium was made. FCS with the serum concentration of 15% was added into each bottle to make DMEM/RPMI1640 complete medium and then it was sealed and preserved at 5°C until use. Trypsin (3 g) and ethylene diamine tetraacetic acid (EDTA, 0.3 g) were dissolved in 1000 mL of phosphate buffer solution (PBS). After mixing, it was filtered and then preserved at -20°C. Each bag of PBS powder was dissolved in 2.5 L of ultrapure water (pH: 7.6) and sterilized at a high temperature and then preserved at 4°C until use.

Cell cryopreservation solution was prepared according to the volume ratio of dimethylsulfoxide (DMSO) and high-quality FCS (1:10) and preserved at 5°C.

Citric acid (4.5 g) and trisodium citrate (14.5 g) were dissolved in 250 mL and 500 mL of ultrapure water, respectively, 8.5 mL and 40.5 mL of which were taken out and preserved at room temperature until use after being added with 500 mL of ultrapure water.

Tissue specimens

A total of 60 osteosarcoma paraffin blocks were randomly selected from the People's Hospital of Liaocheng City from 2013 to 2015, involving 28 males and 32 females (median age: 22.8 years). According to the types of pathological tissues, the patients were divided into chondroblast type (15 cases), osteoblasts type (20 cases), fibroblast type (6 cases) and other types (19 cases). Twenty-three osteoclastoma paraffin blocks were taken as the control group, including 12 males and 11 females (median age: 37.6 years). This study was approved by the ethics committee of the People's Hospital of Liaocheng City and all patients signed an informed consent.

Cell strains

Cell strains MG63, SaOS2 and U2OS were passaged and inoculated.

Cell culture

MG63 cells were cultured in 1640 medium containing

10% of FCS. SaOS2 and U2OS cells were cultured in DMEM medium containing 10% of FCS. Then, all of the cells were cultured in a sterile incubator containing 6% of CO₂ at 37°C.

Cell recovery

Cell microtube was taken out from liquid nitrogen and immediately put into a water bath (37°C). After centrifugation, it was added with culture medium and blown and beaten, and then transferred into a culture flask (120 mL) for culture (6% CO₂) at 37°C. The following day, the culture solution was changed.

Cell passage

Well-conditioned cells in the logarithmic phase were drained and washed three times with PBS and then added with 2.5 mL of trypsin solution (0.30%) and put aside at room temperature for 3 min. Trypsin digestive juice was drained and the culture solution was then added to terminate the digestion when the intercellular space became larger and cells became rounder under a microscope. The culture flask was blown and beaten repeatedly with a sucker and cells were passaged to a new culture flask (1:3) after they were separated from the bottom of the flask. Then, the culture flask was cultured continuously in the incubator (6% CO₂) at 37°C.

Cell cryopreservation

Well-conditioned cells in the logarithmic phase were drained and washed three times with PBS and then added with 2.5 mL of trypsin solution (0.30%) and put aside at room temperature for 3 min. Trypsin digestive juice was drained and the culture solution was then added to terminate the digestion when intercellular space increased and cells became rounder under a microscope. Culture flask was blown and beaten repeatedly and the separated cells were centrifuged at 1200 rpm and then drained. The frozen stock solution was prepared with DMSO and whole serum or 15% of blood serum medium (1: 9) (12) and frozen in a sterile microtube with the tube cap covered at 5°C, -20°C and -80°C respectively and finally put in the liquid nitrogen for a long-term cryopreservation.

RanBP9 protein expression in paraffin tissue specimens by immunohistochemical staining

Paraffin specimens were sliced with the thickness of

3.5 μm and then baked for 25 min under a light and then immediately transferred to xylene I, II and III for 12 min and absolute ethyl alcohol and ethyl alcohol (94%, 80% and 72%) for 6 min, respectively. After that, the slices were soaked in water and rinsed three times with PBS, 6 min each time.

The slices were sealed in 12% of H_2O_2 at 37°C for 17 min to block endogenous peroxidase and then washed three times with PBS (6 min every time) and repaired with citric acid antigen retrieval buffer in a antigen repair box for 3.5 min (750 W) and 17 min (170 W) respectively. Afterwards, slices were put into ice water and then washed three times with PBS, 6 min every time.

The slices were washed three times with PBS (6 min every time) and added with diaminobenzidine (DAB) dropwise. Three minutes later, cells started to show color under a BX-40 optical microscope and the reaction was then terminated by adding distilled water in due time. After hematoxylin counter staining, the slices were differentiated with 0.1% of hydrochloric-alcohol solution for few seconds and then added with distilled water until they returned to blue.

Then, the slices were dehydrated with 72%, 80% and 94% of ethyl alcohol and absolute ethyl alcohol for 3 min, 3 min, 2 min and 3 min respectively, and then baked and finally sealed with neutral gum. Additionally, the slices were observed from five views under a light microscope and images were filmed and scored by pathologists according to immunohistochemical standards.

RanBP9 protein expression in different osteosarcoma cell strains by immunofluorescent staining

Well-conditioned cells were resuspended in culture medium ($3.5 \times 10^5/\text{mL}$) and then added to a 24-well plate and incubated overnight. Then, cells were drained and slices were washed twice with PBS and fixed with 5% of paraformaldehyde for 25 min. The slices were then washed five times with PBS, 6 min every time.

After that, the slices were sealed for 25 min at 37°C. Preliminary experiment sconfirmed the dilution ratio of the antibody (1:100) and RanBP9 antibody was added dropwise. The primary antibody was replaced with PBS, which was considered as the negative control. The slices were put into a wet box overnight and finally observed under a laser scanning confocal microscope and images were filmed.

Content of messenger ribose nucleic acid of RanBP9 in different osteosarcoma cell strains detected by real-time polymerase chain reaction

Cell RNA extraction

Cells were drained and washed three times with PBS and then added with 1 mL of lysis buffer per 10 cm^2 . A petri dish was horizontally put on the operating floor to make lysis buffer fully access cell surface and then cells were decomposed. Lysis buffer was blown and beaten to a centrifuge tube, which was then put aside at room temperature for 6 min after no obvious precipitation was found.

Chloroform was mixed with lysis buffer (1:5) and vibrated for a few seconds and then put aside at room temperature for 6 min until they were fully mixed and emulsified, with no stratification found. After the solution was centrifuged at 12,000 g (5°C) for 20 min, the centrifuge tube was taken out and the solution was separated, with an obvious white layer in the middle. The solution was transferred to a new centrifuge tube after the transparent supernatant on the white layer was carefully sucked out.

Afterwards, isopropyl alcohol was added into the centrifuge tube and fully mixed, and then put aside at room temperature for 15 min. After the solution was centrifuged at 12,000 g (5°C) for 20 min, white RNA precipitation in the bottom of the tube was rinsed out and cells were drained. Then, 1 mL of ethyl alcohol (74%) was added to the centrifuge tube along the wall of the tube without coming into contact with the precipitation and then centrifuged for 5 min (12,000 g, 4°C). Ethyl alcohol was sucked out and the precipitation remained. White precipitation was dried at room temperature for 4 min and preserved at -80°C after RNase-free water was added.

Determination of RNA concentration

Diethylpyrocarbonate (DEPC) buffer was used to dilute 2 μL of RNA sample according to the proportion of 1:25 and optical density (OD) 260/OD280 value was measured by nucleic acid protein quantometer, i.e., RNA concentration. OD260/OD280 ratio was between 1.85 and 1.98, which indicated that RNA was highly concentrated and purified, which could be used to calculate the amount of RNA (μL) during the reverse transcription reaction. After determination, it was preserved at -80°C .

RanBP9 protein expression level in osteosarcoma cell by Western blot

After the culture solution was blotted up, cells were washed four times with pre-cooled PBS and then the culture flask was put on ice after cells were drained. The working solution made up of protease inhibitor and lysis buffer (1:100) was prepared and put on ice. The culture flask was shaken to ensure that cells were fully lysed and the whole process had to be performed on ice for 25 min. Cells were repeatedly scraped from one side of the culture flask to the other side to guarantee all of the cells were scraped away. Then, the lysis buffer, together with cell debris, was transferred to a centrifuge tube (2 mL) and centrifuged at 12,000 rpm for 6 min (5°C). After that, the supernatant was poured into a centrifuge tube (2 mL) and preserved at -20°C.

Protein concentration measured by bicinchoninic acid (BCA)

Pierce BCA Protein Assay Kit was used to detect protein concentration and OD of each tube of protein was read by microplate reader, thus a standard curve was drawn and the protein concentration was calculated.

Protein content detected by Western blot

Sodium dodecyl sulfonate - polyacrylamide gel electrophoresis (SDS-PAGE). The glass plate was brushed over with a small amount of washing powder and washed with distilled water repeatedly and then dried for standby application. Afterwards, glue was prepared and samples were loaded. After electrophoresis apparatus was charged with electricity and electrophoresis conditions and time were set, spacer gel received electrophoresis at 82 V for 28 min and 125 V for 115 min for separation gel. The electrophoresis was terminated when bromophenol blue was about to run out of the bottom of the separation gel.

Membrane transfer

A polyvinylidene fluoride (PVDF) membrane and 6 filter papers were clipped in advance and soaked in the transfer buffer for 25 min. Then, 3 filter papers and the PVDF membrane were put on the electroporator instrument successively and rolled with a glass rod to prevent bubbles in the middle. After the electrophoresis, the spacer gel was cut off and the separation gel was carefully transferred to the PVDF membrane, and the

upper left corner of the PVDF membrane was sheared. Afterwards, the remaining 3 filter papers were spread on the gel and wet with electricity transfer buffer to make a combination of filter papers, gel, PVDF membrane and filter papers. After electrotransformation (65 V, 115 min), the PVDF membrane was taken out.

Sealing

The PVDF membrane was soaked in skim milk powder and shaken slowly. Then, it was sealed for 3.5 h at room temperature.

Antibody incubation

Firstly, the membrane which was washed four times with tris buffered saline tween (TBST) solution was put into the diluted primary antibodies of RanBP9 polyclonal antibody (1:1000) and rabbit-anti-human glyceraldehyde-phosphate dehydrogenase (GAPDH) monoclonal antibody (1:1000) and was kept overnight at 5°C. Secondly, the membrane was soaked in TBST buffer, shaken slowly and then washed four times for 15 min. Thirdly, the membrane was diluted in the diluted secondary antibody according to the proportion of 1:2000 and then incubated for 2.5 h at 37°C. Fourthly, the membrane was soaked into TBST buffer, shaken slowly and then washed four times for 10 min.

Color development and results acquisition

Color was shown by using chemiluminescence chromogenic reagent kit and images were displayed in the Bio-Rad gel imager.

Statistical analysis

SPSS13.0 software, independent sample *t*-test and χ^2 test were used to analyze data, and the data were expressed as mean $\pm$ standard deviation (SD). The difference was considered to be statistically significant if $p < 0.05$.

RESULTS

RanBP9 protein expression in osteosarcoma and osteoclastoma paraffin sample tissues by immunohistochemical staining

RanBP9 was found to be positively expressed in osteosarcoma and osteoclastoma paraffin sample

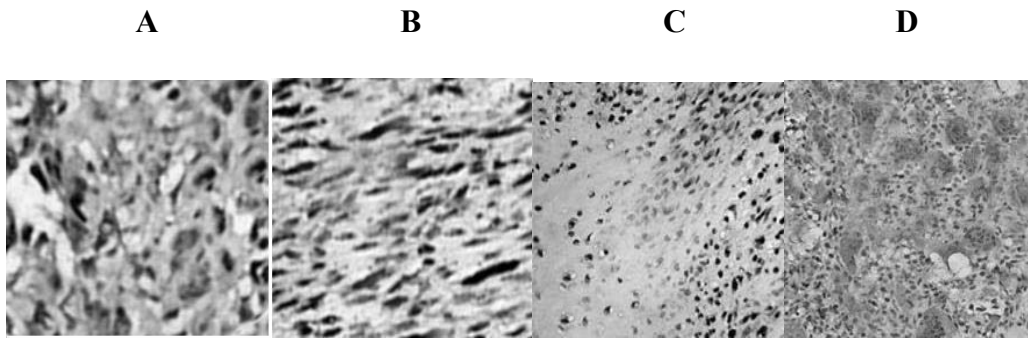


Fig. 1. Expression of RanBP9 in bone tumor paraffin tissues.

Note: the expression of osteosarcoma RanBP9 in osteoblastomas (A), fibroblasts (B), chondroblasts (C) and osteoclastomas (D).

Table I. Expression of RanBP9 in osteosarcoma and osteoclas

Types	Number of cases	RanBP9	
		Positive rate (%)	<i>p</i>
Osteosarcoma	60	81.7%, (49/60)	$p < 0.05$
Osteoclastoma	23	100%,(23/23)	

Table II. Relationship between RanBP9 expression and pathological parameters of osteosarcoma tissues.

Clinical pathologic parameters	Groups	RanBP9			<i>p</i>
		Number of cases	Negative	Positive	
		60	11	49	
Pathological types	Osteoblastoma	20	3	17	0.601
	Chondroblast	15	5	10	
	Fibroblast	6	0	6	
	Other types	19	1	11	
Age	> 20	27	7	20	0.655
	≤ 20	33	9	24	
Gender	Male	28	7	21	0.245
	Female	32	8	24	

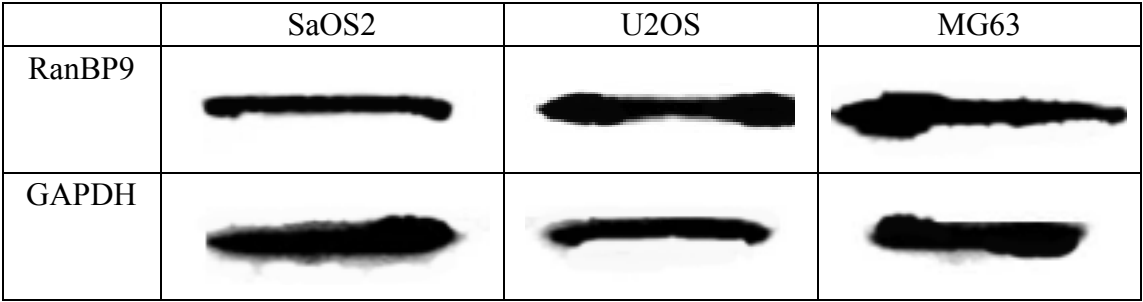


Fig. 2. *RanBP9* protein expression in three osteosarcoma cell strains (MG63, SaOS2 and U2OS) detected by Western blot.

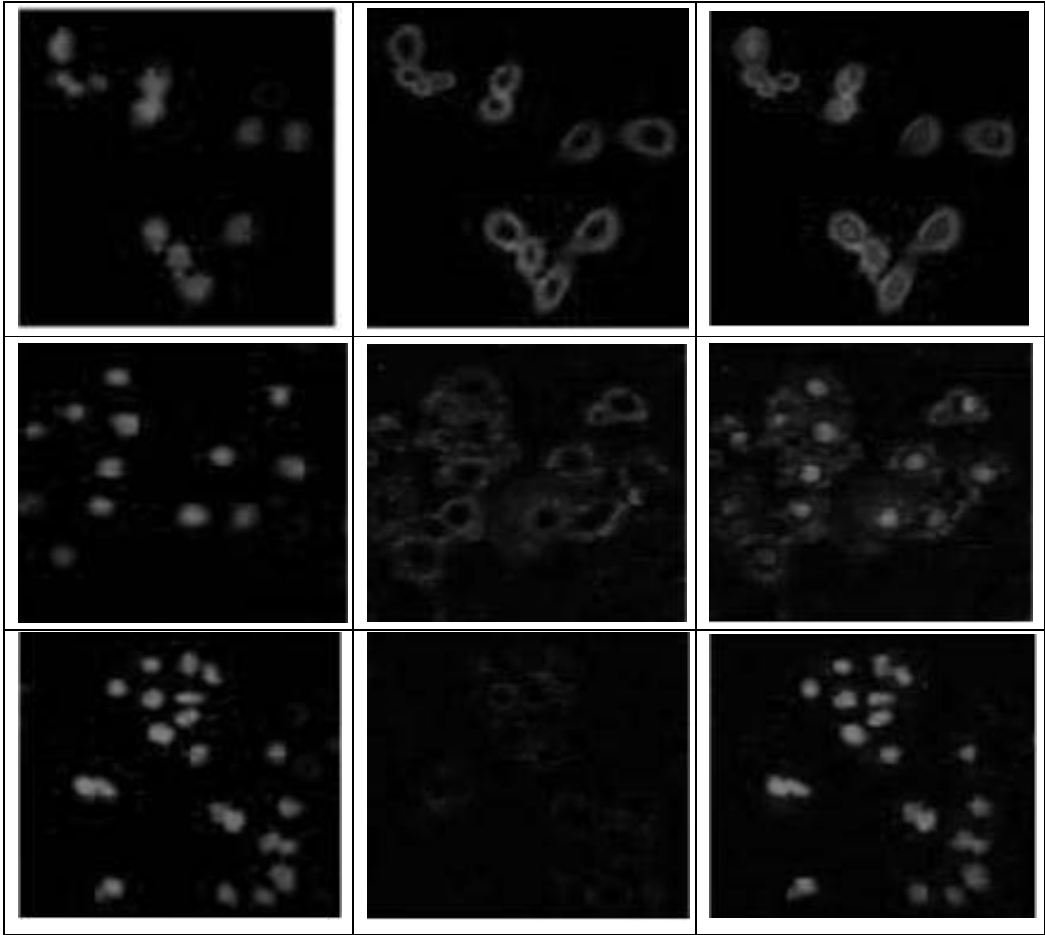


Fig. 3. *RanBP9* protein expression in different osteosarcoma cell strains by immunofluorescence staining.
Note: *RanBP9* protein expression in three osteosarcoma cell strains MG63, SaOS2, and U2OS under a laser scanning confocal microscope from top to bottom, respectively

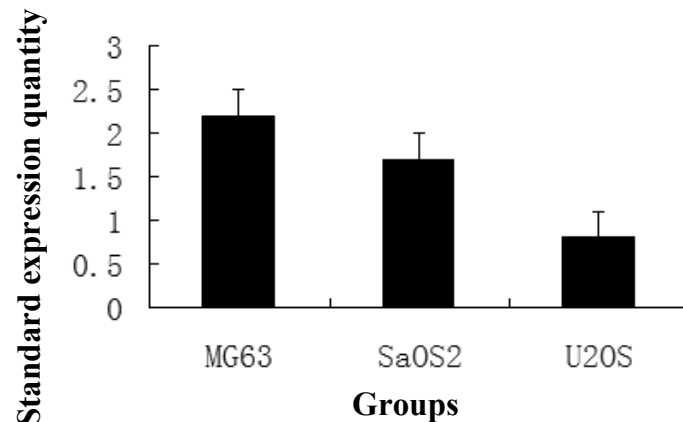


Fig. 4. Content of mRNA of RanBP9 in three osteosarcoma cell strains (MG63, SaOS2 and U2OS) detected by real-time PCR.

tissues. It mainly located in cytoplasm and part of them was in cytoplasm and/or nucleus (Fig. 1). Totally 49 out of 60 osteosarcoma paraffin sample tissues were found with positive RanBP9 expression, with the rate of 81.7%. RanBP9 was strongly positively expressed in all osteoclastoma tissues in the control group (23/23), and the difference was statistically significant ($p < 0.05$) (Table I). Furthermore, no statistical significance was found between relevant clinical pathologic parameters such as patients' age, gender and pathological types, etc ($p > 0.05$) (Table II).

RanBP9 protein expression in different osteosarcoma cell strains by Western blot

As shown in Fig. 2, the same results were obtained by Western blot as acquired by immunofluorescence cell staining and real-time PCR.

RanBP9 protein expression in different osteosarcoma cell strains by immunofluorescence staining

By immunofluorescence staining, it was found that RanBP9 was positively expressed in osteosarcoma cell strains and mainly located in cytoplasm (Fig. 3). Besides, it was highly expressed in osteosarcoma cell strain MG63 of low malignancy compared with cell strains SaOS2 and U2OS of high malignancy.

Content of messenger ribose nucleic acid of RanBP9 in different osteosarcoma cell strains by PCR

The same results were obtained by real-time PCR as acquired by immunofluorescence cell staining (Fig. 4).

DISCUSSION

Ran gene related nucleoprotein is an important transport protein (8-10). To date, at least 10 binding proteins of Ran protein have been found, one of which is RanBP9 (11-12). Some studies revealed that RanBP9 was not only widely expressed in tissues, but also highly expressed in cells (13-15). It was mainly concentrated in cytoplasm and nucleus, and partial expression was found in cell membrane as well.

Based on the studies above, the first part of this experiment aimed to detect the expression of RanBP9 from two aspects (tissues specimens and osteosarcoma cells). We randomly collected paraffin tissue samples from 60 patients who had been pathologically confirmed with osteosarcoma and investigated the expression of RanBP9 by immunohistochemical staining. The expression quantity of RanBP9 increased greatly in 23 osteoclastoma tissues of potentially malignant lesions in the control group. RanBP9 was strongly positively expressed in all osteoclastoma tissues (23/23), which was significantly higher than in osteosarcoma tissues. The differences in RanBP9 expression between the two bone tumors had a statistical significance ($p < 0.05$).

In conclusion, taking paraffin tissue specimens of the two bone tumors (osteosarcoma and osteoclastoma) as well as differently malignant osteosarcoma cell

strains as research objects, this study determines RanBP9 expression from the perspective of RNA and protein level by immunohistochemical staining, PCR, immunofluorescent staining and Western blot and found that RanBP9 is positively expressed in bone tumor tissues. There were differences in the expression of bone tumor tissues and the expression quantity of RanBP9 was significantly higher in potentially malignant osteoclastoma tissues than in malignant osteosarcoma tissues. In addition, no obvious relationship was found between patients' age, gender, pathological types and other pathological parameters.

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ADIPOSE-DERIVED MESENCHYMAL STROMAL (STEM) CELLS DIFFERENTIATE TO OSTEOBLAST AND CHONDROBLAST LINEAGES UPON INCUBATION WITH CONDITIONED MEDIA FROM DENTAL PULP STEM CELL-DERIVED OSTEOBLASTS AND AURICLE CARTILAGE CHONDROCYTES

A. CARBONE^{1,2}, M. VALENTE³, L. ANNACONTINI³, S. CASTELLANI², S. DI GIOIA², D. PARISI³, M. RUCCI³, G. BELGIOVINE², C. COLOMBO⁴, A. DI BENEDETTO³, G. MORI³, L. LO MUZIO³, A. MAIORELLA³, A. PORTINCASA³ and M. CONESE²

¹Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Medical Genetics Laboratory, Milan, Italy; ²Department of Medical and Surgical Sciences, University of Foggia, Foggia, Italy;

³Department of Clinical and Experimental Medicine, University of Foggia, Foggia, Italy;

⁴Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Cystic Fibrosis Center, Milan, Italy

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The potential of adipose-derived mesenchymal stromal (stem) cells (ADSCs) to differentiate into either osteoblasts or chondrocytes is controversial. In this study we investigated the multicapacity potential of ADSCs to differentiate towards adipocyte, osteoblast, and chondrocyte lineages when cells are seeded onto plastic in comparison with incubation with conditioned media (CM) obtained from differentiated cell types. ADSCs, obtained from liposuctions, were characterized for mesenchymal and hematopoietic markers by cytofluorimetry. Their differentiation capacity towards adipocytes, osteoblasts, and chondrocytes was investigated by histochemistry methods (Oil-Red-O staining, Safranin O and Alizarin Red staining, respectively). Dental pulp stem cells (DPSCs) and dedifferentiated auricle derived-chondrocytes were differentiated towards osteoblastic and chondrocytic lineages respectively, and the CM obtained from these cultures was used to induce differentiation of ADSCs. ADSCs were positive for mesenchymal markers (CD29, CD105, CD73, CD44), but not for hematopoietic lineage markers (CD14, CD34, CD45), and this behavior was conserved from the isolation up to the fifth passage. While ADSCs were readily differentiated in adipocytes, they were not towards chondrocytic and osteoblastic lineages, a behavior different from that of bone marrow-derived MSCs that differentiated into the three lineages at two weeks post-induction. Only ADSCs treated with CM from cultured chondrocytes and DPSCs, produced glycosaminoglycans and mineralized matrix. These results indicate that ADSCs need growth/morphogenic factor supplementation from the tissue environment to be appropriately differentiated to mesodermic lineages.

Adipose-derived mesenchymal stromal cells (ADSCs) have attracted much interest in the field of cell therapy and tissue engineering, since they can be easily obtained in large amounts via the liposuction process, using only local anesthesia. The lipoaspirate

is extensively washed and then cells are lysed. The floating adipose tissue is digested with collagenase type IA to break down the extracellular matrix, and then filtered and centrifuged. After spinning down, the resulting pellet, known as the stromal vascular

Key words: adipose-derived stromal cells, osteoblast, chondrocyte, growth factors, morphogenic factors

Mailing address:

Prof. Massimo Conese,
Laboratory of Experimental and Regenerative Medicine,
Biomedical Centre "E. Altomare", Department of Medical and
Surgical Sciences, University of Foggia, c/o Ospedali Riuniti,
Viale L. Pinto 1, Foggia 71122, Italy
Tel.: +39 0881 588019 - Fax: +39 0881 588047
e-mail: massimo.conese@unifg.it

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fraction (SVF), is obtained. Plating of SVF results in eventual selection and expansion of the ADSC population (1). ADSCs can be isolated in numbers at least 8-fold more than MSCs from bone marrow (BM) when similar amounts of lipoaspirate and BM aspirate are compared (2). ADSCs can be induced to differentiate towards mesenchymal lineages, such as adipocytes, osteoblasts, and chondrocytes, as well as towards vascular cells (smooth-muscle cells and endothelial cells) (3-6). The multilineage differentiative capacity of ADSCs has been described: not only are ADSCs capable of differentiating towards cell types belonging to the mesodermal leaflet (including, myogenic, cardiomyogenic, tenogenic, and periodontogenic lineages), but also towards those of endodermal (hepatic and pancreatic-like beta cell lineages) and ectodermal leaflets (retinal pigmented cell, neural, glial, and epithelial lineages) (7). *In vitro* results have been corroborated by *in-vivo* studies, showing the differentiative potential of ADSCs also in animal models (7).

Interestingly, previous works have demonstrated that, nevertheless, BM-MSCs and ADSCs are different: thus, BM-MSCs are more committed towards osteoblastic and chondrogenic lineages than ADSCs. Indeed, they appear to differentiate at genomic, proteomic and functional levels (3-5). On the other hand, ADSCs seem to possess a better angiogenic effect than BM-MSCs (8). However, ADSCs show the same capacity of BM-MSCs to give rise *in vivo* to osteophytes and chondrocytes when injected into the knee of SCID mice (5). Thus, it seems likely that environmental factors present *in vivo* are important for proper differentiation towards mesenchymal lineages of ADSCs.

In this study we investigated the capacity of ADSCs to differentiate towards adipocyte, chondrocyte and osteoblast lineages, comparing those multipotentiality properties on plastic dishes with that induced in the presence of conditioned media (CM) obtained from differentiated cell types. Literature data indicated that dental pulp stem cells (DPSCs) are an excellent source of MSCs, perfectly comparable to those obtained from bone marrow (9, 10). To study whether DPSCs produce

pro-osteogenic factors, ADSCs were cultured in the presence of CM obtained from DPSCs which had undergone osteogenic differentiation.

The presence of a multipotent stem/progenitor cell in the cartilage is still under debate, due to the lack of reliable and specific phenotypic markers (11). This analysis is also hampered by the fact that a high percentage of mature chondrocytes also express these markers thus confounding previous studies (12). Irrespective of this, human chondrocytes in culture dedifferentiate and exhibit multilineage differentiation capacity (12). We therefore exploited the properties of dedifferentiated chondrocytes isolated from human auricle cartilage to differentiate into chondrocytes and induce chondrogenesis of ADSCs by incubating them with the CM-derived chondrocytes.

Our results strongly suggest that paracrine factors are involved in the proper differentiation of ADSCs toward mesenchymal lineages.

MATERIALS AND METHODS

Isolation and culture of human adipose-derived mesenchymal stromal cells

Human adipose tissue was obtained from liposuction procedures under local anesthesia. Fourteen subjects participated in this study (mean age 40.7 years, range 18-72 years). Lipoaspirates were obtained from patients operated in the Plastic and Reconstructive Surgery Department for different morbidity conditions and treated with lipofilling. Each participant gave written informed consent to the study which was performed in accordance with the principles of the Declaration of Helsinki.

We injected the abdominal subcutaneous layer with 500 to 1000 mL lactated Ringer's solution with the addition of 0.5 mL epinephrine and 25 mL 1% lidocaine. After 10 min, waiting for local vasoconstriction induced by epinephrine, two small incisions in the abdominal region (4 mm each) allowed adipose tissue suction using a Ø3-mm cannula with a 10 mL luer lock syringe.

To isolate the SVF, the fat tissue was added with equal volumes of phosphate-buffered saline (PBS), centrifuged at 300x g for 10 min at 18-22°C and the resulting middle phase was digested at 37°C for 45 min with 0.2% collagenase solution. Enzyme activity was

neutralized with Dulbecco's modified Eagle's medium (DMEM), containing 10% FBS, and centrifuged at 300x g for 10 min to obtain an SVF pellet. The final pellet was resuspended in DMEM:F12 media supplemented with 40% (v/v) heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% Antibiotic Antimycotic (ABAM) and 10 ng/ml epidermal growth factor (EGF) (all reagents were purchased from Sigma-Aldrich), plated on T75 flasks for cell culture (Corning) and incubated at 37°C and 5% CO₂. The following day the medium was aspirated to remove the floating cells, the plate was washed with PBS to remove the remaining debris and the attached cells were maintained in DMEM low glucose supplemented with 10% FBS, 1% L-glutamine, 1% ABAM and 10 ng/ml EGF at 37°C and 5% CO₂.

Every time cells reached 80% of confluence, cells were detached with Accutase Solution (Invitrogen), washed, counted with a Bürker chamber and replated in a new plastic flask at a density of 1×10⁵ cells/cm² in order to calculate their growth curve. Doubling time was calculated inserting times and cell counts on the website www.doubling-time.com/compute.php

Characterization of ADSCs by flow cytometry

Flow cytometry analyses were performed on cultured ADSCs at passages 0, I, II, III, IV and V. Briefly, cells were detached from culture flask using Accutase solution and, after washing, were incubated with PBS supplemented with 2% FBS for 10 min at 4°C in order to block non-specific sites on cell membrane. Cells were then stained at 4°C for 30 min with the following monoclonal antibodies (moabs) against CD14, CD29, CD34, CD45, CD73, CD105 (all from Invitrogen), and CD44 (Becton Dickinson Biosciences, BD, Franklin Lakes, NJ, USA). All moabs were conjugated with fluorescein isothiocyanate (FITC), except the moab against CD73 that required an additive incubation with secondary antibody (FITC goat anti-mouse; Sigma-Aldrich) for 30 min at 4°C.

Culture of human dental pulp stem cells

DPSCs were isolated as previously described (13). DPSCs were seeded into T75 flasks and cultured in DMEM low glucose containing 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin. Cultures were maintained at 37°C in 5% CO₂, the supplemented medium was changed every 3 days and the cells were passaged when 80% confluent

using Accutase Solution.

In order to induce osteogenic differentiation in ADSCs, we collected CM from DPSCs for 2 weeks in osteogenic medium: DMEM low glucose supplemented with 2% FBS, 1% penicillin/streptomycin, 50 µg/ml ascorbate-2-phosphate, 10⁻⁸ M dexamethasone, 10⁻² M β-glycerophosphate.

Isolation and culture of human chondrocytes

Human auricular cartilage was acquired from patients hospitalized at the Division of Plastic and Reconstructive Surgery, Hospital of Foggia, and who gave written informed consent. Cartilage fragments were removed from apparently healthy auricular regions by surgical biopsy. The cartilage was transported to the laboratory immediately after harvesting in sterile tubes containing PBS (Ca²⁺ and Mg²⁺ free) with penicillin/streptomycin and ABAM. A sterile scalpel was used to cut the cartilage into small pieces, which were then transferred to a Petri dish and maintained overnight at 37°C, 5% CO₂ in DMEM containing 10% FBS, 100 µg/ml L-ascorbic acid, 1% L-glutamine, 1% penicillin/streptomycin and 1% ABAM (growth medium) to ensure sterility of the specimens prior to enzyme digestion. The day after, the cartilage pieces were washed in PBS (Ca²⁺ and Mg²⁺ free) containing penicillin/streptomycin and ABAM and then incubated in the growth medium containing trypsin (0.25% w/v) at 37°C, 5% CO₂ for 30-60 min (the time of enzymatic digestion was adapted to the size of tissue fragments). Supernatants were discarded and the cartilage was further digested in growth medium containing 1.0 mg/ml collagenase II for 4 h at 37°C, 5%CO₂. The digested tissue was passed through a nitex nylon filter (mesh width 70 µm) and then centrifuged at 1500 xg for 10 min. The cell pellet was washed three times in PBS (Ca²⁺ and Mg²⁺ free) containing penicillin/streptomycin and ABAM and re-suspended in the growth medium.

Cultures were maintained at 37°C in 5% CO₂, and the supplemented medium was changed every 3 days. When chondrocytes reached 70 to 90% confluency, the cells were passaged using Accutase Solution and replated in a new plastic flask.

In order to induce chondrogenic differentiation in ADSCs, we collected CM from chondrocytes cultured for 3 weeks in chondrogenic medium: DMEM low glucose supplemented with 1% FBS, 1% penicillin/streptomycin,

6.25 µg/ml insulin, 10 ng/ml TGF-β3, 50 nM ascorbate-2-phosphate.

Differentiation of hADSCs towards different lineages

Adipogenic differentiation. To induce adipogenic differentiation, ADSCs at passage II were harvested and plated on cell culture plate (Corning) at a density of 5×10^4 cells. Cells were then cultured for two weeks in adipogenic differentiation media: DMEM low glucose supplemented with 2% FBS, 1% antibiotic solution, 0.5 mM isobutyl-methylxanthine, 1 µM dexamethasone, 10 µM insulin, 200 µM indomethacin. The presence of adipose elements in induced cultures was determined by Oil-Red-O (Sigma) staining as follows: cells were washed in PBS, then fixed in 10% formalin for 5 min at room temperature, washed in 60% isopropanol and air-dried. Cells were then incubated with Oil-Red-O staining solution for 10 min, then washed several times in PBS and observed with an inverted microscope Eclipse TS100 (Nikon, Tokyo, Japan) equipped with a DS-FI1 CCD camera (Nikon).

Osteogenic differentiation. In order to induce osteogenesis, ADSCs at passage II plated on cell culture plate at a density of 5×10^4 were treated for 2-4 weeks with osteogenic medium: DMEM low glucose supplemented with 2% FBS, 1% antibiotic solution, 50 µg/ml ascorbate-2-phosphate, 10^{-8} M dexamethasone, 10^{-2} M β-glycerophosphate. The presence of calcium deposits in induced cultures was determined by Alizarin Red (Sigma) staining as follows: cells were fixed in 10% formalin for 10 min, then washed in PBS (pH 4.2) and incubated for 10 min at 37°C with Alizarin Red 2% in water. The cells were finally washed several times to remove the excess of staining and analyzed as described above. In another set of experiments, ADSCs were incubated with CM obtained from DPSCs. CM was filtered and fresh FBS (10%), penicillin/streptomycin (1%) was added.

Chondrogenic differentiation. In order to evaluate the chondrogenetic potential, ADCSs at passage II plated on cell culture plate at a density of 5×10^4 were maintained in chondrogenic induction medium: DMEM low glucose supplemented with 1% FBS, 1% antibiotic solution, 6.25 µg/mL insulin, 10 ng/mL TGF-β3, 50 nM ascorbate-2-phosphate. After two-four weeks the presence of proteoglycans in induced cultures was determined by

Safranin O (Sigma) staining as follow: cells were fixed in 10% formalin for 20 min at room temperature, then washed in PBS and acetic acid and incubated for 5 min at room temperature with 2% Safranin O in water. The cells were finally washed several times to remove the excess of staining and analyzed as described. In another set of experiments, ADSCs were incubated with above CM obtained from differentiated chondrocytes. CM was filtered and fresh FBS (10%), penicillin/streptomycin (1%) was added.

Culture of bone marrow-derived MSCs and their differentiation

Bone marrow (BM) aspirates obtained from two healthy volunteer donors were diluted 1:1 in PBS and subjected to Histopaque 1077 (Sigma, St Louis, MO, USA) density gradient centrifugation at 600 xg for 30 min without brake. The obtained buffy coat cell fraction was centrifuged at 600 xg for 10 min and resuspended in mesenchymal stem cells medium (Stem Cell Technologies) supplemented with 10% FBS (Gibco Limited, Uxbridge, UK), 100 UI/ml penicillin and 100 µg/ml streptomycin, at cell density of 4×10^5 cells/cm². After 24 h the non-adherent fraction was discarded and the BM-derived MSCs obtained from the adherent fraction were expanded at confluence until the second passage (14). For the differentiation toward the mesodermic lineages, the cells at passage II were plated at a density of 1×10^3 /cm² and maintained in differentiating induction medium for two weeks, as described above for the ADSCs.

Image analysis

Images were taken from each culture condition in double, considering 10X fields. In order to perform analysis of differentiation into the three mesenchymal lineages, jpeg image files of samples were accessed in Photoshop 7.0. Each image was divided into three parts, and each of them was analysed first for the total number of pixels by accessing Image > Histogram. Then, the same part was accessed in Select > Color range and analysed for number of pixels corresponding to stained cells and subsequently by Inage > Histogram. The pixel ratio for each image was calculated by dividing the stained number of pixels for total number of pixels. For each culture condition, ADSCs and BM-MSCs obtained from three and two donors, respectively, were used.

Statistical analysis

Data are expressed as mean $\pm$ SD. Comparisons between percentages of phenotypic markers in different passages and pixel ratios under different differentiating conditions were made using ANOVA and Tukey's post hoc test. A p-value of < 0.05 was considered significant.

RESULTS

Isolation and characterization of ADSCs

Upon isolation, ADSCs were characterized after plastic adhesion by a fibroblastic morphology very similar to that described for mesenchymal cells isolated from bone marrow (Fig. 1A). ADSCs adhered and proliferated on tissue culture plastic and could be kept until passage V. All the ADSC cultures at all passages until the V passage presented the same morphology. We did not find any difference in ADSC morphology among the different subjects. We assayed the growth kinetic in three subjects. The average doubling time calculated over 42 days of culture and until passage IV was 11.73 days (Fig. 1B).

ADSC cultures obtained from seven patients were analysed for expression of cell-surface markers from the isolation to the fifth passage (Fig. 2). ADSCs

expressed mesenchymal lineage markers, such as CD29 (92-98%), CD105 (89-93%), CD73 (24-72%), CD44 (71.7-97.8%), but very low levels of hematopoietic lineage markers CD14 (0.5-3.0%) and CD45 (1-4%). CD34, a marker of stem/progenitor hematopoietic and endothelial lineages, was expressed by 10% of freshly isolated cells and its expression lowered to 1% of positive cells in the subsequent passages. No statistically significant differences in the expression of phenotypic markers among different passages was found, except for CD44, that at the first passage was significantly lower as compared with further passages.

Differentiation of ADSCs towards the adipocyte lineage

ADSCs incubated in adipogenic media were reproducibly induced toward the adipogenic lineage as early as 1 week post-induction. Freshly isolated ADSCs showed a clear differentiation pattern in the adipocytes as indicated by a significant fraction of the cells containing multiple lipid-filled droplets that stained with oil red O (Fig. 3). No difference in the adipocytic differentiation was noted in four different subjects.

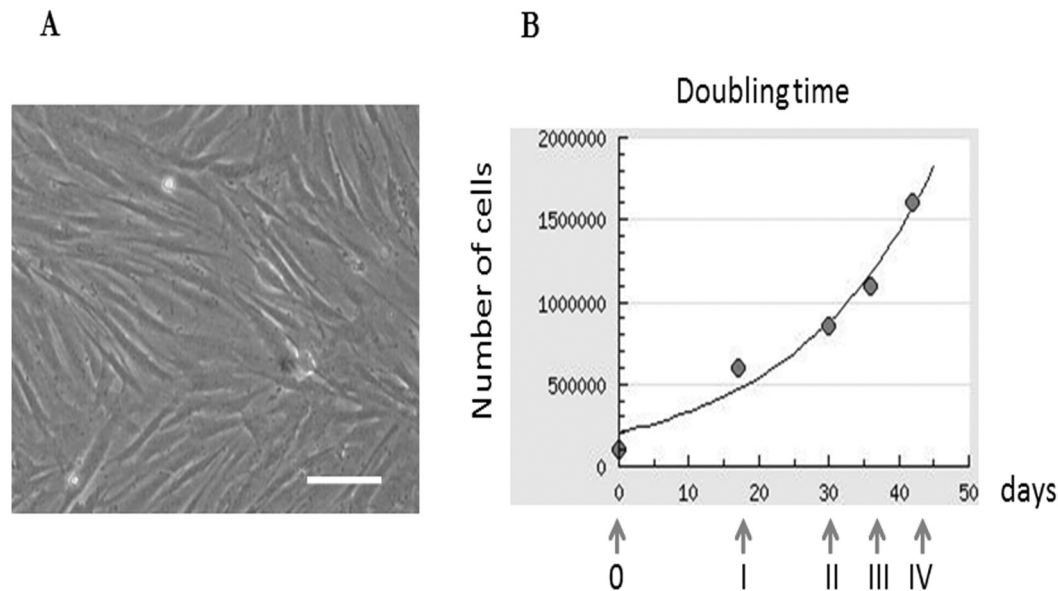


Fig. 1. ADSC morphology and growth. *A)* Cell morphology at passage one, original magnification 10X (Bar = 30 μm). *B)* Growth kinetics of ADSCs in a representative culture obtained from one patient. Three patients gave similar results. 0 = adherent cells after isolation; I, II, III, and IV indicate cells at the first, second, third and fourth passage.

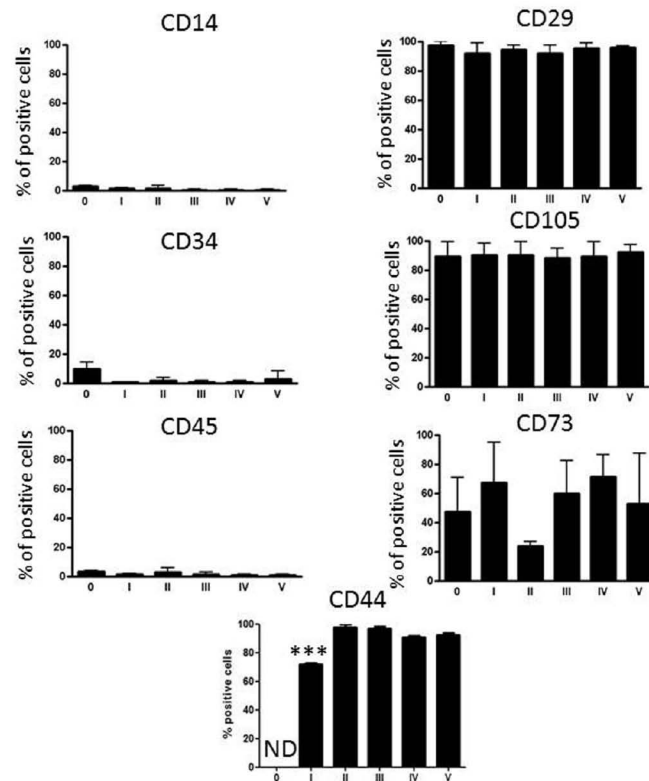


Fig. 2. ADSC surface marker expression. Flow cytometry analysis of the hematopoietic (CD14, CD34, and CD45) and mesenchymal (CD29, CD105, CD73 and CD44) lineage markers in cells from 0 to V passages. Data are shown as mean $\pm$ SD of percentages of positive cells obtained from eight patients. In CD44 panel, *** $p < 0.0001$: passage I vs II, III, IV, and V passages. ND: not determined.

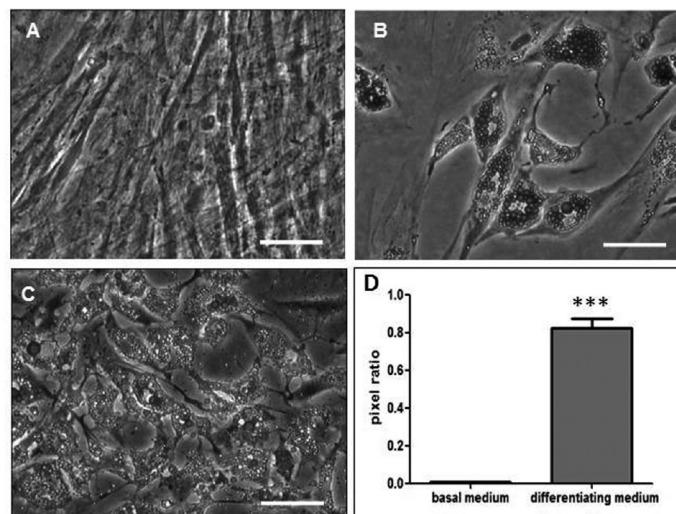


Fig. 3. In vitro differentiation capability of ADSCs towards the adipogenic lineage. Representative images of ADSCs grown in differentiating medium (B and C). A) represents negative controls (i.e. uninduced cells grown in basal medium). Original magnification: A) 10X (Bar = 30 μ m); B) 20X (Bar = 15 μ m); C) 10X (Bar = 30 μ m). Four patients were analysed. Images obtained in one representative experiment are shown. Panel D shows the pixel ratio of ADSCs grown either in basal medium or in differentiating medium. *** $p < 0.0001$.

Differentiation of ADSCs toward the osteoblast lineage

ADSCs cultured in osteogenic medium changed morphology but did not produce calcified extracellular matrix after 2 weeks of induction (Fig. 4A and B). This result was obtained even when ADSCs obtained from other two individuals were cultured in differentiation medium for either 3 or 4 weeks (data not shown). In order to see whether paracrine factors from osteoblasts could be involved in the acquirement of osteogenetic behaviour, ADSCs were cultured in conditioned media from DPSCs. In the first place, DPSCs were induced to differentiate to osteoblasts with a defined osteogenic medium for 2 weeks. After this time, they were able to produce calcified extracellular matrix, as shown by alizarin red S staining (Fig. 4C). ADSCs were treated with the CM from these differentiating DPSCs every 2 days for 2 weeks. Only with this treatment, ADSCs were shown to secrete a calcified matrix (Fig. 4D).

Differentiation of ADSCs toward the chondrocytic lineage

After incubation of ADSCs in chondrogenic

defined medium, the cells showed morphological changes but not proteoglycan deposition (Fig. 5B). This result was obtained even when ADSCs obtained from other two individuals were cultured in differentiation medium for either 3 or 4 weeks (data not shown). Chondrocytes were isolated from auricle cartilage and cultured in a growth medium. After 2 weeks in culture, they dedifferentiated, i.e. they acquired a fibroblastic morphology as previously shown (15). However, these cells exposed to chondrogenic medium for 3 weeks displayed positivity to proteoglycan deposition and formed typical micromasses (Fig. 5C). On the other hand, ADSCs incubated in CM obtained from differentiated chondrocytes show proteoglycan secretion after 2 weeks of induction (Fig. 5D). No micromasses were found in the CM-induced ADSC cultures.

Differentiation of BM-MSCs

As a positive control for differentiation conditions, BM-MSCs were subjected to the same conditions as those for ADSCs promoting adipogenesis,

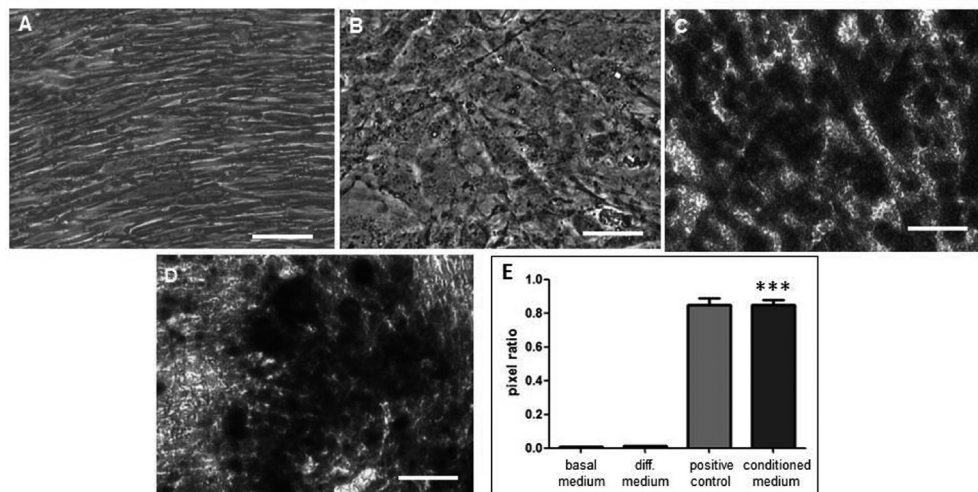


Fig. 4. *In vitro* differentiation capability of ADSCs towards the osteogenic lineage. Cells maintained in basal media did not show any change in their morphology and no calcium deposit (A, magnification 10X. Bar = 30 µm); freshly isolated ADSCs maintained in differentiating medium showed a clear change in their morphology but no calcium deposit (B, magnification 20X. Bar = 15 µm) in contrast to DPSCs (used as positive control) that showed a clear osteogenic differentiation pattern (C, magnification 10X. Bar = 30 µm). ADSCs incubated in conditioned medium obtained from differentiated DPSCs show calcium deposits after 2 weeks of induction (D, magnification 10X. Bar = 30 µm). Four patients were analysed. Images obtained in one representative experiment are shown. Panel E shows the pixel ratio of ADSCs grown in different conditions. *** $p < 0.0001$ of "conditioned medium" versus both "basal medium" and "diff. medium".

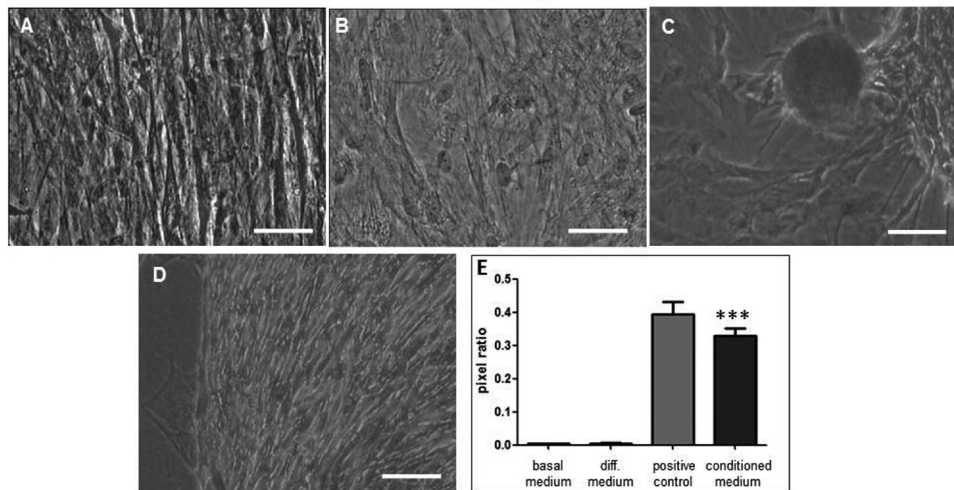


Fig. 5. *In vitro* differentiation capability of ADSCs towards the chondrogenic lineage. Cells maintained in control media did not show any change in their morphology and no proteoglycan secretion (A, magnification 10X. Bar = 30 μ m); freshly isolated ADSCs maintained in differentiating medium showed a clear change in their morphology but no proteoglycan secretion (B, magnification 20X. Bar = 15 μ m) in contrast to differentiated chondrocytes from auricular cartilage (used as positive control) that displayed the formation of a micromass positive to safranin O staining (C, magnification 10X. Bar = 35 μ m). ADSCs incubated in conditioned medium obtained from differentiated chondrocytes show proteoglycan secretion after 2 weeks of induction (D, magnification 10X. Bar = 30 μ m). Four patients were analysed. Images obtained in one representative experiment are shown. Panel E shows the pixel ratio of ADSCs grown in different conditions. *** $p < 0.0001$ of “conditioned medium” versus both “basal medium” and “diff. medium”.

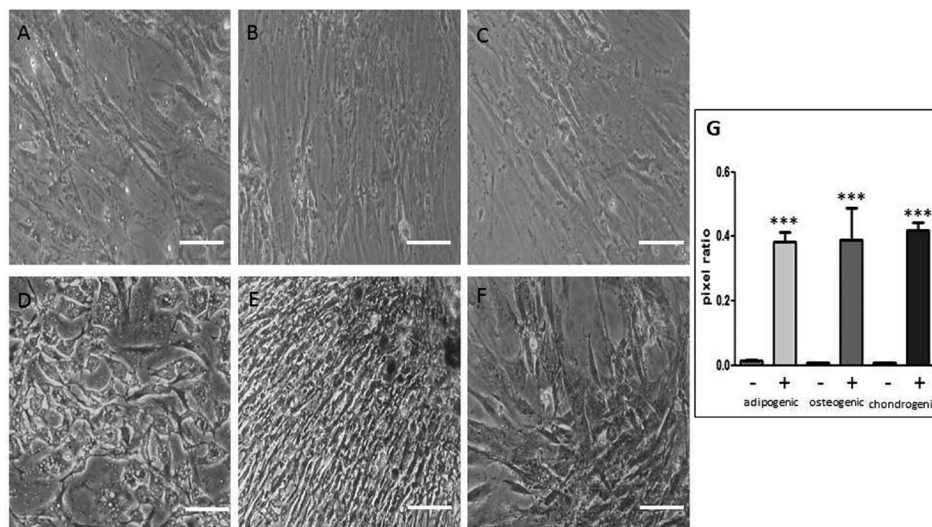


Fig. 6. *In vitro* differentiation capability of BM-MSCs towards the adipogenic, osteogenic, and chondrogenic lineages. Cells maintained in basal media did not show any change in their morphology and no lipid droplets (A), calcium deposits (B), or proteoglycan secretion (C, all with magnification 10X. Bar = 30 μ m). Second passage BM-MSCs maintained in respective differentiating medium showed a clear change in their morphology and show lipid droplets (D), calcium deposits (E), or proteoglycan secretion (F) after 2 weeks of induction (all with magnification 10X. Bar = 30 μ m). Two individuals were analysed, and images obtained from one subject are shown. Panel G shows the pixel ratio of BM-MSCs grown in different conditions. *** $p < 0.0001$ of “differentiating medium” (+) versus “basal medium” (–).

osteogenesis, and chondrogenesis. After two weeks, BM-MSCs exhibited multiple intra-cellular lipid filled droplets in the majority of cells (Fig. 6B). Differentiation of BM-MSCs into osteocytes was demonstrated after two weeks by change in morphology and by the presence of calcium deposits (Fig. 6C). BM-MSCs differentiated in chondrocytes after two weeks of induction as demonstrated by change in morphology and the occurrence of staining for proteoglycans (Fig. 6D). No micromasses were observed.

DISCUSSION

Since the first reports on their isolation in 2001 (1), more than 3,000 papers have been published on ADSCs, including many biological and applicative aspects (7). The reason for this popularity is based on their interesting features that make them more attractive than BM-MSCs in the field of regenerative medicine and tissue engineering. Firstly, human adipose tissue is ubiquitous and easier to obtain in larger quantities than a bone marrow biopsy. Secondly, ADSCs can be obtained in higher amounts than BM-MSCs, as judged by the colony forming unit-fibroblast assay (2). Thirdly, the proliferative capacity of ADSCs seems higher than that of BM-MSCs. Previous reports have shown that the doubling times of ADSCs during the logarithmic phase of growth range from 40 to 120 hours (1, 16), and accordingly our results show a doubling time of approximately 140 hours. Although we did not calculate the doubling time of BM-MSCs, these cells undoubtedly grew more slowly than ADSCs.

ADSC differentiation towards adipogenic, osteogenic, and chondrogenic lineages has been demonstrated by a wealth of assays, including histochemistry, gene expression by RT-PCR, and flow cytometry. In this study, we report that ADSCs show a ready capacity to differentiate towards the adipogenic lineage but not to the osteoblastic and chondrocytic lineages. ADSCs were induced to produce calcified matrix or proteoglycans only when incubated in the presence of CM obtained from differentiated DPSCs or auricle cartilage-derived chondrocytes, respectively. These data strongly

suggest that growth factors or other morphogenic factors produced by bone and cartilage work in inducing and/or favoring the differentiative capacity of ADSCs. When BM-MSCs were analyzed in the same differentiating conditions, we could observe a clear difference, i.e. BM-MSCs already at two weeks of induction differentiated readily into adipocytes, and began to differentiate into osteoblasts and chondrocytes. These data show that ADSCs and BM-MSCs are mesenchymal (stem) lineages with clear differences in growth and differentiation capacity, as pointed out previously by other authors and as discussed below.

The main limit of this study is that we analyzed ADSCs multipotency towards osteoblastic and chondrogenic lineages only by histochemical assays. The study of expression of genes involved in this process would have aided in better comprehending the early and late steps of ADSC differentiation, however our results clearly indicate that ADSCs are not favored in giving rise to osteoblasts or chondroblasts unless stimulated with soluble factors coming from differentiated cells.

Our results are in good agreement with previous studies reporting about the differentiative capacity of ADSCs which suggested that supplemental factors are needed. The osteogenic potential of ADSCs was reported to be inferior to that of BM-MSCs when cultured in the same osteogenic medium (17). Many studies have shown the osteogenic potential of ADSCs by histochemistry with alizarin red, however ADSCs appear to require more factors to achieve osteogenic differentiation comparable to BM-MSCs. Indeed, Elabd et al. (18) reported that two weeks in culture in the presence of 1% FCS, vitamin D₃, dexamethasone, ascorbate and β -glycerophosphate, was not able to induce osteogenic differentiation in ADSCs, while the presence of EGF accelerated the formation of mineralized matrix. Anurag et al. (19) found that distinct foci of mineralization appeared after 28 days of induction only when the osteogenic medium contained vitamin D₃ besides ascorbic acid and β -glycerophosphate. Finally, Song et al. (20) showed that mineralization was found at negligible levels after 2 weeks of induction with dexamethasone, ascorbate and β -glycerophosphate,

while it was greatly enhanced using BMP-2 and vitamin D₃. On this line of evidence, it has been reported that ADSCs exhibited limited efficiency in osteoblast differentiation as compared with BM-MSCs when studied by gene expression (21). Moreover, the transcriptome of ADSCs appears to be much more distant to that of BM-MSCs in comparison with MSCs obtained from skin (21). Overall, it seems that there exists a lineage “imprinting” in different stromal cell compartments that influences the differentiation potential of MSCs. In alternative, it was previously demonstrated that bone marrow harbors heterogeneous populations of pre-osteoblastic and pre-adipocytic cell populations in addition to multipotent MSCs (22). Furthermore, ADSCs have been found to express variable levels of markers suggestive of different cell phenotypes. For example, some authors found the stem/progenitor hematopoietic and endothelial cell marker CD34 expression by flow cytometry (23, 24), while others were not able to show this positivity (1, 2). ADSCs isolated by us expressed the stem/progenitor CD34, in 10% of freshly isolated cells and negligible levels in the further cultures. This heterogeneity may derive from the so far not completely explained origin of ADSCs. Recent reports indicate they may exist within the perivascular tissue since they express similar cell surface antigens to pericytes (25).

As to the chondrogenesis, it was previously observed that the capacity of ADSCs to differentiate into chondrocytes grown as monolayer is inferior to that shown when they are grown in three-dimensional cultures (alginate beads) (26). Indeed, most of the studies have explored the chondrogenic capability of ADSCs by the micropellet method (5, 27). Although we did not use the micropellet method, we could force ADSCs in bi-dimensional culture to acquire a chondrogenic phenotype by incubating them with CM obtained by differentiated chondrocytes. Nevertheless, ADSCs display less capability to differentiate towards the chondrogenic lineage than BM-MSCs. Im et al. (17) showed that, when equal amount of bioactive factors (IGF-1 and TGF- β_2) are given, ADSCs have inferior capacity to differentiate into cartilage as compared with BM-MSCs. These results are paralleled by those presented

by Huang et al. (28), who found that ADSC-derived aggregates underwent significantly less extensive chondrogenesis, as evidenced by lower matrix production when compared with BM-MSCs from the same patient. As pointed out above, ADSCs may represent distinctly different cell populations that are at different stages of lineage-specific commitment compared to BM-MSCs (27). Thus, different conditions are perhaps required to promote more extensive chondrogenic differentiation of ADSCs. A number of growth factors and morphogens including TGF- β_1 , TGF- β_3 , BMP-2, and BMP-6 have been shown to induce chondrogenesis in mesenchymal stem cells (29, 30).

The results obtained in this study implies that only in vivo, ADSCs would find the correct environment which will support their differentiation towards differentiated osteoblasts and chondrocytes. During the repair of a bone or cartilage injury, tissue differentiated cells would supply for growth and morphogenic factors. This issue has been evidenced by the study by Noel et al. (5), who showed that in vivo ADSCs display the same ability of BM-MSCs to differentiate towards chondrocytes/osteoblasts, despite ADSCs and BM-MSCs are fundamentally different cell types and differently committed cells.

In conclusion, in this study we show that only those ADSCs cultured in the presence of CM obtained from differentiated dental pulp stem cells or progenitors of chondrocytes could elicit osteoblast and chondrocyte differentiation of ADSCs, respectively. The findings of this study indicate that in-vitro experiments do not firmly establish the multipotent capacity of ADSCs; these features should be corroborated and confirmed by in-vivo studies. Future works on specific growth and morphogenic factors involved in ADSC differentiation towards osteogenic and chondrogenic lineages are warranted. Regenerative medicine aimed to repair bone and cartilage would benefit from these findings.

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LETTER TO THE EDITOR

DECREASED SERUM LEVELS OF T-CELL IMMUNOGLOBULIN MUCIN-1 AND T-CELL IMMUNOGLOBULIN MUCIN-3 IN SYSTEMIC LUPUS ERYTHEMATOSUS PATIENTSH. YUAN¹, Y-S. YAO¹, G-M.CHEN², J. SHENG³, L. XU³ and H-F. PAN⁴

¹Department of Preventive Medicine, Wannan Medical College, Wuhu, Anhui, China; ²School of Health Management, Anhui Medical University, Hefei, Anhui, China; ³Department of Rheumatology and Immunology, the First Affiliated Hospital of Wannan Medical College, Wuhu, Anhui China; ⁴Department of Epidemiology and Biostatistics, School of Public Health, Anhui Medical University, Hefei, China

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This study aims to investigate the serum T-cell immunoglobulin mucin (TIM)-1 and TIM-3 levels in systemic lupus erythematosus (SLE) patients and analyze their correlations with clinical features. Sixty-one SLE patients and 69 healthy controls were enrolled, serum TIM-1 and TIM-3 levels were detected by ELISA. Results demonstrated that both serum TIM-1 and TIM-3 levels were significantly decreased in SLE patients compared with controls (both $P < 0.05$). Lower serum TIM-3 levels in SLE patients with nephritis were observed when compared to those without nephritis, with a marginal statistical significance ($P = 0.059$). However, both serum TIM-1 and TIM-3 levels had no significant correlation with SLE disease activity (both $P > 0.05$). In summary, decreased serum TIM-1 and TIM-3 levels, and association of TIM-3 with nephritis suggest their possible role in the development and pathogenesis of SLE. However, further studies are needed to confirm these preliminary results.

Systemic lupus erythematosus (SLE) is a chronic connective tissue disease, which often is associated with multiple autoantibody production, inflammatory cytokine secretion, complement activation and immune-complex deposition, causing tissue or organ damage. To date, the etiology and pathogenesis of SLE remain unclear. Dysregulation of Th1 and Th2 cell function have been implicated in the pathophysiology of SLE, a predominance of Th1 may induce autoimmunity, while Th2 can be related with lupus nephritis (LN) (1). T-cell immunoglobulin mucin (TIM) proteins include a family of cell surface molecules expressed on T cells, which regulate T cell-mediated immune responses (2). Genomic analysis

study, combined with positional cloning, identified a cluster of similar TIM genes including 8 members (TIM 1–8) on mouse chromosome 11B1.1 and 3 members (TIM 1, 3 and 4) on human chromosome 5q33.2 (3, 4).

TIM-1 is the first member identified in TIM gene family, it is expressed in both kidney and liver. TIM-1 was originally cloned as the kidney injury molecule 1 (5) and hepatitis A virus cellular receptor (6, 7). Recently, studies have revealed that TIM-1 is also expressed on CD4⁺ T cells after activation and its expression is sustained preferentially in Th2 but not Th1 cells (4). Another TIM family member, TIM-3, was initially identified by screening for Th1 specific

Key words: TIM-1, TIM-3, systemic lupus erythematosus

Mailing address:

Dr. Hai-Feng Pan, M.D,
Department of Epidemiology and Biostatistics,
School of Public Health, Anhui Medical University,
81 Meishan Road, Hefei, Anhui, 230032, PR China
Tel.: +86 551 65161165 - Fax: +86 551 65161171
e-mail: panhaifeng1982@sina.com

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markers, but it has also been found on cytotoxic CD8⁺ T cells, Treg, Th17, monocytes, dendritic cells, mast cells and microglia (2, 8-10). TIM-3 can inhibit aggressive Th1 mediated auto- and allo-immune responses (11).

It has been reported that TIM family are involved in the pathogenesis of inflammatory and autoimmune diseases (3, 8, 11-14). Study on the cerebrospinal fluid mononuclear cells from patients with multiple sclerosis (MS) showed significantly higher expression levels of TIM-1 mRNA compared with normal controls (12). An anti-TIM-3 antibody in vivo significantly exacerbated the pathogenic course of experimental autoimmune encephalomyelitis (EAE) in SJL/J mice, associated with activation and expansion of macrophages (8). Administration of inhibitory anti-TIM-1 in lupus-prone MRL-Fas (lpr) mice attenuated established LN, indicating that manipulating immune system TIM-1 may represent a therapeutic strategy in autoimmune diseases affecting the kidney (15). In addition, aberrant expressions of TIM-1 or TIM-3 in SLE patients have been reported in several recent studies, suggesting that TIM-1 and TIM-3 may play important roles in SLE pathogenesis (16-19).

In the present study, to further explore the role of TIM-1 and TIM-3 in human SLE, we investigated the serum TIM-1 and TIM-3 levels in SLE and analyzed their correlations with disease activity and the development of nephritis.

MATERIALS AND METHODS

Study subjects

Sixty-one patients with SLE (56 females, 5 males; mean age 33.89±10.428 years) were recruited from the Department of Rheumatology and immunology at the first affiliated hospital to Anhui Medical University and Anhui Provincial Hospital. SLE diagnosis was established by the presence of four or more 1997 revised American College of Rheumatology (ACR) classification criteria (20). The involvement of kidney in SLE was defined according to the ACR criteria, i.e, any one of the following: i) persistent proteinuria ≥0.5 g/day; ii) presence of active cellular casts; or iii) biopsy evidence of LN (21). The disease activity was quantified using the

SLE disease activity index (SLEDAI) score (22). More active SLE was defined as SLEDAI score ≥6, less active SLE was defined as SLEDAI <6 (23). Sixty-nine healthy volunteers (64 females, 5 males; mean age 31.32±6.814 years) were included as controls, all of whom did not have any rheumatic disease. All study subjects provided their written consent to participate in the study. Demographic data and clinical data were collected from hospital records or by self-designed questionnaire (23).

Extraction of serum and enzyme-linked immunosorbent assay (ELISA)

Sera were obtained from 6 ml of whole blood at the first visit of the study subjects and stored at -80°C. Serum TIM-1 and TIM-3 levels were detected by ELISA according to the recommendation of the manufacturer (R&D Systems, Inc.) and the results were expressed as pg/ml.

Statistical analysis

The numerical data were expressed as mean± SD, or median (interquartile range, IQR) if they were not in normal distribution. Difference in serum concentration among different groups was compared with Mann-Whitney rank sum test. The Chi-squared test or Fisher's exact test was used to evaluate differences in categorical data between the two groups. All statistical analysis was conducted by the Statistical Package for the Social Sciences (SPSS) statistical software for Windows, Version 10.01 (SPSS Inc, IL, USA). Probability values (P) ≤ 0.05 were considered as significant.

RESULTS

The basic characteristics of the study subjects are shown in Table I, no significant difference in gender and age distribution were observed between SLE patients and normal controls (both P>0.05). Both TIM-1 and TIM-3 levels were significantly decreased in the serum of SLE patients compared with health controls (both P<0.05). Lower serum TIM-3 level in SLE patients with nephritis was observed when compared to those without nephritis, with a marginal statistical significance (P=0.059). However, there was no significant difference in serum TIM-1 level between SLE patients with and without nephritis

($P=0.134$) (Tables II and III); no significant difference in serum TIM-1 and TIM-3 levels were found between less active SLE and more active SLE (both $P>0.05$) (Tables II and III). Both serum TIM-1 and TIM-3 levels had no significant correlation with SLEDAI score (both $P>0.05$) (Figs. 1 and 2).

DISCUSSION

In recent years, there have been increasing studies on the role of proteins of the TIM family in regulating immune responses. TIM-1 and TIM-3 are

expressed on terminally differentiated Th2 and Th1 cells respectively, and participate in regulating Th immune response. To date, the role of TIM family proteins in autoimmune diseases remains unclear. In a recent study, Xiao et al. (24) generated a TIM-1-mutant mouse, TIM-1(Δ mucin), in which the mucin domain was genetically deleted. The mutant mice demonstrated a profound defect in IL-10 production from regulatory B cells. Along with the loss of IL-10 production in B cells, older TIM-1(Δ mucin) mice developed spontaneous autoimmunity associated with hyperactive T cells, with elevated IFN- γ

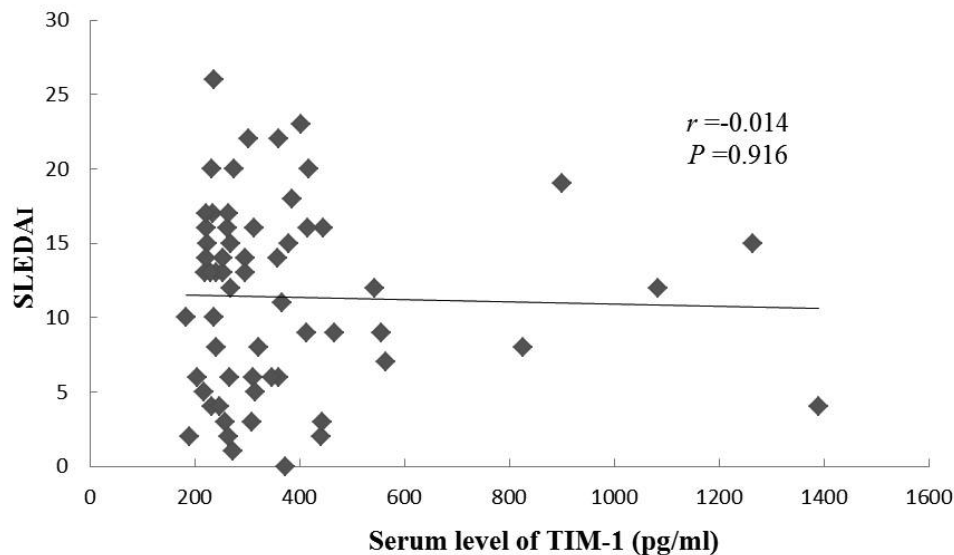


Fig. 1. Correlation between serum TIM-1 levels and SLEDAI in SLE patients.

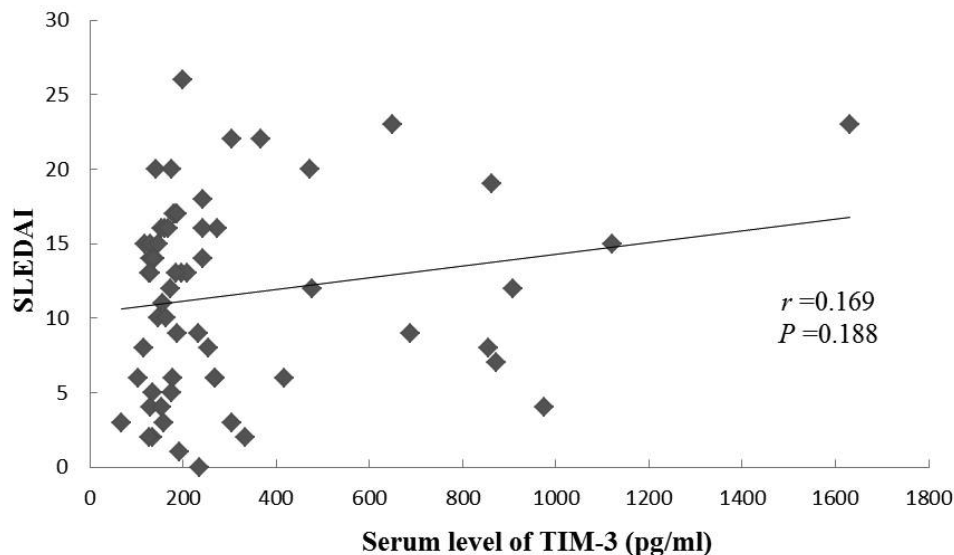


Fig. 2. Correlation between serum TIM-3 levels and SLEDAI in SLE patients.

Table I. *General data of study subjects.*

Parameters	SLE patients (n=61)	Controls (n=69)	P value
Age	33.89±10.428	31.32±6.814	0.105
Sex (male/female)	5/56	5/64	0.860
Disease activity (more active/ less active)	36/25	NA	NA
Nephritis (yes/no)	24/37	NA	NA

NA: not available

Table II. *Comparison of serum TIM-1 levels between different subgroups.*

Group	Number	Serum TIM-1 level Median (interquartile range) pg/ml	P value
Normal controls	69	363.53(300.57-529.34)	
SLE patients	61	296.66(239.11-408.67)	<0.001*
SLE without nephritis	37	267.05(234.09-376.43)	
SLE with nephritis	24	334.87(264.18-417.63)	0.134 [☆]
Less active SLE	13	273.73 (240.54-407.71)	
More active SLE	48	299.76 (238.51-411.41)	0.711 [▲]

*versus normal controls; [☆]versus SLE without nephritis; [▲]versus less active SLE

production and increased serum levels of Ig and autoantibodies. Nevertheless, TIM-1(Δ mucin) mice did not develop frank systemic autoimmune disease unless they were crossed onto the Fas-mutant lpr mice on a C57BL/6 background. TIM-1(Δ mucin)lpr mice developed accelerated and fulminant systemic autoimmunity with accumulation of abnormal double negative T cells and autoantibodies against a number of autoantigens associated with SLE (24). All these results indicate that TIM-1 may play an important role in maintaining suppressive regulatory

B cell function, and also reveal an unexpected role of the TIM-1 mucin domain in the regulation of regulatory B cell function and maintaining self-tolerance (24, 25). Treatment with high- but not low-avidity anti-TIM-1 antibody not only worsens EAE in susceptible mice but also breaks tolerance and induces EAE in a genetically resistant strain of mice (26). The expression of TIM-3 was elevated in the spinal cord during EAE and following antigen restimulation of the encephalitogenic TCRBV8S2+ population (27). Seki et al. reported that galectin-9

Table III. Comparison of serum TIM-3 levels between different subgroups.

Group	Number	Serum TIM-3 level Median (interquartile range) pg/ml	P value
Normal controls	69	299.29(217.95-495.50)	
SLE patients	61	186.09(143.61-289.89)	0.002*
SLE without nephritis	37	175.87(130.51-286.38)	
SLE with nephritis	24	204.61(157.82-423.39)	0.059 [*]
Less active SLE	13	158.62 (132.11-270.41)	
More active SLE	48	187.04 (149.36-297.39)	0.218 [▲]

versus normal controls; ^{}versus SLE without nephritis; [▲]versus less active SLE

ameliorates collagen-induced arthritis (CIA) by suppressing the Th17 generation, promoting the regulatory T cell induction (13). Study on T cell clones obtained from patients with MS revealed significantly lower expression of TIM-3 as compared with normal controls (28). Additionally, TIM gene family is located on the chromosomal region 5q33.2, which has been linked with susceptibility to RA, autoimmune thyroid disease (ATD, Type 1 diabetes (T1D) and Crohn's disease (CD) (29). Collectively, TIM-1 and TIM-3 may play an important role in the induction and development of autoimmune diseases.

SLE is a prototypical autoimmune disease with a wide array of clinical manifestations, which has been reported to be likely both a Th1 and Th17-mediated disease (30). More recently, Nozaki et al. investigated the effects of an inhibitory anti-TIM-1 (RMT1-10) in lupus-prone MRL-Fas (lpr) mice, their results showed that RMT1-10 treatment significantly improved survival, limited the development of lymphadenopathy and skin lesions, preserved renal function and decreased proteinuria, reduced serum anti-DNA antibody levels, and attenuated renal leukocyte accumulation; Th1 and Th17

cellular responses systemically and intrarenally were reduced, but regulatory T and B cells were increased; RMT1-10 treatment also reduced glomerular immunoglobulin and C3 deposition and inhibited cellular proliferation and apoptosis; Urinary excretion and renal expression of TIM-1 was decreased, reflecting diminished interstitial injury. As RMT1-10 attenuated established lupus nephritis, manipulating immune system TIM-1 may represent a therapeutic strategy in autoimmune diseases affecting the kidney (15). All these results support a possible role of TIM-1 and TIM-3 in the pathogenesis of SLE.

In the current study, we investigated the serum TIM-1 and TIM-3 levels in SLE patients. Results showed that both TIM-1 and TIM-3 levels were significantly decreased in the serum of SLE patients compared with healthy controls. In addition, lower serum TIM-3 levels in SLE patients with nephritis were found when compared to those without nephritis. It has been revealed that TIM-3 negatively regulates the activation of macrophages in the kidney during nephrotoxic serum nephritis, and TIM-3 blockade aggravates nephritis by increasing

the number of kidney-infiltrating inflammatory cells, indicating that TIM-3 exerts protective roles in the course of nephritis (31). Therefore, stimulation of TIM-3 expression might be a promising target for therapeutic intervention in autoimmune nephropathy.

It is of interest to compare our results to several previous attempts to explore the expression and clinical associations of TIM-1 and TIM-3 in SLE patients. A very recent study by Cai et al. reported downregulation of TIM-3 mRNA expression in peripheral blood mononuclear cells from patients with SLE, and TIM-3 mRNA expression was negatively correlated with the SLEDAI (18). In addition, another study by the same group suggests that insufficient expression of TIM-3 mRNA may be involved in the pathogenesis of LN (32), which are similar to our results. Guo et al. reported that TIM-3 was expressed in renal tissue from the patients with SLE, but rarely expressed in cases without SLE, TIM-3 expression was associated with the disease activity (17). However, elevated expression of TIM-1 and TIM-3 ligand (galactin-9) mRNA have also been shown in SLE patients (16). These disparities may be influenced by the difference between protein and mRNA expression level, different demographic characteristics, application of different classification and patient selection criteria.

Taken together, decreased serum level of TIM-1 and TIM-3, and association of TIM-3 with nephritis suggest their possible role of in the pathogenesis of SLE. However, further studies are needed to confirm these preliminary results.

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LETTER TO THE EDITOR

DOPPLER LASER IMAGING PREDICTS RESPONSE TO TOPICAL MINOXIDIL IN THE TREATMENT OF FEMALE PATTERN HAIR LOSS

J. McCOY¹, M. KOVACEVIC², M. SITUM³, A. STANIMIROVIC⁴, Z. BOLANCA³
and A. GOREN^{1,2}

¹*Applied Biology, Inc., Irvine, CA, USA;* ²*Department of Dermatology and Venereology, University of Rome "G.Marconi", Rome, Italy;* ³*Department of Dermatology and Venereology, University Hospital Center "Sestre milosrdnice", Zagreb, Croatia;* ⁴*Department of Clinical Medicine, University of Applied Health Sciences, Zagreb, Croatia*

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Topical minoxidil is the only drug approved by the US FDA for the treatment of female pattern hair loss. Unfortunately, following 16 weeks of daily application, less than 40% of patients regrow hair. Several studies have demonstrated that sulfotransferase enzyme activity in plucked hair follicles predicts topical minoxidil response in female pattern hair loss patients. However, due to patients' discomfort with the procedure, and the time required to perform the enzymatic assay it would be ideal to develop a rapid, non-invasive test for sulfotransferase enzyme activity. Minoxidil is a pro-drug converted to its active form, minoxidil sulfate, by sulfotransferase enzymes in the outer root sheath of hair. Minoxidil sulfate is the active form required for both the promotion of hair regrowth and the vasodilatory effects of minoxidil. We thus hypothesized that laser Doppler velocimetry measurement of scalp blood perfusion subsequent to the application of topical minoxidil would correlate with sulfotransferase enzyme activity in plucked hair follicles. In this study, plucked hair follicles from female pattern hair loss patients were analyzed for sulfotransferase enzyme activity. Additionally, laser Doppler velocimetry was used to measure the change in scalp perfusion at 15, 30, 45, and 60 minutes, after the application of minoxidil. In agreement with our hypothesis, we discovered a correlation ($r=1.0$) between the change in scalp perfusion within 60 minutes after topical minoxidil application and sulfotransferase enzyme activity in plucked hairs. To our knowledge, this is the first study demonstrating the feasibility of using laser Doppler imaging as a rapid, non-invasive diagnostic test to predict topical minoxidil response in the treatment of female pattern hair loss.

Female pattern hair loss (FPHL) is the most frequent cause of hair loss in female patients; its prevalence is approximately 50% in women by the age of 50 (1). It is characterized by hair follicle miniaturization and changes in hair cycle dynamics (e.g., shortening of the anagen phase of hair growth cycle and stabilization or increase in the duration of telogen phase) (2). FPHL usually occurs at about 40

years of age in a specific pattern affecting the central portion of the scalp, sparing the frontal hairline (3). It can also present as a more noticeable thinning on the frontal portion of scalp, or as a hair fall pattern similar to male pattern hair loss involving the frontal and frontoparietal hair line (4, 5). Independent of age, patients with this disorder may have significant impairment in quality of life. Hair loss has a major

Key words: female pattern hair loss, minoxidil, alopecia

Mailing address:

John McCoy, PhD
Applied Biology, 17780 Fitch, Suite 192,
Irvine, CA 92614, USA
Tel.: +1 (949) 387 4526
e-mail: johnm@appliedbiology.com

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impact on patients' self-esteem, social perceptions, and psychosocial functioning (6).

A number of products have been proposed as hair regrowth therapies, however, topical minoxidil is the only drug approved by the US FDA for the treatment of FPHL. Unfortunately, its efficacy is relatively low; less than 40% of patients regrow hair following 16 weeks of daily application of 5% minoxidil (7). Although the exact mechanism of action of minoxidil is not completely clear, it is suggested that minoxidil reverses follicular miniaturization, triggers follicles in the latent part of telogen into anagen and prolongs the duration of anagen in humans (8). Additionally, minoxidil may decrease epidermal growth factors (EGFs) and open cellular potassium channels, leading to impaired entry of calcium into the hair follicle cells (3).

In vitro studies have demonstrated that minoxidil sulfate is the active metabolite of minoxidil (9). Conversion of minoxidil to minoxidil sulfate is catalyzed by minoxidil sulfotransferase enzymes in the hair follicle (10). A correlation between sulfotransferase expression in the scalp and minoxidil response has been reported (10, 11). In addition, Goren et al. reported that a colorimetric activity assay for sulfotransferase can be used as a rule-out for non-responders to topical minoxidil (12, 13).

As minoxidil sulfate is the active metabolite that stimulates hair regrowth, other methods to detect the conversion of minoxidil to minoxidil sulfate in

the scalp would have similar diagnostic capabilities as the colorimetric assay reported in Goren et al. One such method is the detection of increased scalp blood flow after the application of minoxidil. Vasodilation is reported to be induced by minoxidil sulfate and not minoxidil (14). It would follow that the detection of increased scalp blood perfusion after the application of minoxidil would indicate the conversion of minoxidil to minoxidil sulfate. The direct detection of increased scalp blood flow after the application of minoxidil has been measured by the method of laser Doppler velocimetry (15). However, the connection between predicting minoxidil response and measured blood flow has never been established. We hypothesized that laser Doppler velocimetry measurement of scalp blood perfusion, subsequent to topical minoxidil application, would correlate with sulfotransferase enzyme activity in plucked hair follicles.

MATERIALS AND METHODS

Ten female subjects diagnosed with FPHL (Sinclair scale 2-4) were included in the study. Ten hairs with intact bulbs were plucked from the top of the head of each subject. Sulfotransferase enzyme activity was determined from the hair samples utilizing methods previously described (12, 13). Subsequent to hair sample collection, a target area of thinning hair on the top of the scalp was marked with a surgical pen. At baseline (t=0), the laser Doppler velocimeter (PeriFlux System 5000, Jarfalla,

Table I. Enzyme Activity (AU) versus Laser Doppler Velocimetry (% increase).

Patient ID	Laser Doppler Velocimetry (% increase from baseline)						Enzyme Activity Assay (AU)
	T0	T15	T30	T45	T60	Max	
1	--	-4.2%	59.6%	46.1%	19.4%	59.6%	0.027
2	--	41.3%	69.2%	73.3%	51.1%	73.3%	0.074
3	--	42.7%	61.6%	26.4%	-19.7%	61.6%	0.115
4	--	-44.3%	-1.7%	-48.3%	-21.0%	-1.7%	0.228
5	--	-45.8%	-5.6%	32.9%	19.2%	32.9%	0.315
6	--	-14.7%	35.8%	-18.3%	2.1%	35.8%	0.362
7	--	65.9%	28.0%	16.6%	81.9%	81.9%	0.463
8	--	197.0%	279.4%	254.9%	254.0%	279.4%	0.493
9	--	-8.6%	91.0%	180.1%	90.5%	180.1%	0.503
10	--	11.2%	366.6%	75.7%	137.6%	366.6%	0.638

Sweden) was used to measure blood perfusion in the target area. Subject perfusion data was collected until a continuously stable signal was obtained for a minimum length of 30 seconds. The average perfusion within the 30 s interval was calculated with the perfusion software analyzer (PeriFlux System 5000, Jarfalla, Sweden). After the baseline measurement, a 5% topical minoxidil solution was applied to the target area. Subsequent perfusion measurements in the target area were made at 15, 30, 45, and 60 minutes.

RESULTS

Data from the study are shown in Table I. Perfusion data are displayed as percent increase from baseline at times 15, 30, 45, and 60 minutes. Perfusion data are shown in line with the results from the sulfotransferase activity assay for each patient. Data were tabulated according to the sulfotransferase activity measured with the colorimetric assay.

Six of the ten patients studied were below the previously reported cutoff, 0.4 absorbance units (AU), for determining responder/non-responders to minoxidil with the colorimetric assay (12). Correlating the results of the colorimetric assay to the increase in perfusion in the target area, within the 60 minutes sampled, we found that patients having less than a 75% increase ($r = 1.0$) in scalp blood perfusion are unlikely to respond to 5% topical minoxidil.

DISCUSSION

Minoxidil is a pro-drug. To elicit hair regrowth, minoxidil must be converted to its active form, minoxidil sulfate, by sulfotransferase enzymes found in hair follicles (9, 10). In previous works we have shown that sulfotransferase activity in plucked hair follicles is a powerful diagnostic tool for ruling out non-responders to 5% topical minoxidil (12, 13). However, patients' discomfort with the procedure and the time required to perform the enzymatic assay make the diagnostic test less desirable than the rapid, non-invasive method described here. Consistent with our hypothesis, this study demonstrated a correlation ($r=1.0$) between sulfotransferase activity in hair follicles and scalp

blood perfusion as measured by laser Doppler velocimetry.

Interestingly, the laser Doppler velocimetry method for identifying non-responders to 5% topical minoxidil may present an opportunity to provide an alternative treatment. Patients identified as non-responders to the 5% topical minoxidil present with low perfusion following the application of the drug, owing to low sulfotransferase activity. These patients may produce a clinically effective amount of minoxidil sulfate with a higher dosage of minoxidil (Le Châtelier's principle). With the rapid diagnostic method described here it is possible to assess the response of each patient to 5% minoxidil and qualify non-responders for higher dosages. Additionally, the efficacy of a higher dosage minoxidil can also be diagnosed with the same method. Laser Doppler velocimetry can be used to identify the optimal topical minoxidil dosage (above 5%) that will convert each non-responder to a responder. We are currently conducting a larger trial to test this hypothesis using 15% minoxidil.

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LETTER TO THE EDITOR

EFFECTS OF SODIUM FERROUS CHLOROPHYLL TREATMENT ON ANEMIA OF HEMODIALYSIS PATIENTS AND RELEVANT BIOCHEMICAL PARAMETERS

XF. XU¹, JP. HU¹, X. CHENG¹, GJ. YU², F. LUO³, GS. ZHANG¹,
N. YANG⁴ and P. SHEN¹

¹Nephrology Division, Zhengzhou TCM Hospital, Zhengzhou, China; ²Nephrology Division, Affiliated Hospital, Henan Province, Chinese Medicine Research, Institute, Zhengzhou, China; ³Endocrinology Division, Zhengzhou Central, Hospital, Zhengzhou, China; ⁴Science and Education, Department, Zhengzhou TCM Hospital, Zhengzhou, China

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This study explores the effects of sodium ferrous chlorophyll treatment on the anemia of maintenance hemodialysis (MHD) patients, as well as the relevant biochemical parameters. We selected 72 patients who had received regular MHD treatment two or three times a week for more than 3 months in the Hospital of Traditional Chinese Medicine of Zhengzhou City of Henan Province from March 2014 to March 2015. They were equally divided into a treatment group and a control group. Haemoglobin (HB) and hematocrit (HCT) of the treatment group increased significantly after treatment ($p < 0.01$), but less in the control group ($p < 0.05$); Also serum ferritin (SF) and transferrin saturation (TAST) of the treatment group increased significantly after treatment ($p < 0.01$); SF of the control group also increased significantly ($p < 0.01$) and TAST of the control group increased ($p < 0.05$) but less than in the treatment group. No obvious changes of serum creatinine (SCR), blood urea nitrogen (BUN), C-reactive protein (CRP) and superoxide dismutase (SOD) were found in either groups after treatment ($p > 0.05$). Albumin (ALB) dosage of the treatment group increased after treatment ($p < 0.05$) while hemopoietin (EPO) decreased significantly ($p < 0.01$). ALB and EPO of the control group had no obvious changes after treatment ($p > 0.05$). ALB level of the treatment group increased more significantly than in the control group ($p < 0.05$), while EPO dosage decreased more significantly than in the control group ($p < 0.05$). Therefore, the combination of conventional western medicine and sodium ferrous chlorophyll can effectively improve anemia conditions of MHD patients and their quality of life.

Kidney failure is a disease that causes damage to body organs; replacing kidney functions by treating with hemodialysis has been used for more than 50 years (1-3).

Although it is the most used therapy at present,

it is not a perfect substitution for renal functions (4).

Renal anemia (RA) (5-7), a common complication in hemodialysis, can result in cardiomegaly, ventricular hypertrophy, stenocardia, increase of cardiac output, congestive heart-failure and immune

Key words: sodium ferrous chlorophyll, anemia, maintenance hemodialysis

Mailing address:

Dr Guojun Yu
Nephrology Division
Affiliated Hospital of Henan Province
Chinese Medicine Research Institute,
65, Wenhuaogong Road,
Zhengzhou 450000, China
e-mail: guojunyuyy@163.com

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response barrier, etc. (8-9). These complications severely affect patients' quality of life as well as increasing the death rate of MHD patients. Therefore, it is very useful to study relevant factors that influence RA (10-11).

Song et al. (12) discovered that levocarnitine could reduce the dose of rhEPO in MHD patients, which can be associated with improvements of the micro-inflammation and erythropoietin resistance in MHD patients. Nissenson et al. (13) found that heme iron polypeptide successfully replaced intravenous (IV) iron therapy in the majority of hemodialysis patients and maintained target hematocrit (HCT) with no concomitant use of IV iron

This study mainly explored the effects of sodium ferrous chlorophyll treatment on the anemia of MHD patients and relevant biochemical parameters. Moreover, this study aims to suggest better methods that can improve the anemia and quality of life of patients through observation of the effects of sodium ferrous chlorophyll on the anemia of MHD patients.

MATERIALS AND METHODS

Study subjects

Seventy-two patients who received maintenance hemodialysis treatment in the Hospital of Traditional Chinese Medicine of Zhengzhou City of Henan Province from March 2014 to March 2015 were selected. The study used a primitive analysis method that was approved by the Ethics Committee of the same hospital. All the patients signed informed consent. All the patients were diagnosed with RA and had been under dialysis two or three times a week for more than three months. They were divided into a treatment group and a control group, 36 in each group (12). Results were comparable as the two groups had no significant differences in general information, test parameters, EPO dosage or traditional Chinese medicine score, etc. ($p > 0.05$).

Methods

Basic treatment: patients received dialysis 2 or 3 times a week for approximately 4 hours each time. They were also asked to have hypotensor according to their conditions. Patients who had pathoglycemia were given antidiabetics and patients who had disorders of calcium-

phosphate metabolism were given a calcium phosphate regulator based on their conditions (13-14).

Medication: both groups received r-HuEPO and the dosage was adjusted during the experiment. The dosage of EPO was decreased 25% if the hemoglobin (HB) of patients reached 110 g/L. After being observed for half to one month, patients were re-examined. EPO dosage was lowered by another 25% if HB had not reduced or had even increased. Patients were examined at least once a month to establish the minimum dosage of EPO for maintaining HB at target value. Patients were also given chalybeate, folic acid, vitamin B12 based on their examination results. On the basis of the above treatments, the treatment group was given sodium ferrous chlorophyll three times a day. During the treatment, all patients were forbidden to take hormone drugs, cytotoxic drugs or drugs that could affect iron metabolism.

Parameter observation

Anemia parameters [HB, hematocrit (HCT)], iron metabolism parameters [serum ferritin (SF), transferrin saturation (TAST)] and other biochemical parameters (serum creatinine (SCR), blood urea nitrogen (BUN), C-reactive protein (CRP), albumin (ALB) and superoxide dismutase (SOD)] as well as EPO dosage before and after treatment were recorded.

Statistical analysis

Results were statistically analyzed by using SPSS19.0 software. Measurement data were examined by t-test and all data were presented using mean $\pm$ SD. Enumeration data were examined using Chi-squared test.

RESULTS

Comparison of general information

The treatment group and the control group both had 36 patients, consisting of 19 males and 17 females. The treatment group and the control group were aged 45.59 ± 5.64 and 46.3 ± 5.79 , respectively. Dialysis duration and dry weight of the treatment group were 4.55 ± 1.96 and 46.3 ± 5.79 respectively; while dialysis duration and dry weight of the control group were 4.66 ± 1.82 and 56.45 ± 7.67 ($P > 0.05$, compared with control group).

Comparison of HB and HCT before and after treatment

Intra-group comparison: HB and HCT of the treatment group increased significantly after treatment ($p<0.01$) and HB and HCT of the control group increased after treatment ($p<0.05$), as shown in Table I.

Comparison of SF and TAST as well as Scr and BUN before and after treatment

Intra-group comparison: after treatment, SF and TAST of the treatment group increased significantly ($p<0.05$); SF of the control group increased significantly ($p<0.01$) and TAST of the control group increased ($p<0.05$). Comparison between the two groups: after treatment, SF and TAST of the treatment

group increased more significantly than that of the control group ($p<0.05$).

Intra-group comparison: Scr and BUN of both groups had no significant changes after treatment ($p>0.05$). Comparison between the two groups: no significant difference of SCR and BUN was found between the two groups after treatment ($p>0.05$). Details are shown in Table II.

Comparison of CRP and SOD dosage as well as ALB and EPO dosage before and after treatment

Intra-group comparison: CRP and SOD dosage of both groups had no significant changes after treatment ($p>0.05$). Comparison between the two groups: no

Table I. Comparison of Hb and Hct before and after treatment.

Groups	Treatment time	Hb(g / L)	Hct(%)
Treatment group	Before treatment	82.73±11.12	26.81±3.42
	One month before treatment	90.12±11.64	28.85±3.77
	Two months before treatment	101.61±11.59	30.92±5.21
	Three months before treatment	110.37±11.85**●	33.21±5.32**●
Control group	Before treatment	83.71±10.73	27.13±5.19
	One month before treatment	89.59±12.55	28.80±5.74
	Two months before treatment	97.65±13.29	30.12±5.54
	Three months before treatment	103.35±12.15*	31.23±5.55*

* $P<0.05$ and ** $P<0.01$ in comparison before and after treatment;

● $P<0.05$ and ●● $P<0.01$ in comparison between treatment group and control group.

Hb: haemoglobin; Hct: hematocrit

Table II. Comparison of SF and TAST as well as Scr and BUN before and after treatment.

Groups	Treatment time	SF(ng/ml)	TAST (%)	Scr(mmol/l)	BUN(mmol/l)
Treatment group	Before treatment	169.31±101.62	19.44±9.21	895.61±168.49	22.61±2.73
	After treatment	268.41±122.27**●	31.60±10.16**●	887.47±172.63	22.51±2.80
Control group	Before treatment	158.49±98.71	19.65±9.19	903.44±172.56	22.73±2.52
	After treatment	223.48±131.49**	27.24±9.40*	897.44±169.86	22.65±2.47

* $P<0.05$ and ** $P<0.01$ in comparison before and after treatment;

● $P<0.05$ and ●● $P<0.01$ in comparison between treatment group and control group.

SF: serum ferritin; TAST: transferrin saturation; Scr: serum creatinine; BUN: blood urea nitrogen

Table III. Comparison of SF and TAST as well as Scr and BUN before and after treatment.

Groups	Treatment time	CRP(mg/L)	SOD(U/L)	ALB(g/L)	EPU dosage [U/(kg·w)]
Treatment group	Before treatment	6.61±3.08	44.84±2.21	32.71±5.29	127.25±21.67
	After treatment	5.99±2.81	41.12±2.24	36.30±4.25*●	100.08±22.61**●●
Control group	Before treatment	6.23±2.55	45.18±2.11	33.22±6.63	125.41±21.79
	After treatment	6.41±2.29	42.47±2.38	33.51±6.19	120.64±22.58

* $P<0.05$ and ** $P<0.01$ in comparison before and after treatment within group;

● $P<0.05$ and ●● $P<0.01$ in comparison between two groups.

CRP: C-reactive protein; SOD: superoxide dismutase; ALB: albumin; BUN: blood urea nitrogen

significant changes of CRP and SOD dosage were found between two groups ($p>0.05$).

Intra-group comparison: ALB dosage of the treatment groups increased after treatment ($p<0.05$) and EPO dosage of the treatment group decreased significantly after treatment ($p<0.01$). ALB and EPO dosage of the control group after treatment had no significant differences compared with before treatment ($p>0.05$). Comparison between the two groups: ALB dosage of the treatment group increased more significantly than in the control group after

treatment ($p<0.05$); EPO dosage of the treatment group decreased more significantly than in the control group ($p<0.05$). Details are shown in Table III

Comparison of clinical effects

Obvious effective rate, effective rate and ineffective rate of the treatment group after treatment were 66.7% ($n=24$), 22.2% ($n=8$) and 11.1% ($n=4$), respectively, while the corresponding rates in the control group were 47.2% ($n=17$), 38.9% ($n=14$) and 13.9% ($n=5$), respectively. Total effective rates of the

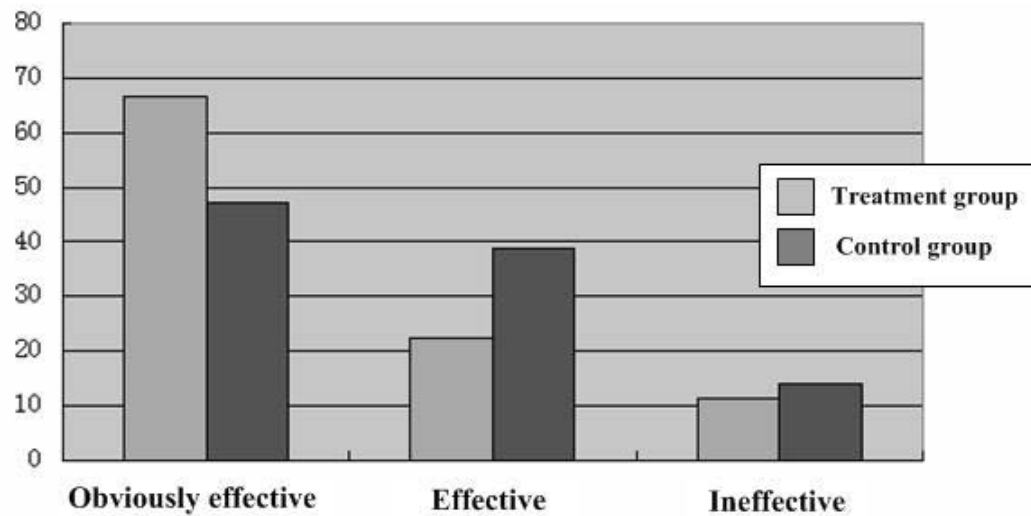


Fig. 1. Clinical efficacy comparison before and after treatment.

treatment group and the control group after treatment were 88.9% and 86.1%, respectively, which had no significant difference ($p>0.05$). Obvious effective rate of the treatment group was higher than for the control group ($p<0.05$). Treatment was considered to be obviously effective if anemia was relieved significantly and HB increased more than 30 g/L or HCT increased more than 10%. Relief of anemia, increase of HB between 15-30 g/L or increase of HCT between 5%-10% was held to be effective. Insignificant relief of anemia, increase of HB less than 15 g/L and increase of HCT less than 5% were considered to be ineffective (Fig. 1).

DISCUSSION

HB and HCT are the two major parameters for diagnosing RA and can also directly reflect the anemia condition of the patient (15). This study indicates that the combination of conventional western medicine treatment and sodium ferrous chlorophyll can significantly improve MHD patients' HB, HCT, ferritin and TSAT ($p<0.01$). SF and TAST are the two most widely applied parameters used to evaluate iron condition in current clinical practice. This study verified that sodium ferrous chlorophyll could effectively improve the anemia condition of MHD patients and their iron metabolisms. CRP and SOD are two reliable parameters that can reflect microinflammation of MHD

patients (16), while CRP is the main marker protein of chronic microinflammation in MHD patients as well as a reliable parameter used for diagnosing inflammation and evaluating treatment. This study also showed that sodium ferrous chlorophyll had no significant influence on microinflammation and oxidative stress reaction parameters of CRP, SOD, etc. ($p>0.05$). Besides, much evidence indicates that CRP is closely related to hypertensive cardiovascular remodeling and ventricular remodeling after myocardial infarction and can predict the death of hemodialysis patients (17). ALB is an important parameter of the nutritional condition and liver function, and it also plays an important role in blood colloid osmotic pressure maintenance, metabolic substance transfer and nutrition (18-19). Moreover, this study revealed that sodium ferrous chlorophyll can improve ALB ($p<0.05$), which indicates the effect of sodium ferrous chlorophyll on MHD patients' nutrition. Many studies in recent years have proved that besides improving anemia condition, r-HuPEO can also protect organs, which is not only beneficial in treating acute kidney damage, but can also relieve chronic kidney diseases. It has been confirmed that, early application of r-HuPEO can correct a mild anemia condition of chronic kidney disease (CKD), relieve CKD and finally reduce the probability of renal transplantation (20). Therefore, sodium ferrous chlorophyll is considered to have effects that are similar to hemopoietin.

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LETTER TO THE EDITOR

CURATIVE EFFECT OF MECHANICAL HEART VALVE REPLACEMENT
AND ANTICOAGULANT THERAPY AFTER SURGERYJ.B. CHUAI¹, L. SHI², X.Y. MA², D. WU³, K. KANG¹ and S.L. JIANG¹

¹Department of Cardiovascular Surgery, the Second Affiliated Hospital of Harbin Medical University, Harbin, China; ²Department of Cardiothoracic Surgery, Daqing Oilfield General Hospital, Daqing, China; ³Department of Pathology, the First Affiliated Hospital, Heilongjiang University of Chinese Medicine, Harbin, China

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The first two authors contributed equally to this work

This study was carried out to determine the curative effect of low-intensity anticoagulant therapy by observing the oral administration of warfarin (anticoagulant therapy) on patients who had undergone mechanical heart valve replacement (MHVR) surgery with subsequent anticoagulation complications. Fifty patients who underwent MHVR in the Second Affiliated Hospital of Harbin Medical University and 52 patients in the Cardiovascular Surgery of Daqing Oilfield General Hospital between January 2013 and January 2015 were selected (63 males and 39 females, ages 26-77 years). They took warfarin after treatment and were followed-up by means of outpatient review and telephone after leaving the hospital. The effect of warfarin and the occurrence of anticoagulation complications were analyzed. The operations lasted 230 ± 106 min, extracorporeal circulation for 110 ± 50 min and aorta occlusion for 82 ± 23 min. During post-operation 3 patients developed skin purpura and one patient died. During follow-up we found 3 cases of anemia caused by excessive menstruation, 4 cases of hematuria, 3 cases of repeated epistaxis, 1 case of gastrointestinal bleeding, 1 case of cerebral hemorrhage, 1 case of embolism in the lower limbs and 1 case of cerebral infarction, although they all improved or were totally cured. Therefore, the incidence of complications can be reduced significantly by the correct administration of warfarin as well as timely monitoring of interference factors after MHVR.

Valvular heart disease is caused by a disorder in the intracardiac haemodynamics due to an abnormal structure and function of the heart valve, which affects life quality and can endanger a patient's life. According to statistics (1), approximately 100,000 patients worldwide with rheumatic valvular disease or degenerative valvular disease receive valve replacement annually. These patients are required to take warfarin as an anticoagulation therapy for their entire life, as an artificial valve

implanted into the human body can lead to cerebral embolism, body-embolism and other short-term and long-term complications. It is demonstrated that both thromboembolism caused by insufficient anticoagulation and hemorrhage induced by excessive anticoagulation treatment are the main factors that determine the postoperative survival of patients (2).

Allou et al. (3) introduced the heparin intravenous injection anticoagulant therapy and

Key words: mechanical heart valve replacement surgery, warfarin, anticoagulant therapy, prothrombin time, international normalized ratio

Mailing address:

Dr Kai Kang and Shuli Jiang,
Department of Cardiovascular Surgery,
The Second Affiliated Hospital
Harbin Medical University,
Harbin, China
e-mail: kkang163@163.com

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believed that within 3 h after mechanical heart valve replacement (MHVR) it could reduce the risks of embolism. Whitlock et al. (4) suggested using heparin in combination with other antiplatelet drugs for anticoagulant therapy after MHVR. A good anticoagulant treatment compliance has proved to be decisive in reducing or even avoiding relevant complications (5).

For this study we selected 50 subjects who underwent heart valve replacement in the Second Affiliated Hospital of Harbin Medical University and 52 subjects in Cardiovascular Surgery of Daqing Oilfield General Hospital from January 2013 to January 2015. Warfarin was administered as a low-intensity anticoagulant therapy.

MATERIALS AND METHODS

Clinical materials

A total of 102 subjects (63 males and 49 females; ages 26-77 years) who had undergone heart valve replacement were enrolled in this study. Eighty-three patients had rheumatic heart disease, 11 patients had a degenerative disease, 5 patients had infectious endocarditis and 3 patients had congenital heart disease. Before surgery, patients were graded based on heart function: II level (22 cases), III level (67 cases) and IV level (13 cases). This experiment was approved by the medical ethics committee and verified by pathologists, and all patients signed informed consent.

Patients undergoing surgery had no abnormal liver function or hematological system disorders and were not influenced by other anticoagulant drugs. Patients who did not meet above requirements or who had stopped taking warfarin without reason were excluded.

Operation and anticoagulation methods

All patients received heart valve replacement in general anesthesia. Sixty-nine patients underwent mitral valve replacement, 20 patients sustained aortic valve and mitral valve replacement and 13 patients aortic valve replacement. Before surgery they all recorded an average plasma prothrombin time (PT) of 13.9 ± 2.68 s, prothrombin time - international normalized ratio (PT-INR) of 1.13 ± 0.30 , an average value of alanine aminotransferase (ALT) of 23.9 ± 17.6 , an aspartate aminotransferase (AST) of 26.3 ± 13.9 , an average value of urea nitrogen of 5.8 ± 2.6 and an average value of creatinine of 73.88 ± 18.3 . Mitral valve replacement was performed through the right atrium to interatrial septum and aortic valve replacement was completed with a transverse incision under the aortic root.

Follow-up data

Follow-up included PT, INR drug dose, cardiac function recovery and diet. PT and INR values of patients as well as the dose of warfarin were followed through by telephone and outpatient service regularly. Patients were divided into hemorrhage group, normal group and embolism group according to the conditions, and measured with PT and INR value when complications occurred. Dosage of warfarin was recorded.

Statistical analysis

Measurement data were expressed with mean $\pm$ SD, *t*-test was applied to compare the mean value between two groups and average values between multiple groups were compared with analysis of variance. The data measured the variance and results were tested with χ^2 test. SPSS 17.0 software was used for statistical processing. Difference was considered to be statistically significant if $P < 0.05$.

Table I. Measurement of postoperative PT and INR values.

	The 3rd day after surgery	The 5th day after surgery	The 7th day after surgery	The 9th day after surgery	Hospital discharge
Warfarin (mg/d)	2.51 ± 0.52	$2.54 \pm 0.56\#$	$2.52 \pm 0.75\#$	$2.55 \pm 0.88\#$	$2.52 \pm 0.92\#$
PT-INR value	1.16 ± 0.24	$1.42 \pm 0.65^*$	$1.75 \pm 0.75^*$	$1.85 \pm 0.74^*$	$1.80 \pm 0.45^*$

#: more than 0.05 compared with the 3rd day after surgery; *: less than 0.05 compared with the 3rd day after surgery.

Table II. Occurrence of complications using warfarin for anticoagulant therapy.

Complications	Case number	PT-INR value	Follow-up time	Anticoagulant therapy	Results
Skin purpura ecchymosis	3	1.95~2.16	1 month	decrement	cured
Anemia	3	1.90~2.85	3 months to 5 years	decrement	
Hematuresis	4	2.35~2.85	1 month	decrement	cured
Epistaxis	2	2.34~2.55	3 months to 2 years	decrement	cured
Gum bleeding	1	2.23~2.52	3 months to 2 years	decrement	cured
Gastrointestinal bleeding	1	2.62	6 month	intramuscular vitamin K1	cured
Intracranial hemorrhage	1	2.95	2 month	intramuscular vitamin K1	cured
Limb thrombus	1	1.62	1 month	thrombus taken and increment	cured
Cerebral infarction	1	1.73	1 month	increment	improved

RESULTS

Overall post-operative results

The operations lasted for 230 ± 106 min, extracorporeal circulation for 110 ± 50 min and aorta occlusion for 82 ± 23 min. During post-operation 3 patients developed skin purpura and one patient died. During follow-up we found 3 cases of anemia caused by excessive menstruation, 4 cases of hematuresis, 3 cases of peated epistaxis, 1 case of gastrointestinal bleeding, 1 case of cerebral hemorrhage, 1 case of embolism in the lower limbs and 1 case of cerebral infarction, although they all improved or were totally cured.

Post-operative complications

Anticoagulant drugs can induce imbalance of the coagulation system. Table II shows the conditions of complications due to anticoagulation therapy.

PT-INR fluctuation at different time points after operation

Analysis of variance of measured PT-INR values demonstrated that the difference of dispersion degree of PT-INR distribution at different time points was statistically significant ($P < 0.05$). Fig. 1 reveals the changes of PT-INR value at different time points after surgery.

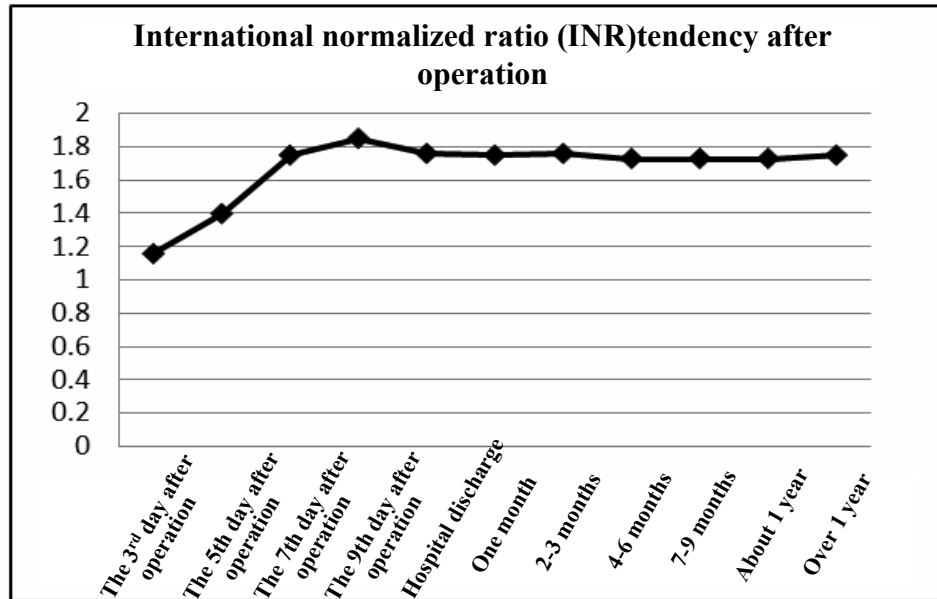


Fig. 1. Changes of PT-INR value in different periods after surgery.

Table III. Distribution of cases with different kinds of bleeding in different PT-INR scope.

	PT-INR scope	No. of bleeding case	No. of non-bleeding case
Anemia induced by excessive amount of menstrual blood volume	1.5-1.9	0	102
	>1.9	3	99
Hematuresis	1.6-2.1	0	102
	>2.1	4	98
Repeated epistaxis or gingival bleeding	1.6-2.1	3	99
	>2.1	0	102
Gastrointestinal bleeding	1.6-2.1	1	101
	>2.1	0	102
Cerebral hemorrhage	1.6-2.1	1	101
	>2.1	0	102

Postoperative bleeding

Light bleeding neither affects organ functions nor induces blood composition changes, i.e. hematoma of bulbar conjunctiva, skin purpura and ecchymosis, gum bleeding and microscopic hematuria. Heavy bleeding involves a larger area and requires special clinical treatment. We found 3 cases of anemia induced by excessive amount of menstrual blood volume, 4 cases of hematuria, 3 cases of repeated epistaxis or gingival bleeding, 1 case of gastrointestinal bleeding and 1 case of cerebral hemorrhage. Table III shows bleeding conditions of patients.

DISCUSSION

It is clinically recognized that a long-term anticoagulation treatment is required after MHVR, and oral administration of warfarin is considered important (6-9). The dosage of warfarin for anticoagulant therapy varies from person to person and patients with irregular anticoagulation are likely to have post-operative complications.

Insufficient dosage could result in thrombus, cause embolism and in severe cases it could lead to sudden death. Excessive dosage usually generates bleeding. The dosage is usually determined by gender, age, weight and genetic factors. Generally speaking, patients are advised to take 5 mg per day in the first two days as a standard loading dosage. A unified standard for an ideal PT-INR level has not yet been agreed on although the warfarin anticoagulation detection index has been widely accepted (10-13). Individual reactions to warfarin anticoagulant therapy vary and some patients could spend a longer time in achieving anticoagulation effectiveness while others can achieve it shortly after surgery. Heparin can be added to assist anticoagulation if patients are in a hypercoagulable state after surgery (14).

Bleeding and thromboembolism associated with warfarin anticoagulation are the main complications after MHVR, although bleeding is more likely than thromboembolism. Low-intensity anticoagulation treatment has proved to be effective and safe in most patients who have undergone MHVR. Postoperative PT-INR should be detected early to prevent the occurrence of complications.

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LETTER TO THE EDITOR

C-REACTIVE PROTEIN CORRELATES TO FUNCTIONAL SCORE SYSTEMS OF PATIENTS IN GENERAL SURGICAL INTENSIVE CARE UNITJ. TIAN¹, X. LIU² and D. LIU¹¹*Intensive Care Unit, The Second Hospital of Jilin University, Changchun, Jilin, People's Republic of China;* ²*Clinical Medicine College, Jilin University, Changchun, Jilin, People's Republic of China**Received September 3, 2015 - Accepted November 6, 2015*

The aim of our study was to document the relationship between inflammation indicated by the serum levels of widespread acute-phase proteins and scoring systems used in assessing patient's functional condition. We therefore assessed serum levels of c-reactive protein (CRP) and four clinical tests [acute physiology and chronic health evaluation (APACHE) II score and sequential organ failure assessment (SOFA) score, functional independence measure (FIM) and Barthel index of activities of daily living (BI)]. We tested the hypothesis that results of functional tests correlate with infections and consecutively influence the length of stay (LOS) in non-specialized general intensive care units (ICUs). One hundred and twenty patients from non-specialized general ICU were enrolled in the study group. We scored patients consecutively on admission (APACHE II-initial; SOFA-initial, FIM initial and BI initial) and on the fifth day of stay (APACHE II-5th day; SOFA-5th day, FIM 5th day and BI 5th day). At the same time points, serum levels of CRP were assessed. FIM index did not correlate to the CRP at any time point (FIM- initial=0.027; FIM- 5th day=0.024; respectively) at discharge, total number of hospital days, i.e. LOS was recorded. Serum CRP values and APACHE II, SOFA, and BI scores correlated positively both on admission and on the fifth day of stay in the general surgical ICU (APACHE II- initial $r=0.289$; SOFA- initial $r=0.305$; BI - initial $r=0.291$; APACHE II 5th day $r=0.728$; SOFA-5th day $r=0.725$; BI 5th day $r=0.792$). Therefore, pending further studies, functional scoring in general surgical ICUs might prove useful in assessing LOS.

Applying functional score systems in intensive care units (ICUs) is habitually used for specific patients as well as for evaluation and measurement of different ICU success rates (1, 2). There are obvious limitations as, at least to date, different ICUs showed significant variations in length of stay (LOS) that were attributed to individual patients and to selected institutional characteristics (3).

Moreover, we evaluated different scoring systems, each having its strengths and weaknesses. Some of the functional scoring systems such as Acute Physi-

ology and Chronic Health Evaluation (APACHE) II and Sequential Organ Failure Assessment (SOFA) score, functional independence measure (FIM) and Barthel index of activities of daily living (BI) could be useful indicators of infection-complicated hospitalization and, therefore, reliable in evaluating LOS in various ICUs. APACHE II is a severity-of-disease scoring system used for audit and research purposes and SOFA score is used to track a patient's status, i.e. the extent of organ function or rate of failure during the stay in ICU. The advantages of APACHE II

*Key words: correlation, C-reactive protein, intensive care unit**Mailing address:*

Dr Dexin Liu,
Intensive Care Unit,
The Second Hospital of Jilin University,
Changchun, Jilin 130041, PR China
e-mail: dexin.liuicu@gmail.com

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score are that it is easy, widespread and for long-term use which enables extensive retrograde statistical evaluation, within and between ICUs. Comparison of different ICUs is burdened with significant variations in patients' and institutional characteristics. Furthermore, its validity has been questioned for not taking into consideration a patient's medical therapy or the course of the disease following the initial 24 hours in the ICU (4). On the other hand, SOFA score is one of the most common organ failure scores used in the ICU with advantages of assessing the intensity of organ failure and organ support during the patient's stay in ICU when compared to the APACHE II score (5).

Most concisely, BI had been used to measure physical functioning and was improved by the FIM. FIM scoring refers to measuring one's ability to function with independence while BI measures performance in everyday activities (ADL) (6, 7).

As a measure of infection, various indicators may be used, but for this study – serum levels of C-reactive protein (CRP) was selected – namely, it is an infective and inflammatory marker in intensive care medicine (8).

The aim of our study was to establish the correlation of inflammation (indicated by the serum levels of widespread acute-phase protein) and scoring systems used in assessing a patient's functional condition. The term "correlation" refers to any of a broad class of statistical relationships involving dependence. In this paper, we focused on examining the usefulness of implementing scoring systems used in assessing patient's functional condition when estimating LOS extended by the infection in the surgical ICUs.

We hypothesized that correlation between scoring for estimating LOS extended by the infection is more likely to be established for APACHE II and SOFA tools since they are more commonly used in ICU scoring systems.

MATERIALS AND METHODS

The study included all patients consecutively admitted to surgical ICUs in the Intensive Care Unit of The Second Hospital of Jilin University, Changchun, Jilin from October to December 2014. The general surgical

ICU admits patients after major abdominal, plastic, maxillofacial, or trauma surgery.

Anesthesiology and intensive care residents, members of the ICU staff scored patients on admission (APACHE II-initial; SOFA- initial, FIM – initial and BI - initial) and on the fifth day of stay (APACHE II-5th day; SOFA- 5th day, FIM- 5th day and BI- 5th day) according to the scoring forms. At the same time points, plasma levels of CRP in mg/L were measured/quantified using enzyme-linked immunosorbent assay kits (Phoenix Pharmaceuticals, Burlingame, CA, USA), according to the manufacturers' instructions.

APACHE II was calculated following the instructions from referenced literature, through 12 physiological variables, with additional weighting for previous health related to emergency on admission and age. SOFA score was calculated considering six major organ systems (i.e. cardiovascular, respiratory, renal, hepatic, central nervous system, and coagulation) (9). BI (10) was used to measure physical functioning and was improved upon by the FIM (11). BI measures the capacities divided into groups that relate to self-care and mobility. It gives a quantitative estimate of patient's level of dependency, scoring from 0 (totally dependent) to 100 (totally independent) with somewhat broader scale in FIM score. Both tools have been reported to have high reliability (12).

As scoring is routine activity in ICUs, we did not ask for additional Institution's nor patient's consent.

Statistical analysis

Statistical analysis was performed with SPSS software for Windows, version 11.0 (SPSS Inc., Chicago, IL, USA). Apart from APACHE II, SOFA, FIM and BI scores, data on gender, age, type of admission/discharge (elective/urgent surgery; alive/died) and LOS. Data were expressed as mean $\pm$ standard deviation (SD) and frequencies as well as percentages. Mann-Whitney U test was used for differences between groups. Categorical data were expressed as n (%) and compared using χ^2 test. Spearman's rank correlation coefficient was used to correlate APACHE II, SOFA, FIM and BI scores with CRP where $P < 0.05$ was considered statistically significant.

RESULTS

Tables I and II show characteristics of all 120 patients included in this study, regarding the gender,

Table I. *Patients' characteristics.*

Demographic characteristics		
Gender	n	%
male	81	67.5
female	39	32.5
	X ± SD	MIN-MAX
Age (years)	58.70 ± 17.39	1-93
ICU LOS (days)	6.19 ± 3.74	2-95
Type of admission		
	n	%
nonoperative – from Emergency Department	3	2.5
nonoperative – from ward	4	3.3
urgent surgery	24	20
elective surgery	89	74.2
Type of discharge		
alive	116	96.7
died	4	3.3

ICU: intensive care unit; LOS: length of stay

Table II. *Scorings and plasma CRP values in the ICU on admission and on 5th day given as mean ± standard deviation (SD) with extreme values (minimum and maximum).*

	X ± SD	MIN-MAX
APACHE II - initial	9.31 ± 5.19	0-34
SOFA - initial	2.28 ± 2.25	0-11
FIM- initial	118.26±12.2	98-126
BI - initial	95.66±4.3	80-100
CRP - initial (mg/L)	9.61 ±2.47	5.65-11.21
APACHE II - 5th day	27.54±12.23	12-51
SOFA - 5th day	1.79±0.39	0-6
FIM- 5th day	121.48±8.19	119-126
BI - 5th day	97.12±0.67	90-100
CRP - 5th day (mg/L)	9.76±1.02	7.52-12.8

APACHE II: acute physiology and chronic health evaluation; SOFA: sequential organ failure assessment; FIM: functional independence measure; BI: Barthel ADL index; CRP: C-reactive protein

Table III. Correlations between APACHE II, SOFA, FIM and BI on admission and on 5th day with plasma CRP concentrations at the same time point.

Functional score	r	p
APACHE II - initial	0.289	< 0.01
SOFA - initial	0.305	< 0.01
FIM- initial	0.027	< 0.01
BI - initial	0.291	< 0.01
APACHE II - 5th day	0.728	< 0.01
SOFA - 5th day	0.725	< 0.01
FIM- 5th day	0.024	< 0.01
BI - 5th day	0.792	< 0.01

APACHE II: acute physiology and chronic health evaluation; SOFA: sequential organ failure assessment; FIM: functional independence measure; BI: Barthel ADL index

age, type of admission/discharge (elective or urgent surgery; alive or dead) and LOS in addition to APACHE II, SOFA, FIM and BI scores on admission as well as on the 5th intrahospital day.

When comparing initial and 5th day APACHE II scores, the U-value is 6, and the Z-Score is -2.4881, as calculated by Mann-Whitney test. The p-value is 0.00639, thus significant at $p \leq 0.05$. SOFA scores on admission and the day 5th had the Z-Score 2.7081. The p-value is 0.00672. The result is considered significant at $p \leq 0.05$.

Initial and 5th day BI values differed with the p-value of 0.00076 ($p < 0.05$).

As for the FIM scores from admission in comparison to fifth intrahospital day, the Z-Score is -1.9925. The result is significant at $p \leq 0.05$.

Furthermore, when comparing initial and 5th day plasma CRP concentrations, values differ statistically significant (the U-value is 16. The critical value of U

at $p \leq 0.05$ is 16. Therefore, the result is significant at $p \leq 0.05$).

Correlations of APACHE II, SOFA, FIM and BI scores with plasma CRP concentrations are given in Table III with statistical significance of $p \leq 0.01$.

DISCUSSION

Scoring systems used in surgical ICUs vary greatly. In the present study, plasma CRP concentration was strongly positively correlated with APACHE II, SOFA and BI scores on admission, and APACHE II, SOFA as well as BI scores on the 5th intrahospital day. Not fully in accordance to our hypothesis; BI score, though not a classical ICU scoring system, was strongly correlated with infection and LOS extended by the infection.

Elevated CRP concentrations represent an acute-phase response to acute tissue injuries when plasma

CRP can reach 1000-fold normal concentrations. The conditions of acute tissue injuries can be of infectious, acute inflammatory or surgical origin and CRP was therefore used to monitor disease activity. They indicate the body's attempt to neutralize the inflammatory agent and promote healing of the injured tissue (8, 13, 14).

CRP values and experimental scores were not hidden from the treating physicians to reflect actual functioning of a specific ICU. Moreover, they were mostly not intricately, nor participated in the design, purpose and methods of this study.

Length of stay

Relation of intrahospital stay to plasma CRP levels was, almost exclusively, investigated in pediatric (15, 16) and cardiosurgical patients (17), association with longer postoperative hospital stays was found in both populations.

Studies indicate a stay average of 6.1 days when care is provided by an intensivist versus 9.3 days when an attending physician provides care. This finding is consistent in medical and surgical ICUs where reductions range from 11% to 30% (18).

We found a similar range of 2 to 95 days spent in ICUs (with an average of 6.19 ± 3.74). As the minimal stay was below five days, second scoring was made on discharge for those patients being discharged alive before the 5th day (transfer to another ward, voluntary discharge). Not having the exact number of these patients could be considered the main drawback of this work.

Plasma CRP concentrations and APACHE II scores

Plasma concentrations of CRP were positively correlated with APACHE II scores in the present study, in agreement with other Authors (19). Despite contradictory studies, tendencies in CRP concentrations during ICU hospitalization are frequently used to determine whether or not further, more invasive, interventions are required. An increase in CRP levels has been described as a crucial indicator for the diagnosis of postoperative complications in surgical patients (20).

In our study, initial APACHE II score moderately correlated with plasma CRP concentrations ($r=0.289$)

which was elevated in the following days to $r=0.728$.

Plasma CRP concentrations and SOFA scores

Organ dysfunction over time was estimated based on SOFA score. This simple scoring system describes 1 to 4 points for each of the six organ systems examined (pulmonary, cardiovascular, coagulation function, hepatic, renal and neurological) (5).

SOFA score varied between 0 and 11 points on admission and 0 and 6 points on the 5th day of hospitalization. On admission, a mean SOFA score of 2.28 ± 2.25 points was found, which improved till the 5th day (1.79 ± 0.39 points) of stay. We found a significant and strong ($r=0.725$) positive correlation with plasma CRP concentrations on the 5th day.

Plasma CRP concentrations correlation to and FIM and BI scores

Among the most common scales in literature used in a general ICU are the following: BI and FIM. The BI (10) has been used to measure physical functioning and was improved upon by the FIM (11). BI proved a valid and reliable measure of ADL dependency in stroke patients, and has been recommended as 'gold standard' for other ADL rating scales and the benchmark for evaluating newer scales (12). Both scores are used for measuring nursing dependency after a certain event (12).

Proper connection between CRP concentrations and FIM score had been assessed in specialized ICUs and post-stroke wards studying the correlation between inflammatory markers and Subtypes, severity, and risk factors of ischemic stroke, i.e. predictive value of admission CRP levels for functional disability (21-23).

Average values of BI of 95.66 ± 4.3 and 97.12 ± 0.67 and FIM $118.26 \pm 12.2/121.48 \pm 8.19$ in five days, were found to be a significant improvement ($p<0.05$).

By the nature of facts, on admission, BI moderately ($r=0.291$) correlated with plasma CRP concentrations while FIM showed a low degree of correlation ($r=0.027$). On the 5th intrahospital day, BI improved to strong correlation ($r=0.792$), but FIM score remained at a low degree of correlation ($r=0.024$). This could be due the comprehensiveness of FIM test, i.e. correlation would probably be

significant if only the motor component of FIM score was considered.

In conclusion, scoring in general surgical ICUs might prove useful in assessing LOS. Plasma CRP concentrations were positively correlated with APACHE II, SOFA and BI scores in patients in general surgical ICU throughout this study, therefore this correlation on the 5th day is not sufficient to draw valid conclusion regarding intrahospital infections.

Since positive correlation from our study increased over time, further work is needed to understand this interdependence. In future studies, other inflammatory markers also need to be correlated with functional scores during hospitalization in order to draw the conclusion that scoring in ICU can be used in assessing intrahospital infections.

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LETTER TO THE EDITOR

EFFECTS OF WUZHI CAPSULE ON BLOOD CONCENTRATION OF TACROLIMUS
AFTER RENAL TRANSPLANTATION

K. WANG, QS. QU, YX. ZHANG, SZ. MIAO and X. JIANG

*Nephrology Center, People's Hospital of Zhengzhou, Zhengzhou City, Henan Province, China**Received September 10, 2015 – Accepted November 6, 2015*

This study aimed to investigate the effects of Wuzhi capsule on blood concentration of tacrolimus after renal transplantation. Sixty patients after allogenic renal transplantation were enrolled in this study and randomly divided into an experimental group and a control group. One oral Wuzhi capsule was taken in the morning and evening for patients in the experimental group, while none was given to the control group, maintaining the trough blood concentration of tacrolimus in the normal range. After 3 weeks, the changes of tacrolimus dosage and hepatorenal function in the two groups were compared. Comparisons of drug dosage and blood concentration C0 value of tacrolimus before initiating the experiment showed that there was no statistically significant difference ($P > 0.05$) between the two groups. The differences of blood concentration of tacrolimus and hepatorenal function for patients in both two groups after 3 weeks' treatment also showed no statistical significance ($P > 0.05$), whereas a statistically significant decrease was demonstrated in the tacrolimus dosage of the Wuzhi capsule group compared with that of the control group ($P = 0.0083$). After renal transplantation, Wuzhi capsules were added so as to enable tacrolimus to reach a suitable blood concentration, which can prevent the occurrence of renal transplantation rejection, thus alleviating the economic burden of patients and producing larger social benefits.

Renal transplantation rejection is a major threat to health and may be life-threatening. Immunological mechanisms are involved in transplantation rejection. Effects of traditional Chinese medicine Wuzhi capsules on the pharmacokinetics of tacrolimus have been investigated in rats by Wei et al. (1). Tacrolimus (FK506), as the first-line medicine in immunosuppressor therapy after renal transplantation, has the advantages of less side effects and strong anti-rejection effects compared to ciclosporine. However, due to the higher economic cost for the patient, applying a large dosage of tacrolimus capsules after surgery, blood concentration is unable to be kept in the normal range, thereby the occurrence rate of acute renal allograft rejection is

enhanced. Studies have shown that Wuzhi capsule, the clinical common Chinese patent medicine for liver-protection, can affect the blood concentration of FK506 (2-4). Specific to some patients in our hospital, the blood concentration of tacrolimus was enhanced by the combined use of Wuzhi capsules, which achieved a better clinical efficacy.

MATERIALS AND METHODS

Study subjects

Sixty chronic renal failure patients with allogenic renal transplantation in the People's Hospital of Zhengzhou between January in 2013 and June in 2015 were enrolled in this study. The inclusion and exclusion criteria were as

Key words: Wuzhi capsule, renal transplantation, tacrolimus concentration

Mailing address:

Dr Kai Wang,
Nephrology Center, People's Hospital of Zhengzhou,
Zhengzhou City, Henan Province, 33, Huanghe Road,
Zhengzhou City, 450000, China
e-mail: wangk1056@163.com

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described previously (1, 2). All the subjects gave informed, signed consent to participate in the study, or in the case of children or deceased, informed consent was given by the family. In this study, the donors as well as the recipients of the kidneys signed informed consent.

Among primary diseases, there were 28 cases of chronic nephritis, 15 of hypertensive kidney lesion, 9 of diabetic nephropathy, 3 of polycystic kidney and 5 with unknown pathogens. The patients were randomly divided into two groups: an experimental group (Wuzhi capsule group) of 30 subjects, 18 male, 12 female, ages 18-56 years (average 33.7 ± 10.9 years), (average weight 50.3 ± 10.7 kg) and postoperative time of renal transplantation of 4.36 ± 2.86 months; and a control group of 30 subjects, 17 male, 13 female, ages 20-57 years, (average 34.24 ± 9.76 years), (average weight of 53.40 ± 11.74 kg) and postoperative time of 4.42 ± 2.49 months. No statistically significant differences were identified in respect to age, weight and postoperative time for patients in the two groups ($P > 0.05$). The study subjects had undergone surgery 1-12 months previously, with stable renal allograft function as already described (1, 2). We measured renal function by serum creatinine. Exclusion criteria were as follows: patients with hepatitis B, hepatitis C, acute rejection, infection, abnormal glucose tolerance, posttransplantation diabetes mellitus. The drugs which may affect the blood concentration of FK506, such as diltiazem, macrolides, antifungal drugs and rifampicin were avoided during the experimental process.

Application of immunosuppressor

During the renal transplantation, 1000 mg methylprednisolone was applied in the vein, and intravenous drip of methylprednisolone in 500, 500 and 250 mg/d was administered at the 3 d after surgery. Triple immunosuppressive anti-rejection therapy with tacrolimus (Astellas Pharma Manufacturing) 1 mg, 0.5 mg/plate + mycophenolate mofetil (Roche) 0.25 g/grain + prednisone (Zhejiang Xianju Pharma) 5 mg/plate was adopted on the first day after surgery. The initial dose of tacrolimus capsule was $0.1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, taken orally two times in the morning and evening, respectively; Mycophenolate mofetil capsule was 1 g/d, taken orally two times, 0.75 g in the morning and evening, respectively, in the first month after surgery, and reduced to 0.5 g long-term oral administration in the morning and evening, respectively, in the second month after surgery; Prednisone tablet was $0.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, reducing into and maintaining at 10 mg/d within 1 month. Detecting the blood concentration of tacrolimus to regulate the dosage enabled the blood trough

concentration of tacrolimus in the ranges of therapeutic window of the corresponding period ($8\text{-}12 \mu\text{g/L}$ within 3 months after transplantation and $5\text{-}8 \mu\text{g/L}$ over 3 months). Prednisone tablet was taken $0.5 \text{ mg}/(\text{kg} \cdot \text{d})$ as initial dose, reducing to and keeping at 10 mg within 1 month.

Grouping

The enrolled cases in this study were all Han nationality, thus avoiding that CYP3A5 polymorphism may lead to the blood concentration differences of tacrolimus (5-6). The study subjects were randomly divided into two groups: (i) 30 subjects in the experimental group. After the renal transplantation, on the basis of triple immunosuppressive therapy of tacrolimus + mycophenolate mofetil + prednisone, a Wuzhi capsule (Sichuan Hygien Pharmaceutical Co., Ltd) was given in combined use. Each capsule contained 11.25 mg deoxyschizandrin, with chemical structural formula of $\text{C}_{24}\text{H}_{32}\text{O}_6$. Wuzhi capsules and tacrolimus were taken simultaneously, with 1 capsule/time and 2 times/d; (ii) 30 subjects in the control group. After the renal transplantation, triple immunosuppressive therapy of tacrolimus + mycophenolate mofetil + prednisone was administered, without using Wuzhi capsules. After the combination with Wuzhi capsules in 3-5 d, the blood trough concentration of tacrolimus started to be detected. The tacrolimus dosage was regulated in accordance with its concentration, which enabled it to stabilize in the therapeutic window. Moreover, the other therapeutic methods were the same for the two groups.

Detection of various indicators

Rapid blood concentration analyzer IMX (American Abbott Laboratories) was used to detect the concentration of tacrolimus (monoclonal antibody kit). Hitachi 7170A automatic biochemistry analyzer was employed to detect serum ALT, AST, total bilirubin (TB), direct bilirubin (DB), total plasma protein (TP), albumin (A), globulin (G), BUN, Cr and blood glucose (GLU).

Statistical analysis

SPSS 17.0 statistical software was adopted for treating all data. Quantitative data were expressed as $\bar{x} \pm s$, and *t*-test was employed for comparisons between groups. The study included an univariable and if appropriate a multivariate statistical analysis, comparing demographics and including donation characteristics, HLA-matching and CYP450 genotyping. Differences were considered statistically significant if $P < 0.05$.

RESULTS

Changes in the blood concentration C₀ value of tacrolimus

Comparisons of drug dosage and blood concentration C₀ value of tacrolimus before taking Wuzhi capsules between the Wuzhi capsule group and the control group showed that there was no statistically significant difference ($P > 0.05$). After three weeks of treatment, the differences of blood concentration of tacrolimus for patients in both two groups showed no statistical significance ($P > 0.05$), whereas a statistically significant decrease was found in the tacrolimus dosage of the Wuzhi capsule group compared with the control group ($P = 0.0083$) (Table I).

Changes in hepatorenal function

There were no significant changes in hepatorenal function before and after medication for patients in the Wuzhi capsule group ($P > 0.05$), basically keeping the original level. Compared with the control group, no significant differences were manifested in serum ALT and Cr values ($P > 0.05$) (Table I).

Other adverse effects

During the observation process, after Wuzhi capsules were used on renal transplant recipients, there was no record showing that drug-related

adverse effects had led to suspend the treatment. The blood concentration C₀ value of tacrolimus for recipients was in the ranges of therapeutic window, without medicamentous liver lesion, drug-related neurotoxicity or hyperglycemia (Table I).

DISCUSSION

Tacrolimus, a macrolide antibiotic extracted from streptomycin fermentation, has a great immunosuppressive effect. As the calcineurin inhibitor, it can be used to inhibit T lymphocyte mainly by inhibiting the release of interleukin-2 (IL-2), which is extensively administered to renal transplantation recipients in long-term. Due to the major metabolism by liver CYP3A4 isozyme, the large individual differences of bioavailability and narrow therapeutic window, many foods and drugs can have interactions with it (1). *Schisandrae sphenantherae* is a magnoliaceous plant, which has been used in traditional Chinese medicine for thousands of years. In recent years, its extract has been used in various patent medicines applied in a clinic. The Wuzhi capsule is refined from the derivatives of lignan extract from the schizandra fruit and used as cytochrome P4503A4 enzyme inhibitor, with the main component of deoxyschizandrin which gives a certain protection

Table I. Comparisons of hepatorenal function as well as concentration and dosage of tacrolimus before and after 3 weeks of medication for patients in the two groups.

	Experimental group		Control group	
	Before combined Wuzhi capsule	After combined Wuzhi capsule	Before experiment	After experiment
FK506 concentration (ng/mL)	5.34±1.21	8.47±2.53	5.19±1.03	8.23±2.78
Tacrolimus dosage (mg)	6.50±0.28	4.31±0.42	6.26±0.24	8.38±0.51
Cr (μmol/L)	146±78	149±81	150±76	156±85
ALT (U/L)	45±29	38±27	49±31	53±28
AST (U/L)	48±26	35±23	43±24	47±26

to liver cells. Deoxyschizandrin is also able to promote the synthesis of hepatic protein, speed up the regeneration of liver tissues and help mitochondria recover and increase, as well as promoting enhancement of the corresponding enzymatic activity, thereby the liver cells can be recovered and regenerated. Studies (7-8) have demonstrated that for renal transplantation recipients administered with tacrolimus as immunosuppressor, taking Wuzhi capsules at the same time significantly and safely enhanced the blood concentration of tacrolimus. Wuzhi capsules have a remarkable effect on hepatic microsome cytochrome P450 system and deoxyschizandrin expresses a dose-dependent inhibiting effect on CYP3A subfamily (9). For renal transplantation recipients taking tacrolimus with combined application of Wuzhi capsules, a statistically significant increase is shown in the blood concentration of tacrolimus. However, during increasing or decreasing the Wuzhi capsule dosage, blood concentration of tacrolimus must be strictly monitored, as well as hepatorenal function, electrolyte and blood glucose. The tacrolimus dosage is timely regulated to protect the renal transplant function and the homeostasis.

In this paper, we did not group and analyse patients considering type of donation (deceased-donor versus living donor, non-heart beating donor versus encephalic death donor), either by HLA-matching or pre-existence of preformed antibodies in the recipient, which is a major limitation. We realize that it is well known that these conditions, especially in renal transplantation, influence the subsequent graft outcome and may be skewing the renal function evaluated for which we indicated that there were no significant differences between patients receiving Wuzhi capsule and the control group.

In this study, the patients suffering from acute rejection have been excluded and are consistent with the exclusion criteria. We only evaluated patients who may develop chronic rejection, but the follow-up monitoring period was limited to that assessment because during the second half of the year post-transplantation late acute rejection and/or chronic early rejection events could occur. Furthermore, considering the following-up period limitation,

monitoring renal function exclusively through serum creatinine levels seems to be a suboptimal marker. Creatinine clearance and BUN determination levels for renal function evaluation would be more accurate. Because of limitations, we did not have reference data of control-programmed renal biopsies and the corresponding derived immunological markers.

Cut-off time for comparisons was established 3 weeks post-treatment, which were described in Table I and they were comparisons of absolute parameter means between the first three weeks post-treatment and the remaining following-up period. These comparisons were analyzed and are shown in Table I. It may be more representative and appropriate comparing global means of shorter periods and display the data as dot-plot graphs, where Tacrolimus C_0 , drug dosage and creatinine values oscillation for each group is represented throughout the monitoring period. The use of global means may not be suitable for this type of pharmacokinetic monitoring indicators, as this statistic parameter could be skewing discordant values, which is a limitation. It may be possible to perform a statistical analysis of time-series. Related to Tacrolimus metabolism, we suggest that selection of Han nationality population allows avoiding bias related to polymorphism of CYP3A5 genotypes. The CYP3A5*1 allele induces a greater metabolism of the drug while the CYP3A5*3 allele reduces the metabolism, both alleles results from a single nucleotide variation. The data provided by 1000 Genomes Project database for this variant describe a minor allele frequency around 30% in this population, which does not agree with our results in which we assume the absence of this variant in this population. Possibly, there have been genotypic determinations of CYP3A5 allele to confirm the absence of the effect of this SNP on Tacrolimus metabolism. Secondly, the CYP3A4 genotype also affects Tacrolimus metabolism, notwithstanding that no minor allele frequencies have been described in the 1000 Genomes Project for Han population for the SNP responsible of Tacrolimus metabolism inhibition. The above points are limitations. In order to verify the statement described in the paper, pharmacogenetics of CYP3A4 and CYP3A5 should be performed and included in the study because these inhibitory effects

of Tacrolimus metabolism by Wuzhi capsule could be due to differences in metabolism caused by the genetic profile of these alleles.

We did not evaluate neurotoxicity even when we carried out the longer-term glucose intolerance assessment. Wuzhi capsule has a hepatoprotective effect, so the literature included referred to the data on liver transplantation. It could be of interest to perform this study in these transplant recipients. This approach would allow to identify whether these hepatoprotective effects observed were due to the Wuzhi capsule itself or due to CYP450 Tacrolimus-metabolism influencing genotypes.

The study design involved an observation period of only three weeks, which is technically too short, substantially inadequate for evaluation both of the effectiveness and safety of the treatment, which is a limitation. The risks of using a cytochrome P4503A4 enzyme inhibitor in renal transplant population should be explored long-term.

After renal transplantation, Wuzhi capsule was added to the treatment to enable tacrolimus to reach a suitable blood concentration, which can prevent the occurrence of renal transplantation rejection. In this way, the function of transplant kidney is protected, liver function is improved and tacrolimus dose for renal transplantation recipients is reduced, further ensuring the drug safety and alleviating patients' economic burden. Post-renal transplantation combined with Wuzhi capsule may be a safe and effective approach in future for popularization and application, producing benefits and economic benefits.

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LETTER TO THE EDITOR

LIPDEMA – LACK OF EVIDENCE FOR THE INVOLVEMENT
OF TYROSINE KINASESN. SCHNEBLE¹, R. WETZKER¹ and U. WOLLINA²

¹*Institute of Molecular Cell Biology, Center for Molecular Biomedicine (CMB), University Hospital of Jena, Jena, Germany;* ²*Department of Dermatology and Allergology, Academic Teaching Hospital Dresden-Friedrichstadt, Dresden, Germany*

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Lipedema is a chronic disorder characterized by abnormal distribution of subcutaneous adipose tissue on the proximal extremities, pain and capillary fragility. Its etiology is unknown but in analogy to central obesity chronic low-level inflammation in adipose tissue has been suggested. There seems to be an increased propagation of pre-adipocytes into mature adipocytes contributing to the massive enlargement of subcutaneous adipose tissue. We investigated whether tyrosine kinases might be involved. Proteins from adipose tissue harvested during microcannular tumescent liposuction in lipedema and in lipomas were subjected to 10% polyacrylamide-gel, transferred to a polyvinylidenfluorid membrane and immunoblotted with indicated P-Tyr-100 antibody followed by enhanced chemiluminescence reaction. A survey of all blots did not reveal tyrosine-phosphorylated proteins with a molecular weight > 100 kD in lipedema tissue and controls. These investigations suggest absence of activated growth factor receptors. Some signals indicating unspecific tyrosine-phosphorylation of smaller proteins were detected in tissue of both lipedema patients and controls. The present data suggest that there is no enduring activation of tyrosine kinase pathways of adipogenesis in lipedema as in lipoma controls.

Lipedema is a chronic disease found almost exclusively in women. It is characterized by abnormal bilateral hypertrophy of subcutaneous adipose tissue with a characteristic distribution on the extremities sparing the most distal parts. Trunk and distal parts of extremities (hands, feet) are slender in the typical young patient. Pain and capillary fragility with a propensity to hematoma formation after minor trauma are the most common associated symptoms. Gait disturbances and osteoarthritis are seen later on (1). Genetic studies suggested X-linked dominant inheritance with sex limitation (2).

The pathophysiology of lipedema is not well understood. In analogy to obesity,

immunohistochemical studies of lipedema tissue demonstrated crown-like structures of necrotizing adipocytes surrounded by macrophages (CD68-positive). In addition, there was a proliferation of adipose-tissue-derived stem cells, progenitor cells, and stromal cells positive for both, Ki67 and CD34. Based on these observations, stimulated adipogenesis followed by ongoing inflammation with the recruitment of macrophages and hypoxia has been suggested as a possible mechanism (3).

Tyrosine kinases (TKs) are key players of cellular homeostasis involved in many pathways (4, 5). Adipogenesis or adipocyte differentiation is also regulated by TKs (6). Adipose-tissue-derived

Key words: lipedema, inflammation, stem cells, adipose tissue, tyrosine kinases

Mailing address:

Prof. Dr. med. Uwe Wollina,
Department of Dermatology and Allergology,
Academic Teaching Hospital Dresden-Friedrichstadt,
Friedrichstrasse 41, 01067 Dresden, Germany
Tel: +49 351 4801285 - Fax: +49 351 4801709
e-mail: wollina-uw@khdf.de

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stem cells are regulated by receptor tyrosine kinases (RTKs), including ErbB receptors and the downstream-regulated serine/threonine protein kinase B (Akt) and phosphatase and tensin homolog (PTEN) activity. Metabolic effects of activated RTKs are mediated by the modulation of Akt and extracellular signal-regulated protein kinase 1 (Erk-1) signaling. Akt activity sustains survival and adipogenic differentiation of ASCs, whereas Erk-1 appears downregulated. The inhibition of FGFR-1, EGFR and ErbB2 reduced proliferation, but only FGFR-1 inhibition reduced Akt activity and adipogenesis. Adipogenesis and neovascularization are also chronologically and spatially coupled processes and RTK activation and downstream targets are also involved in ASC-mediated angiogenesis (6).

Affecting GAS6 signaling through receptor antagonism impairs adipocyte differentiation and reduces adipose tissue development in a murine model of nutritionally-induced obesity (7). The nonreceptor tyrosine kinase Abelson murine leukemia viral oncogene (c-Abl) was found to be activated during the early phase of mouse 3T3-L1 preadipocyte differentiation (8).

We investigated whether TKs may be involved in adipose tissue remodeling in lipedema.

Patients and methods

Adipose tissue harvested during therapeutic microcannular liposuction of lipedema of legs and upper arms (n= 20) was subjected to TK analysis. We compared the results to patients with lipomatosis (n = 2).

All lipedema patients were female (mean age was range 21 to 74 years). In the control group there was one male and one female, aged 74 and 51 years, respectively. The classification of lipedema was performed according to Meier-Vollrath and Schmeller (2004): Stage I (n=1), stage II (n=8) and stage III (n=11) (9).

Liposuction was performed in tumescent anesthesia as described elsewhere (10, 11). Lipid tissue was stored at -80°C and thawed at 37°C in a water bath before starting the assay of tyrosine-phosphorylated proteins. Lipid tissue suspension was homogenized by vortexing. Afterwards 5 ml of

the homogenized suspension were transferred into a new 15 ml falcon and 5 ml dissociation solution was added. Dissociation solution contained 5 ml 1x PBS buffer (100 mM NaCl; 20 mM NaH₂PO₄*H₂O; 80 mM Na₂HPO₄*2H₂O) and 5 mg collagenase (collagenase type 1, N° C1-22, Biochrom, Berlin, Germany). The mixture was incubated for 30 min at 37°C in a water bath and inverted 2 to 3 times in the meantime. Following incubation digested tissue was filtered through a 250-µm cell strainer (B&D, Franklin Lakes, New Jersey, USA). Suspension was concentrated (Amicon® Ultra Centrifugal Filters, 10 kD, Merck Millipore, Darmstadt, Germany), 5 x sample buffer (5% SDS, 33% glycerol, 25% -mercaptoethanol) was added and samples were heated to 95°C for 5 min. Protein samples were separated on 10% polyacrylamide-gel, transferred to a polyvinylidenfluorid membrane and immunoblotted with indicated P-Tyr-100 antibody (#9411, Cell Signaling Technology, Danvers, MA, USA) followed by enhanced chemiluminescence reaction (12).

A survey of all blots did not reveal tyrosine-phosphorylated proteins with a molecular weight > 100 kD in lipedema tissue and controls. These investigations suggest absence of activated growth factor receptors. Some signals indicating unspecific tyrosine-phosphorylation of smaller proteins were detected in tissue of both lipedema patients and controls.

DISCUSSION

Liposuction removes primarily intact adipocytes and shows a low content of vasculature and other tissue components (13). Adipose tissue-derived stem cells are found in both the fatty and liquid part of liposuction aspirates (14). Tyrosine kinases are involved in the regulation of stem cell transformation into mature adipocytes (6-8).

The present data suggest that there is no enduring activation of tyrosine kinase pathways of adipogenesis in lipedema as in lipoma controls. Lipomas are characterized by massive enlarged adipocytes in contrast to lipedema with moderate hyperplasia. In lipomas an accelerated adipogenesis

with non-enhanced apoptosis was demonstrated by immunohistochemistry and real-time polymerase chain reaction (15). These authors found an upregulation of leptin but downregulation of adiponectin, tumor necrosis factor- α , and glucose transporter 1 mRNA.

Lipematous adipocytes are not uniform in size as in normal adipose tissue. Ki67 staining argues for a higher proliferative activity of CD34-positive adipocyte progenitors/stem cells but not mature adipocytes (3).

Adipose tissue-derived stem cells are multipotent and capable of secreting angiogenic growth factors. Increased proliferation of these stem cells is in one line with increased adipogenesis as a key factor for massive enlargement of subcutaneous adipose tissue in lipedema. Lipoaspirates from wet liposuction – as in our case – contain less stem cells than dry lipoaspirates (16).

The lack of evidence for TK activation in lipedema suggests that other pathways might be involved in uncontrolled subcutaneous adipose tissue enlargement. Further studies are needed.

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LETTER TO THE EDITOR

EXPRESSION PROFILES OF SMAD1 PROTEIN IN LUNG CANCER TISSUES AND NORMAL TISSUES AND ITS EFFECT ON LUNG CANCER INCIDENCEYQ. GAO¹, M. LIU² and H. ZHANG¹

¹*Respiratory Intensive Care Unit, Zhengzhou Central Hospital Affiliated to Zhengzhou University, Zhengzhou city, PR China;* ²*Cardiovascular internal medicine, Zhengzhou Central Hospital Affiliated to Zhengzhou University, Zhengzhou city, PR China*

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The aim of this study is to detect the expression profiles of Smad1 protein in lung cancer tissues and normal tissues to investigate its effect on the incidence of lung cancer. The expression profiles of Smad1 protein in 60 cases of lung cancer tissues (lung cancer group), 25 cases of normal alveolus tissues (alveolus control group) and 29 cases of normal bronchial tissues (bronchial control group) were detected by adopting immunohistochemical analyses, and their relationships with clinicopathologic data were analyzed. The expression of Smad1 protein in the lung cancer group and the lung squamous cell carcinoma group was significantly lower than that in the alveolus control group and the bronchial control group ($P < 0.01$). The expression of Smad1 protein in the lung adenocarcinoma group was significantly lower than that in the alveolus control group and the bronchial control group ($P < 0.01$); The expression Smad1 protein showed a significant correlation with lung cancer differentiation and lymphatic metastasis ($P < 0.05$), but not with genders, ages, tumor sizes and histological types of lung cancer patients ($P > 0.05$).

Lung cancer is a malignant tumor of the respiratory system, severely threatening human health. The incidence and mortality rates of lung cancer are ranked at the top of the malignant tumors (1, 2). At present, it is known that certain factors, such as smoking, heredity, occupational exposure and air pollution are associated with the incidence of lung cancer, but the mechanism remains to be investigated.

Smad is a cytoplasm transmitter of transforming growth factor β (TGF- β), playing an important role in the TGF β /Smad signal transduction pathway (3). It has been reported that the deficiency or over-expression of Smads has a close relationship with the incidence of various malignant tumors (4, 5). To study the expression profile of Smad protein

in lung cancer may be of crucial significance in revealing the incidence of lung cancer. This study, through immunohistochemistry, aimed to detect the expression profiles of Smad1 protein in lung cancer tissues and normal tissues to investigate its effect on lung cancer incidence.

MATERIALS AND METHODS

Sixty patients with lung cancer (without chemotherapy or radiotherapy prior to surgery) who received operative treatment in Zhengzhou Central Hospital Affiliated to Zhengzhou University between June 2011 and June 2014, were enrolled in the study. The subjects were 41 males and 19 females, with ages ranging from 42 to 81

Key words: lung tumor; alveolus; bronchia, Smad1 protein, immunohistochemistry

Mailing address:

Dr Yanqiu Gao,
Zhengzhou Central Hospital Affiliated to Zhengzhou
University, No. 195, Tongbai Road,
Zhengzhou, 450007, PR China
e-mail: gaoyanqiu1135@163.com

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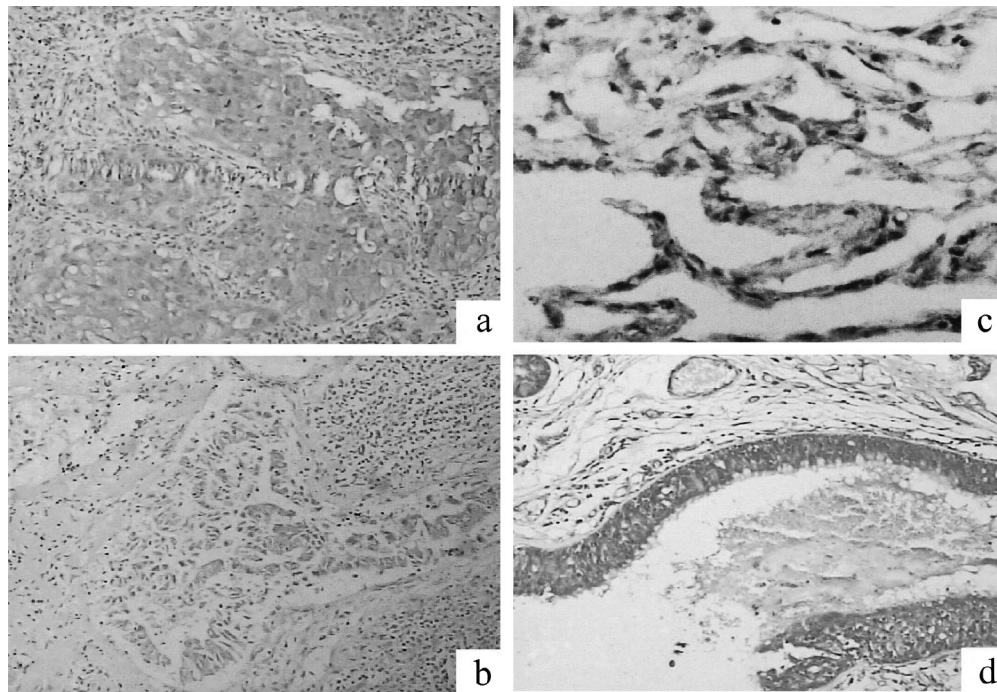


Fig. 1. Weakly positive and Strongly positive expressions of Smad1 protein (SP, $\times 100$). **a)** Lung squamous cell carcinoma in poor differentiation. **b)** Lung adenocarcinoma in high differentiation. **c)** Alveolus tissues. **d)** Bronchial tissues.

years (average 63.57 ± 9.06) years. All the subjects gave written informed consent to participate in the study. In the lung cancer specimens from these patients, 33 cases were with squamous-cell carcinoma and 27 cases with adenocarcinoma. At the same time, 17 cases were in poor differentiation and 43 cases were in moderate and high differentiation. Moreover, 26 cases were accompanied with peripheral lymphatic metastasis and 34 cases were not. Twenty-nine cases with normal bronchial tissues [20 males, 9 females, ages ranging from 48 years to 78 years (average 63.19 ± 7.58) years] were enrolled as the bronchial control group and 25 cases with normal alveolus tissues [18 males, 7 females, ages ranging from 44 years to 68 years (average 56.32 ± 6.16) years] were taken as the alveolus control group. The samples in these two control groups were selected from the alveolus of lung cancer ipsilateral distal and the bronchial normal tissues. All samples were diagnosed and confirmed by 2 senior pathologists. The study was approved by the Ethics Committee of Zhengzhou Central Hospital Affiliated to Zhengzhou University.

Rabbit anti-human Smad1 protein (A-4) monoclonal antibody (sc-7965) was purchased from America Santa Cruz Biotechnology, Inc; Immunohistochemistry kits

and concentrated DAB kit were purchased from Beijing ZSGB-BIO Co., Ltd.

All samples were fixed with 10% formaldehyde solution, routine paraffin was used to embed sections and HE staining was performed for pathological observation. Tumor grades referred to the 2010 WHO lung cancer histologic criteria.

Immunohistochemical assay

Staining was conducted by adopting SP immunohistochemistry and repairing was conducted by citrate buffer solution boiling, in accordance with the manufacturer's instruction. DAB was developed and hematoxylin was stained. The known Smad1 protein positive sections were considered as positive control and PBS solution instead of primary antibody was negative control.

Results determination

Results were observed by microscope adopting the double-blind method. If the results were not in conformity, they were finally determined by another researcher. Smad1 protein was the cell cytoplasm staining. Brown yellow cytoplasm was regarded as positive and no staining was regarded as negative. Positive determination was

comprehensively decided by positive cell number and staining intensity. Percentage scoring of positive cells was defined as the ratio of positive cells accounting for total cell number: that with no larger than 5% was 0 point, that ranging from 6% to 25% was 1 point, that ranging from 26% to 50% was 2 points, and that with no less than 51% was 3 points; staining intensity was determined as no-staining = 0 point, canary yellow = 1 point, brown yellow = 2 points, and sepia = 3 points (6). Five 400 - time visions of every section were randomly selected. With two combinations, that with no less than 3 points was negative and that larger than 3 was positive.

Statistical analysis

SPSS 16.0 statistical software was applied and χ^2 test was adopted. $P < 0.05$ was considered as significant difference.

RESULTS

Comparison of Smad1 protein expression

Smad1 protein appeared grayish yellow in the

cytoplasm of the lung cancer cells, expressing weakly positive (Fig. 1a). Cytoplasm appeared gold brown yellow in the alveolar cells and the bronchial epithelial cells (Fig. 1b). The expression of Smad1 protein in the lung cancer group, the lung squamous cell carcinoma group and the lung adenocarcinoma group was significantly lower than that in the alveolus control group and the bronchial control group ($P < 0.01$) (Tables I, II and III). Grouped pairwise comparisons showed that the expression of Smad1 protein had no statistically significant difference between the alveolus control group and the bronchial control group ($\chi^2 = 0.006$, $P = 0.940$).

Relationship between the expression of Smad1 protein in lung cancer and the clinicopathologic data

Expression of Smad1 protein in the lung cancer group was significantly correlated with differentiation degrees, and its positive expression in the poor differentiation group was higher than that in the moderate and high differentiation group ($P < 0.05$); Expression of Smad1 protein had a significant

Table I. Expression profile of Smad1 protein in lung cancer group

Group	Cases	Positive expression of Smad1 protein	χ^2 value	P value
Lung cancer group	60	13 (21.67)		
Alveolus control group	25	24 (96.00)*	68.287	<0.001
Bronchial control group	29	29 (100.00)*		

* Compared with lung carcinoma group, $P < 0.01$

Table II. Expression profile of Smad1 protein in lung squamous cell carcinoma group and two control groups [cases (%)].

Group	Cases	Positive expression of Smad1 protein	χ^2 value	P value
Lung squamous cell carcinoma group	33	7 (21.21)		
Alveolus control group	25	24 (96.00)*	56.747	<0.001
Bronchial control group	29	29 (100.00)*		

* Compared with lung squamous cell carcinoma group, $P < 0.01$

correlation with the lymphatic metastasis and its positive expression in those without lymphatic metastasis was remarkably higher than that in those with lymphatic metastasis ($P < 0.05$). However, the expression of Smad1 protein was not relative to the patients' genders, ages, tumor size or histological types of lung cancer ($P > 0.05$) (Table IV).

DISCUSSION

Smads are newly discovered proteins in the screening process of drosophila genes, with molecular weight ranging from 45 to 70 kD. To date, 8 kinds of Smad proteins have been discovered, and they are divided into three categories in accordance with function and structure, that is, receptor regulated Smad protein (Smad1, Smad2, Smad3, Smad5 and Smad8), general regulated Smad protein (Smad4) and inhibitory protein (Smad 6 and Smad7). Studies have found that Smad protein in different types may play different roles in the incidence of various malignant tumors. Studies by Zhang et al. (7) showed that Smad4 protein was related to the development and metastasis of colorectal cancer cells of mice. Shen et al. (8) pointed that Smad2 protein may be related to the infiltration and metastasis of lung cancer. At present, studies on lung cancer and Smad mostly focused on Smad2, Smad3 and Smad 4 protein (9, 10), whereas, studies on the correlation between Smad1 protein and Lung cancer tissues are rare.

The lung cancer samples in this study, mainly including squamous cell carcinoma and adenocarcinoma, are the common pathological type of non-small cell lung cancer, usually occurring in the epithelial cells of bronchus mucous membrane

and rarely in the epithelial cells of alveolus. Therefore, the bronchial and alveolus normal control groups were particularly chosen in order to analyze the profile of Smad1 protein in grouped expression, thus enhancing the reliability of the research data. The results indicated that the expression of Smad1 protein in the lung cancer group was significantly lower than that in the alveolus control group and the bronchial control group ($P < 0.01$), suggesting that the expression of Smad1 protein was suppressed in the occurrence of lung cancer, which probably played a role similar to that of the cancer suppression factor. Smad1 protein showed a poor differentiation in the lung squamous cell carcinoma group and the lung adenocarcinoma group, which was remarkably lower than that in the alveolus control group and the bronchial control group ($P < 0.01$), suggesting that Smad1 protein, as a cancer suppression factor, probably had the same or similar action mechanism in the occurrence process of the two types of lung cancer. Yang et al. (11) found that the expressions of Smad1/2/3 protein and p-Smad2/3 in seborrheic keratosis, basal cell carcinoma and squamous cell carcinoma had a significant reduction compared to those in the normal epidermis, beneficial to the occurrence of the above diseases. Studies by Zhang (12) found that positive expressions of Smad1 protein and Smad5 protein were able to suppress the production of tongue cancer cells. Kim et al. (13) found that the expressions of Smad1/2/3, Smad2/3 and Smad4 protein were enhanced in the disease tissues before gastric carcinoma, reduced in the gastric carcinoma tissues and closely relative to the early stage of gastric carcinoma development. The results of the above mentioned studies were similar

Table III. Expression profile of Smad1 protein in lung adenocarcinoma group and two control groups [cases (%)].

Group	Cases	Positive expression of Smad1 protein	χ^2 value	P value
Lung adenocarcinoma group	27	6 (22.22)		
Alveolus control group			52.559	<0.001
Bronchial control group	25	24 (96.00)*		
	29	29 (100.00)*		

* Compared with lung carcinoma group, $P < 0.01$

to those in this study, which further confirmed the cancer suppressor factor role of Smad1 protein in the various malignant tumors. Whereas, the action mechanism in detail was still unclear, therefore worth in-depth studying.

The expression characteristics of Smad1 protein in different clinicopathological factors were analyzed by applying the immunohistochemical method, showing crucial significance for judging the local biologic behavior, relapse and prognosis of

Table IV. Relationship between the expression of Smad1 protein in lung cancer and the clinicopathologic data [case (%)].

Clinicopathological data	Cases	Positive expression of Smad1 protein	χ^2 value	P value
Genders			0.000	1.000
Male	41	9 (21.95)		
Female	19	4 (21.05)		
Ages			3.711	0.054
≤ 60	32	10 (31.25)		
> 60	28	3 (10.71)		
Maximum diameter of tumor			0.227	0.633
≤ 3 cm	38	7 (18.42)		
> 3 cm	22	6 (27.27)		
Differentiated degree			11.220	0.001
Poor differentiation	17	9 (52.94)		
Moderate and high differentiation	43	4 (9.30)		
Histological type				
Squamous cell carcinoma			0.009	0.925
Adenocarcinoma	33	7 (21.21)		
Lymphatic metastasis	27	6 (22.22)		
Yes			5.279	0.022
No	34	11 (32.35)		
	26	2 (7.69)		

lung cancer. The results in this study demonstrated that the expression of Smad1 protein in the lung cancer tissues has a significant correlation with the differentiation degree and lymphatic metastasis of lung cancer ($P < 0.05$), but not with the genders, ages, tumor size or histological types of lung cancer patients. The expression of Smad1 protein in the poorly differentiated lung cancer group was distinctly higher than that in the moderately and the highly differentiated group ($P < 0.01$), suggesting that the expression of Smad1 protein was related to the malignant degrees of lung cancer. Therefore, detection in the expression profile of Smad1 protein presented a certain value in the efficacy prediction and prognosis for lung cancer. The expression of Smad1 protein in group without lymphatic metastasis was significantly higher than that in the group with lymphatic group ($P < 0.05$), suggesting that Smad1 protein was related to the infiltration and metastasis of lung cancer, which immediately played anti-tumor effect on the early lung cancer. Moreover, its anti-tumor effect was weakened with the progress of cancer. However, suppressed expression of Smad1 protein in the group with lymphatic metastasis further confirmed its cancer suppressor factor identity, and regulating it could be used in the study of lung cancer treatment. Chen et al. (14) pointed out in their study that the expression of Smad1 protein was relative to the infiltration, movement and attachment of lung adenocarcinoma and squamous cell carcinoma cells, which was consistent with the results in this study. Another study indicated that both Smad2 and Smad3 were related to the metastasis of lung adenocarcinoma, suggesting that receptor-regulated Smad protein played roles in lung cancer metastasis probably by the similar signal pathway (15-17).

A greater number of samples should be collected to further confirm the findings, which was a limitation of the study because of the extreme difficulty to collect more samples. Furthermore, we are also in accordance on the point that other IHC markers should be analyzed, such as smad 5, smad 2, P38 MAPK, TGF- β , endoglin and bone morphogenetic protein 7, which was another limitation of this study because of the same reason. IHC staining is not a good quantitative measurement. We tried our best to

confirm our conclusion by using Western blot and PCR. However, it was impossible to exclude the inferences of other tissues or samples, especially blood. Therefore, we believe that Western blot and PCR has more limitations.

In conclusion, the expression of Smad1 protein in the lung cancer group was significantly lower than that in the bronchial control group and the alveolus control group and related to the differentiation degree and the lymphatic metastasis of lung cancer.

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LETTER TO THE EDITOR

TOTAL HIP REPLACEMENT FOR DEVELOPMENTAL DYSPLASIA OF HIP AND POSTOPERATIVE NURSING

SJ. ZONG, F. WANG and SL. HU

*Department of Orthopedics, People's Hospital of Zhengzhou, Zhengzhou, Henan, China**Received August 12, 2015 – Accepted January 14, 2015*

This study was designed to determine the clinical effect of total hip replacement for the treatment of developmental dysplasia of the hip (DDH) and analyze the postoperative nursing. Sixty patients (78 hips) aged 18-75 years (average 58.6 ± 2.31 years) who received total hip replacement for treatment of DDH at the Zhengzhou People's Hospital, Henan, China, from April 2013 to June 2015 were selected as research subjects. Twenty-four patients were male (30 hips) and 36 were female (48 hips). Of the 60 patients, according to Crowe typing, 24 were type I (30 hips), 26 were type II (34 hips), 6 were type III (8 hips) and 4 were type IV (6 hips). According to the Harris hip score system, the score of all hips was 39.46 ± 3.56 points average (18-56 points) before treatment and resulted as 89.60 ± 4.25 points (79-98 points) at the last follow-up, showing a statistically significant difference ($P < 0.05$). Complications such as wound infection, dislocation, fracture of femoral shaft, femoral nerve and injury of sciatic nerve were not found after treatment. A total of 48 cases (58 hips) obtained excellent curative results (93.33% recovery), 8 cases (14 hips) good (92.31% recovery), and 4 cases (6 hips) medium. Total hip replacement proved to be effective in treating DDH and secondary osteoarthritis. Moreover, soft tissue release and an optimum degree recovery of anatomic form and physiological function of the diseased hip is an important basis for reconstructing the acetabulum and stabilizing acetabulum prosthesis.

Developmental dysplasia of the hip (DDH) includes a series of related diseases such as hip instability, subluxation, dislocation and acetabular dysplasia. Many patients act normally in childhood but gradually develop subluxation with age, and some are likely to evolve to complete dislocation if no treatment is adopted (1, 2), whilst others may develop osteoarthritis of the hip in the young and middle aged stage which represents as fatigue, pain after walking for a length of time and claudication. Hip osteoarthritis develops rapidly and in severe cases causes great pain, which leads to many patients being unable to work.

Adult hip developmental dysplasia is usually

induced by misdiagnosis, or by omitted, delayed or improper treatment in childhood (3, 4). The patient often refers to a hospital for diagnosis and treatment only when the disease has worsened become severe joint hip osteoarthritis (5). Over time, aggravating arthropathy induces severe arthralgia and dysfunction, which influence normal work and quality of life of the patients, hence needing treatment for adult hip developmental dysplasia. As technique and design of artificial prostheses and surgical technology improves, total hip replacement has become an effective method for treating adult hip developmental dysplasia (6). This operation can greatly enhance joint movement, eliminate pain,

Key words: total hip replacement, developmental dysplasia of hip, bone transplantation, computed tomography, X-ray

Mailing address:

Ms Shujun Zong
No.33, Huanghe Road,
Department of Orthopedics, People's Hospital of Zhengzhou
Zhengzhou, Henan, 450000, China
e-mail: zongsj3823@163.com

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improving the patient's lifestyle and work. Great importance must be given to hip replacement in adults with severe DDH due to the great difficulty of the operation, large amount of bleeding, and high incidence of postoperative complications (7).

This study analyzed the clinical effects of 60 cases with Harris hip score system and discusses the application of total hip replacement in treating dysplasia of the hip joint as well as its impact on postoperative nursing.

MATERIALS AND METHODS

General data

Sixty adults (78 hips) who received total hip replacement for treating dysplasia of the hip joint in Zhengzhou People's Hospital, Henan, China, between April 2013 and June 2015 were selected as research subjects.

Patients aged between 18 and 75 years, who had developed hip pain and functional activity disorder, had scored less than 70 points by Harris scoring, had been examined for osteoarthritis by X-ray, had undergone coordinated treatment, and had complete follow-up data were included in the study. All participating subjects signed an informed consent.

Those who were found with systematic or local infection, who could not undergo treatment due to severe internal medical diseases, had incomplete follow-up data or refused to sign informed consent were excluded.

Selection of prosthesis

The diameter of the acetabular prostheses used in this study ranged from 37 mm to 60 mm. Except for 6 hips which applied small acetabular prosthesis and 8 hips with complete acetabular structure, the other hips all underwent bone transplantation: 52 hips for particulate bone graft, 8 hips for structural bone graft and 4 hips for mixed bone graft (particulate bone graft in combination with structural bone graft).

Selection principles of the prostheses were: for patients who were young and had good bone quality or who could achieve initial stability, uncemented prostheses were selected; while those who were elderly and had osteoporosis or who could not achieve initial stability, cemented prostheses were chosen.

Preoperative preparation

Patients received comprehensive and systematic examination after being admitted to hospital. Complications such as heart disease, hypertension and diabetes were positively controlled and treated. Length of double limbs and length after traction were measured to confirm the extended length of limbs during surgery and provide evidence for confirming bone cutting plane. Normotopia bilateral hips and side position of upper femora were X-rayed. Patients with severely abnormal acetabulum also underwent CT scanning and even three-dimensional reconstruction to confirm the severity of bone defect as well as size and depth of acetabulum. The position of acetabulum was confirmed using Ranawat triangle. (10-12). The position of acetabular prosthesis was determined by comparing the affected hip and healthy hip by applying template technology, and the size and rotation center of the prosthesis was confirmed based on the anteroposterior diameter of the acetabulum. Moreover, the size of femoral prosthesis, cutting site of femoral neck and length of femoral head prosthesis were calculated. One to two days before treatment, patients were given antibiotics. One day before surgery, skin preparation was carried out and the anesthetists visited all patients to evaluate their tolerance to surgery. No food was allowed eight hours prior to surgery, and no liquid for six hours before treatment.

All operations were performed using the hip joint posterior-lateral approach.

Postoperative medication

Patients were given antibiotics for days to prevent infection after operation, injected with 5,000 units low-molecular-weight heparin for 6 days, and meanwhile took traditional Chinese medicines which are effective in activating blood, removing stasis, inducing diuresis and alleviating edema to prevent deep venous thrombosis (13). The affected limb was fixed with elastic bandage to promote vein and lymphatic return in lower limb and prevent edema of the lower extremity. After treatment, changes of hemoglobin were closely monitored; patients were supplemented with blood (8) and took oral non-steroidal anti-inflammatory and analgesic drugs (9).

Observation method

Supplementary examinations were carried out

before surgery. History of disease, symptoms and signs before treatment, especially joint pain, malformation, joint function, muscle force and movement scope, were recorded in detail. The follow-up lasted for 6 to 18 months (average 13.1 ± 0.84 months) and was carried out by regular re-examination and by telephone. Changes of symptoms, signs and X-ray results were also recorded.

Clinical evaluation method

Evaluation was based on clinical effects and imaging results. Replacement of the hip was evaluated by the Harris scoring system (Table I) which evaluates the aspects of pain, functional activities, improvement of limb malformation and movement range of limbs. The score is divided into four levels, excellent (90~100 points), good (80~89 points), medium (70~79 points) and poor (below 70 points). If the Harris score had an increase lower than 20 points or cases required revision surgery after treatment, then the treatment was determined as having failed.

Statistical analysis

SPSS 17.0 software was used to process data. Statistical data were analyzed by *t*-test. All data were expressed as mean $\pm$ SD. Difference was considered to be statistically significant if $P < 0.05$.

RESULTS

All 60 cases (78 hips) were successively followed-up for 6 to 18 months (average 13.1 ± 0.84 months). Harris score before treatment was 39.46 ± 3.56 (average 18~56 points), and the score resulted as 89.60 ± 4.25 points (average 79~98 points) at the last follow-up, showing a statistically significant improvement compared to before treatment ($P < 0.05$). Complications such as wound infection, dislocation, femoral shaft fractures and injury of ischiadic nerve were not observed after treatment. Moreover, hips were without pain and had an improved stability after treatment. Patients were able to walk by themselves without walking sticks. Lower limb malformation was remarkably improved and even disappeared. X-rays demonstrated that acetabular and femoral prostheses were located in correct positions, without loosening or infection, and the bone grafting site healed well. Our results showed that 48 cases (58 hips) obtained

excellent curative effect, 8 cases (14 hips) obtained good effect and 4 cases (6 hips) had medium effect. The excellent and good rate reached 93.3% (92.31%). Furthermore, no heterotopic ossifications were observed after analyzing X-rays of normotopia hips with Brooker heterotopic ossification grading standard. Detailed data are shown in Table II.

Case analysis

Case 1 was a 54-year-old female who developed DDH (Crowe level II) on the left side (0.5 cm shortening) and had a preoperative Harris score of 44 points. CT confirmed poor containment of acetabulum (Fig. 1A) before surgery. The length of double lower limbs was basically the same after acetabulum was deepened during surgery (Fig. 1B). In a 5-month follow up after surgery, no bright zone was found by X-ray (Fig. 1C), position and stability of prosthesis were both in good state and Harris score reached 95 points.

Case 2 was a 56-year-old female who developed DDH (Crowe level II) and osteoarthritis on the right side and had a Harris score of 46 points. CT confirmed abnormal hypertrophic femoral head and obvious osteoarthritis (Fig. 2A) before treatment; Harris score was 84 points after structural bone graft (Fig. 2B) and 91 points one year after treatment (Fig. 2C).

Case 3 was a 63-year-old male who developed DDH (Crowe level III) and had secondary osteoarthritis on the left side for several years, and had a Harris score of 21 points. Before treatment, CT confirmed upward displacement, abnormally hypertrophic femoral head and thickened acetabular bottom (Fig. 3A). Harris score was 87 points after he received total hip replacement on the left side (Fig. 3B). Six months after treatment, the prosthesis showed good stability and Harris score resulted as 92 points (Fig. 3C).

DISCUSSION

Surgical indications

To prevent revision surgery, total hip replacement is not recommended to be performed on young

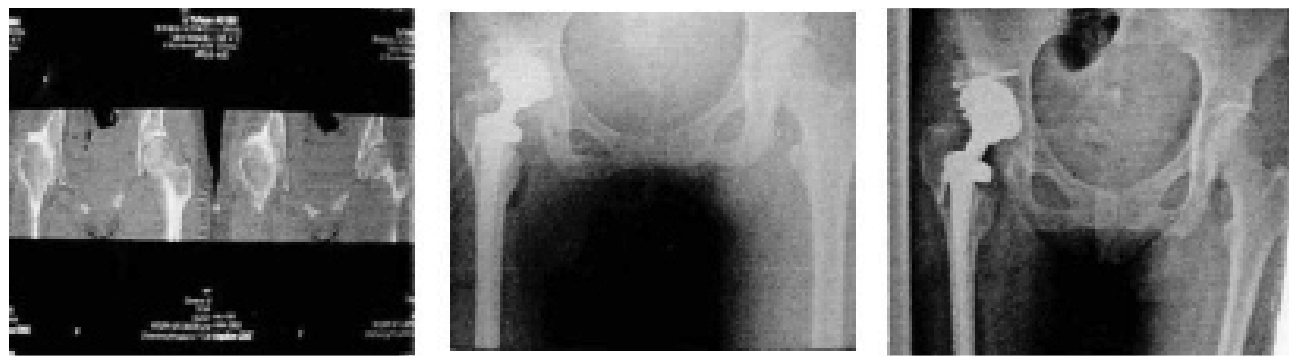
Table I. Harris scoring criteria for curative effect of hip replacem

Pain degree (44 points)		Performance	Score
No	No pain or pain that can be ignored		44
Weak	Sometimes painful, but the pain has no influence on activity		40
Mild	Mild pain which has no influence on daily life; pain becomes medium after excessive activity and needs to be relieved with aspirin		30
Medium	Pain that can be tolerated but limits daily activity; aspirin needs to be taken		20
Intensive	Severely restricted activity		10
Disable	Intensive pain even in bed; claudication; bedridden for long periods		0
Function (47 points)		Daily life	Score
Stairs	One step for one stair, without help of handrail		4
	One step for one stair, with help of handrail		2
	Able to go upstairs by some means		1
	Unable to go upstairs		0
Traffic	Able to enter public transport		1
Sitting	Able to sit on any chairs, without uncomfortableness		5
	Able to sit on high chair, without uncomfortableness		3
	Feel uncomfortable on all chairs		0
Footgear	Convenient to wear socks and shoes		4
	Has difficulty in wearing socks and shoes		2
	Unable to wear socks and shoes		0
Gait	Without claudication		11
	Mild claudication		8
	Medium claudication		5
	Severe claudication		0
Walking assistor	In no need		11
	Need single walking cane in long-distance walking		7
	Need single walking cane most of the time		5
	Need single walking stick		3
	Need double walking canes		2
	Need double walking sticks		0
	Unable to walk		0
Walking distance	Unlimited		11
	Six blocks		8
	Two or three blocks		5
	Indoor activity		2
	Lie in bed or sit on chair		0
Malformation	Without the following malformation		Score
	A Fixed contracture deformity < 30		4
	B Fixed adduction deformity < 10		
	C Fixed extension adduction deformity < 10		
	D Limb shortening < 3.2		
Range of activity (5 points)	Total score is the product of sum of all index values and 0.5		
	Flexion		
	Abduction		
	Extension extorsion		
	Extension intorsion		

Table II. Statistical table of patients' information. (mean $\pm$ SD)

Item	Level I	Level II	Level III	Level IV	Average value
Number of cases	24 cases (30 hips)	26 cases (34 hips)	6 cases (8 hips)	4 cases (6 hips)	-
Average age	54.3 $\pm$ 1.28	61.5 $\pm$ 2.54	62.1 $\pm$ 1.97	59.6 $\pm$ 3.54	58.6 $\pm$ 2.31
Follow-up duration	12.1 $\pm$ 1.04	13.9 $\pm$ 0.97	13.5 $\pm$ 0.65	12.7 $\pm$ 0.58	13.1 $\pm$ 0.84
Harris score before treatment	48.26 $\pm$ 2.13	37.54 $\pm$ 3.45	26.24 $\pm$ 3.17	18.95 $\pm$ 2.64	39.46 $\pm$ 3.56
Harris score after treatment	92.21 $\pm$ 4.28*	90.09 $\pm$ 5.34*	86.34 $\pm$ 4.58*	75.63 $\pm$ 2.98*	89.60 $\pm$ 4.25*

(mean $\pm$ SD)

**Fig. 1.** Female, 54 years, left DDH, Crowe II.**Fig. 2.** Female, 56 years, right DDH, Crowe II.**Fig. 3.** Male, 63 years, left DDH combined with secondary osteoarthritis, Crowe III.

patients too early. Advantages and disadvantages of total hip replacement and other methods which can extend the service life of the hip joint should be carefully evaluated. Methods that are effective in extending the service life of the hip joint include rest, weight loss, walking with double sticks, oral administration of non-steroidal anti-inflammatory and analgesic drugs (14) and physical therapy. Treatment for secondary osteoarthritis occurring to the hip joint aims at changing the pathological environment (15) and delaying progress of secondary osteoarthritis.

Acetabulum reconstruction

Acetabulum reconstruction is the key of joint replacement. For patients with DDH, acetabular prosthesis is stabilized by deepening the acetabulum. However, true acetabulum of patients with DDH was elliptical (thin bone in the middle of acetabulum and thickened bone in the posterosuperior quadrant), which provides an important security for placement of acetabular prosthesis in the middle of the posterosuperior quadrant of the real acetabulum (16). Hence, acetabulum is suggested to be reconstructed in the position of real acetabulum, which is beneficial for recovering body length and normal rotation center of the hip joint, reducing contact capacity of the joint, improving function of the abductor, correcting claudication and avoiding accelerated wear of the prosthesis in an unphysiological state. Confirmation of the rotation center before surgery can make surgery smoother. After the acetabular prosthesis is inserted, the edge of the prosthesis is allowed exposure. Stability of the prosthesis will not be impacted if the uncovered area of acetabular cup is no more than 30%; but if it exceeds 30%, then application of bone grafting and shelf operation can be considered, but the area of acetabular cup covered by grafted bone should be lower than 30% ~ 40% (17). It has been proved that bone cement has no obvious influence on the grafting effect of autologous bone graft and allograft, therefore, the uncemented press-fit method is suggested to fix the acetabular cup after the acetabular defect is repaired with autologous bone graft or allograft. It is correct to fix with bone cement if 40% or more of the acetabular cup area is covered by grafted bone (18, 19).

Femur reconstruction

Patients whose femur grows in an approximately normal state can be treated with conventional femur replacement. But problems such as increased collodiaphyseal angle and anteversion angle, shortened and thickened femoral neck, narrow proximal medullary space of femur and double lower limbs in different length complicates total hip replacement. For those patients, femoral anteversion angle and absolute length of double lower limbs can be measured with the help of imaging data, thus avoiding different lengths of the double lower limbs. During surgery, adjustment of the anteversion angle of DDH patients required attention, as excessive anteversion of the femoral neck usually produces misleading effects. Therefore, the prosthesis cannot be inserted according to the previous anteversion angle, but should be inserted in a neutral position or slightly anteverted position based on 15° anteversion angle confirmed by taking the frontal plane of the femoral shaft or knee joint as reference.

Extension of limbs varies from person to person. For patients with 4 cm or more more dislocation, osteotomy of the femur is suggested by most scholars. Osteotomy of the femur can be typed into subtrochanteric osteotomy and distal femoral osteotomy. For patients with severe dislocation, skeletal traction through the supracondylar femur can be performed before surgery. Femur reconstruction in the position of true acetabulum is not difficult to carry out and lowers the risks induced by femoral osteotomy and forced traction.

To sum up, total hip replacement can remove degenerative changes of the hip joint, recover normal matching correlation of the hip joint, eliminate pain, partially or completely restore the length of the affected limb, and relieve claudication, thus greatly enhancing the quality of life of patients with DDH. Therefore, total hip replacement is considered as an ideal choice.

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LETTER TO THE EDITOR

RELATIONSHIP BETWEEN SERUM INTERLEUKIN-17 LEVEL AND
INFLAMMATORY BOWEL DISEASEQL. LIU¹, L. HUANG², QJ. ZHAO³, Q. LI⁴ and Z. HE⁵¹Department of Clinical Laboratory, Tangshan Fengrun District People's Hospital, China;²Department of Clinical Laboratory, The Fifth People's Hospital of Chengdu, Chengdu, China;³Department of Clinical Laboratory, The Central Hospital of Baoji, Baoji, China; ⁴Clinical Laboratory Center, Xiyuan Hospital, China Academy of Chinese Medical Sciences, Beijing, China;⁵Beijing Center for Physical and Chemical Analysis, Beijing, China

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The first two authors contributed equally to this paper

By detecting expression of interleukin (IL)-17A, IL-10 and interferon- γ (IFN- γ) in serum of inflammatory bowel disease (IBD) patients, this study aims to analyze the effects of these factors on the pathogenesis of IBD. According to illness status, selected patients were divided into Crohn's disease (CD) group (28 patients), ulcerative colitis (UC) group (74 patients) and normal control group (36 patients); enzyme-linked immunosorbent assay (ELISA) was used to detect IL-17A, IL-10 and IFN- γ levels in serum; immunohistochemical assay was used to detect local IL-17A expression in the colonic mucosa of each group. Clinical results showed that IL-17A content of the UC group and CD group was significantly higher than that of the normal control group ($p < 0.05$); IL-17A content of the CD group was higher than that of the UC group ($p > 0.05$). The UC group had the highest IL-10 content, and the difference between the UC group and other two groups had statistical significance ($p < 0.05$); the difference of IL-10 content between UC group and normal control group had no statistical significance ($p > 0.05$). There was no significant difference of IFN- γ level between the CD group and the UC group and normal control group ($p > 0.05$), and no significant difference of IFN- γ level was shown between the CD group and the UC group ($p > 0.05$). Both the CD and UC groups showed IL-17A positive staining in cytoplasm of lymphocyte, however no positive staining was found in any layer of intestinal mucosa of the normal control group. IL-17A was locally expressed in the colon of IBD patients in remission; furthermore, it also had high expression in serum; thus, there still existed high expression of pro-inflammatory factor, which might be related to relapse of IBD. Therefore, prevention of IL-17A may become a feasible therapy for IBD in the future.

Inflammatory bowel disease (IBD), a chronic, non-specific and inflammatory disease which develops in the intestinal tract with unknown pathogenesis, includes ulcerative colitis (UC) and Crohn's disease (CD), both of which are accompanied with intestinal mucosa inflammation. The disease mostly develops among people aged

from 20~40 years of age (1-3), and is a long-course disease that varies in disease levels among patients; in addition, it is easy to relapse and has a certain correlation with pathogenesis of colon cancer (4-6).

UC is one of the chronic and non-specific colonic inflammatory diseases, which mostly

Key words: interleukin-17A, interleukin-10, colonic mucosa, pro-inflammatory factors, inflammatory bowel disease

Mailing address:

Dr Qiuqian Zhao,
No.8 Jiangtan Road, Baoji,
The Central Hospital of Baoji,
Shanxi, 721008, China
e-mail: zhaoqiuqianzqj@163.com

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develops in colonic mucosa and submucous layer. Lesion of UC usually starts from the distal end of the colon and then adversely develops to the proximal end; moreover, diseased areas are in continuous distribution, which will even affect the entire colon and the distal ileum. CD is a chronic and granulomatous inflammatory disease that mainly develops in distal ileum and neighboring colon; lesion of CD is in segmental and symmetrical distribution. Some countries already have a high incidence rate of IBD, however, with the development of China's economy as well as the improvement of people's quality of life, reported IBD incidence rate in China is increasing year by year (7).

Interleukin (IL)-17 is a pro-inflammatory cytokine and its family members include IL-17A - IL-17F. IL-17 can lead to expression or release of chemotactic factors, colony stimulating factors and adhesion factors, as well as attracting and activating inflammatory cells, especially neutrophilic granulocytes (8-9); therefore it can induce inflammation and affect the pathological process of infection, tumor and autoimmunity. Usually, IL-17 is considered as IL-17A which is

the earliest found molecule in the family by the subtractive clone method. T helper type 17 (Th17) cells can produce IL-17A which has powerful abilities to attract and activate neutrophilic granulocytes, as well as inducing and activating various pro-inflammatory mediators generated by T cells, and stimulate macrophage, fibroblast and epithelial cells. In addition, IL-17A can have synergistic effect along with inflammatory factors, such as tumor necrosis factor- α (TNF- α) (10), which can intensify the inflammation-causing effect.

Research, as well as the latest findings on effects of immune cells and cytokines on IBD, enable us to reevaluate the effects of Th1 and Th2 cells on autoimmune diseases (11). Among IBD patients in remission, interferon- γ (IFN- γ) secreted by pro-inflammatory Th1 cells, and IL-10 secreted by inhibiting inflammatory Th2 cells are unbalanced. IL-17A is the main effector molecule of Th17 cells. This study mainly detected IL-17A changes of IBD patients in remission, and discusses the relationship between IL-10 level and IL-17A, and the effect of Th1 cells, Th2 cells and Th17 cells on pathogenesis of IBD.

Table I. Basic information of inflammatory patien

	CD (28)	UC (74)	Control group (36)
Male	12	34	16
Female	16	40	20
Average age	39.24	41.25	40.06

Table II. Activity indexes of Sutherland disease.

Items	Scoring			
	0	1	2	3
Diarrhea	Normal	1-2 times over normality /d	3-4 times over normality /d	5 times over normality /d
Hematochezia	None	A little	Obvious	Blood mainly
Mucous membrane expression	Normal	Mild fragility	Moderate fragility	Severe fragility with exudation
Disease condition evaluation from doctor	Normal	Mild	Moderate	Severe

Total points are the sum of each item. Remission of disease is ≤ 2 scores; mild activity is 3 to 5 scores; moderate activity is 6 to 10 scores; severe activity is 11 to 12 scores.

MATERIALS AND METHODS

Study population

CD patients and UC patients in remission who were hospitalized in the Peoples Liberation Army General Hospital from March, 2014 to July, 2015 were selected as research subjects. The normal control group had 36 healthy volunteers whose colonoscopy results were negative; The IBD group consisted of 36 patients, whose ratios of age and gender matched with that of the control group. All patients signed an informed consent. Detailed information of the patients is shown in Table I.

Diagnostic criteria were based on the Standard of Diagnosis and Treatment of Inflammatory Bowel Disease in China which was established by Digestive Disease Association of Chinese Medical Association at Jinan in May, 2007. This study used 5-amino salicylic acid (5-ASA) as the maintenance treatment for patients in remission. Disease activity indexes (DAI) of Sutherland are shown in Table II.

Exclusion criteria

The following types of IBD patients were excluded: IBD patients in remission who had recently been treated with hormones; patients diagnosed with enteritis diseases with unknown pathogenesis; patients with severe heart, liver, lung or kidney disease; patients who could not bear colonoscopy or refused to have biopsy or blood sampling; patients who refused to sign informed consent.

Reagents

The main reagents were IL-17A antibody; IL-17A enzyme-linked immuno sorbent assay (ELISA) kit; immunohistochemical SP kit; phosphate buffer saline (PBS), hematoxylin, methylbenzene, neutral balsam, xylene and eosin solution.

Statistical analysis

All patients underwent venous blood sampling in the morning before taking food. After centrifugation, sediment removal and serum separation, blood samples were conserved at -80 °C until use. Biopsy specimens were fixed by 10% neutral buffered formalin solution, and were then cut into 4- μ m serial sections and embedded in paraffin.

Immunohistochemistry

Double antibody sandwich - ELISA was used to detect

IL-17A, IL-10 and IFN- γ levels in serum: blood samples were collected and processed by centrifugation, sediment was removed and serum separated; then ELISA was used to detect content of all cytokines in serum; finally the results were calculated.

Detection of IL-17A expression in biptic tissues of colonic mucosa of patients in the UC group and CD group was carried out by immunohistochemistry: samples were processed by dewaxing and hydration; then antigens were repaired by pressure cooker thermal remediation; next the activity of endogenous peroxidase was inhibited and then immunohistochemical results were obtained by staining. The occurrence of yellow or yellowish-brown particles or staining in cytoplasm was regarded as positive result.

Statistical analysis

Statistic software SPSS17.0 was used for statistical analysis. Statistical data were analyzed by *t*-test and data statistics were presented by mean $\pm$ SD. *p* < 0.05 indicated that the difference was statistically significant.

RESULTS

Immunohistochemistry

Clinical results showed that staining of lymph cytoplasm of colonic mucosa of patients in the CD group and UC group were both positive; as shown in Fig. 1, positive cells of the UC group were mainly found in lamina propria lymphocytes of epithelium around glands, and they were yellowish-brown. As shown in Fig. 2, positive cells of the CD group could be seen in all layers of intestinal wall, and the distribution of positive cells in each layer had no significant difference; no positive staining was found in any layer of intestinal mucosa of patients in the normal control group.

Changes of cytokines in serum

As shown in Table III and Fig. 3, no IL-17A was found in the normal control group; IL-17A content of the UC group was 14.67 ± 3.98 pg/ml and IL-17A content of the CD group was 16.78 ± 3.24 pg/ml. Thus, the UC group and CD group both had significantly higher IL-17A content than that of the control group, and the CD group had higher IL-17A content than that of the UC group; however,

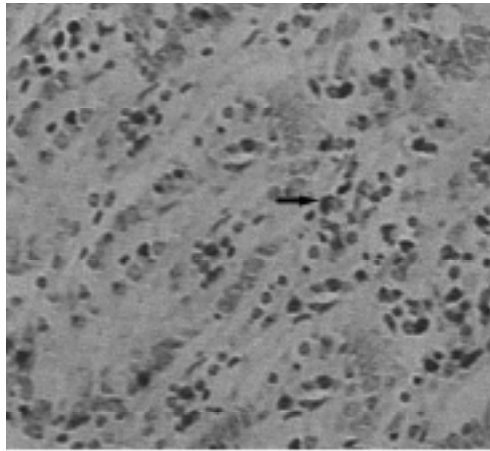


Fig. 1. *IL-17A positive staining lymphocytes in mucous layer of UC patients.*

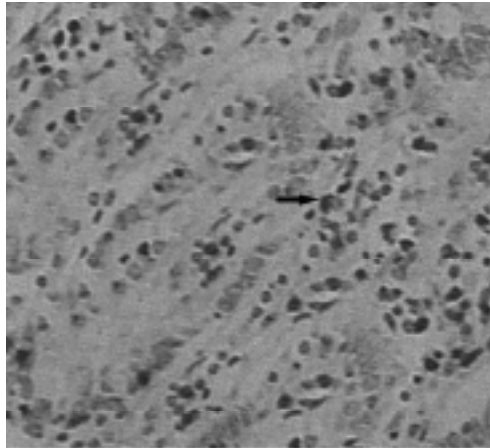


Fig. 2. *Visible positive staining lymphocytes in mucous layer of CD patients.*

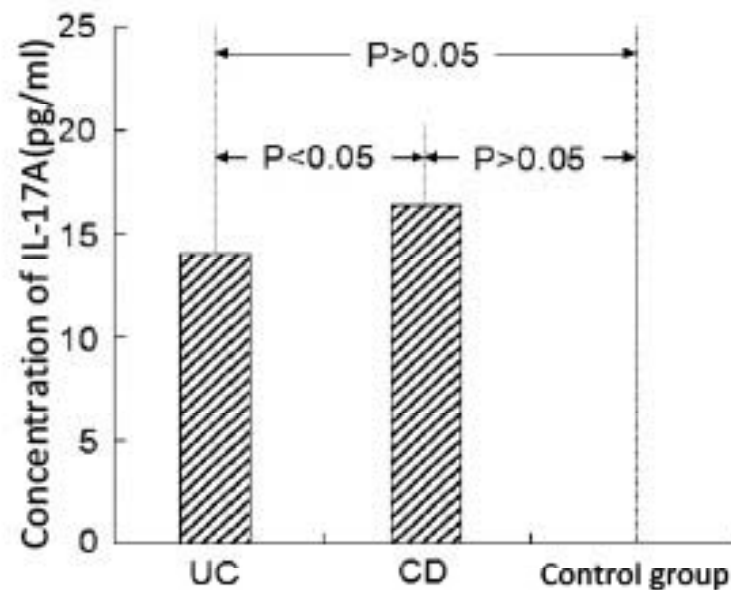
the differences had no statistical significance ($p > 0.05$). The results were the same as IL-17A staining results of colonic mucosa tissues. Other studies have already verified that IL-17A expression of IBD patients in active stage increased, which was in direct proportion with the severity of the disease (12). Data in this study showed that the IL-17A level of IBD patients in remission also increased significantly, thus 5-ASA treatment had no effect on IL-17A level. The reason was that IL-17A had effects on IBD through effector immune response IL-13/IL-17 pathways instead of Th1 response and Th2 response, but 5-ASA could not have immunosuppression effect in these pathways. Whether this was the reason that caused easy relapse of IBD is

still under research.

Table III and Fig. 4 show that IL-10 content in serum of the AC group was 72.36 ± 8.16 pg/ml and IL-10 content in serum of the UC group was 43.58 ± 5.24 pg/ml; IL-10 content of the control group was 41.23 ± 10.24 pg/ml. The CD group had the highest IL-10 content and the difference between the CD group and other two groups had statistical significance ($p < 0.05$). The difference between the UC group and normal control group had no statistical significance ($p > 0.05$). The increase of IL-10 expression was due to that under normal conditions, pro-inflammatory factors and anti-inflammatory cytokines in the body were dynamically balanced, and they were restricted mutually to keep the

Table III. ELISA results of IL-17A, IL-10 and IFN- γ in serum of patients in each group.

Groups	IL-10 (pg/ml)	IL-17 (pg/ml)	IFN- γ (pg/ml)
UC group (n=74)	43.58 $\pm$ 5.24	14.67 $\pm$ 3.98	29.08 $\pm$ 4.38
CD group (n=28)	72.36 $\pm$ 8.16	16.78 $\pm$ 3.24	28.46 $\pm$ 2.64
Control group (n=36)	41.23 $\pm$ 10.24	-	29.84 $\pm$ 2.12

**Fig. 3.** Detection of IL-17A level of each group using ELISA.

balance of the body environment; the unbalance of these two could lead to inflammation of tissues and organs. IL-10 is mainly secreted by Th2 cells, and as an anti-inflammatory cytokine, it restrains antigens of macrophage and monocyte as well as reducing the production of IFN- γ to play an anti-inflammatory role. The experiment of mice without IL-10 showed that the lack of IL-10 could cause IBD, and mice in the experiment began to have symptoms of IBD: all stomach and intestinal tract of mice were affected; diseased intestinal mucosa had epithelial proliferation; ulceration occurred (13-14). The increase of IL-10 is the secondary change of the increase of pro-inflammatory factor, which could reduce activity of immune and non-immune

cells, reduce pro-inflammatory factor production and relieve inflammation of IBD patients; therefore it is a protective factor.

IFN- γ is a kind of pro-inflammatory factor, which is mainly secreted by T cells and natural killer (NK) cells. Th1 cells are main cells that secrete IFN- γ and have a wide immunoregulatory effect. Table III shows that IFN- γ content of the CD group was 28.46 $\pm$ 2.64 pg/ml and IFN- γ content of the UC group was 29.08 $\pm$ 4.38 pg/ml, while IFN- γ content of the control group was 29.84 $\pm$ 2.12 pg/ml. Fig. 5 shows that IFN- γ content of the CD group and UC group had no significant difference from that of the normal control group ($p > 0.05$); IFN- γ content of the CD group and IFN- γ content of the UC group

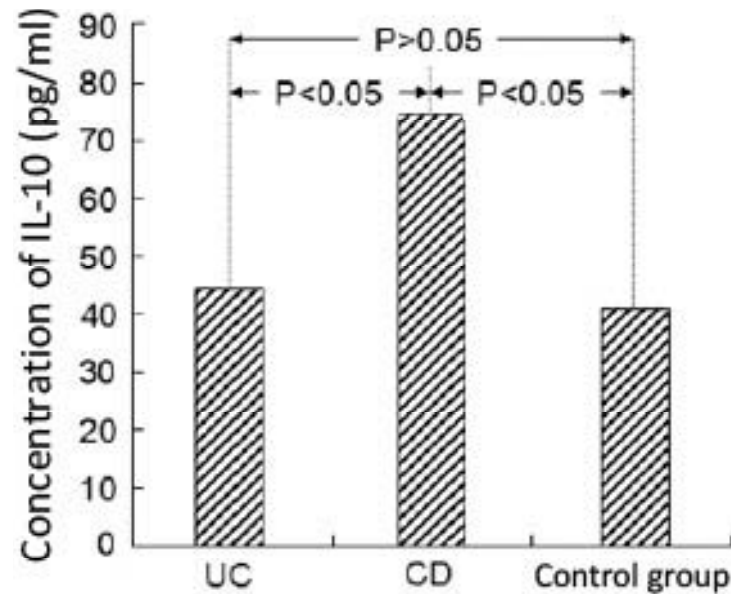


Fig. 4. Detection of IL-10 level of each group using ELISA.

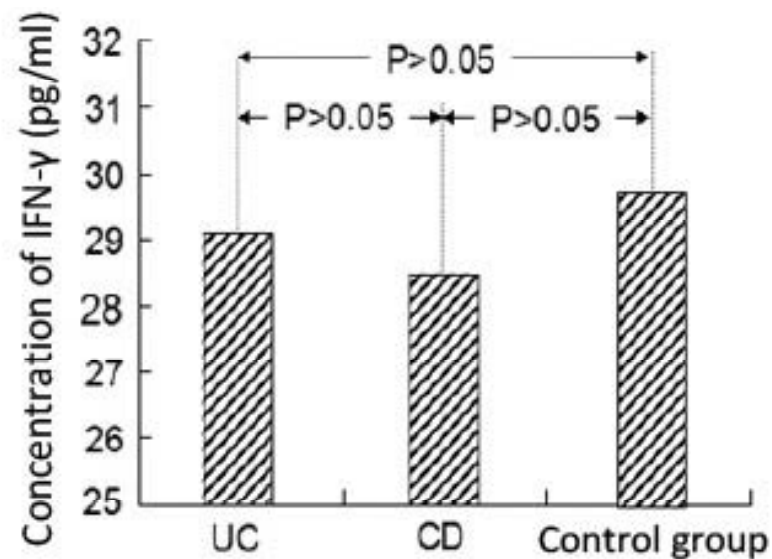


Fig. 5. Detection of IFN- γ level of each group using ELISA.

also had no significant difference ($p > 0.05$). The results may be related to the immunosuppressive effect of 5-ASA treatment which could reduce the pro-inflammatory factor level.

DISCUSSION

The etiology and pathogenesis of IBD are still unknown. According to research on IBD in the

past decades, the pathogenesis of IBD may be related to genetic predisposition, barrier function, intestinal flora, innate immunity and adaptive immunity (15-16). Researchers have begun to realize that cytokines are closely related to IBD, and the unbalance between pro-inflammatory factors and anti-inflammatory factors is regarded as an important link in IBD pathogenesis. Th17 cells may have certain effects on the pathogenesis

of IBD. IL-17 is a downstream regulatory molecule of IL-21, IL-23 and other inflammatory factors, and IL-23/IL-17 pathways are main immune response pathways of IBD pathogenesis, which may be in key position in chronic intestinal inflammation. IL-17 participates extensively in body inflammation and immune response, and its high expression in serum and target tissues is closely related to inflammation, autoimmunity disease, graft rejection, tumor and other diseases (17-18). This study found that IL-17A had local expression in colon of IBD patients in remission; furthermore, it also had high expression in serum, which indicated that there still existed a large amount of pro-inflammatory factor expression in IBD patients in remission, and the reason could be related to the relapse of IBD. Therefore, the prevention of IL-17A may become a feasible therapy for IBD in the future.

Relevant studies (19-20) showed that INF- γ and IL-12 can enhance advantages of Th1 cells through stimulating differentiation of Th1 cells and restraining differentiation of Th2 cells; IL-4 and IL-13 enhance advantages of Th2 cells through stimulating differentiation of Th2 cells and restraining differentiation of Th1 cells. IL-10 is an anti-inflammatory cytokine that is multi-functional and participates in various kinds of immunosuppressive effects: it can restrain monocyte, neutrophil and eosinophilic granulocytes to produce pro-inflammatory factors and chemotactic factors; it can restrain the production of IFN- γ to control activity of NK cells; it can also restrain the conversion of TH0 cells to Th1 cells and restrain the transfer of inflammatory cells. Obviously, the balance between Th1 and Th2 is determined by the balance between pro-inflammatory factors and anti-inflammatory factors (21); thus the unbalance of these two factors is one of the reasons that cause IBD.

This study verified that IL-17A expression was high in bioptic tissues of colonic mucosa of the UC group and CD group, while no expression of IL-17A was found in bioptic tissues of colonic mucosa of the normal control group; thus, Th17 cells might be found in locally infiltrated lymphocytes in the colon of IBD patients. Positive staining was only found in mucous layer of UC patients, which indicated

mucous layer inflammation; CD patients showed whole-layer positive staining, which indicated whole-layer inflammation. IL-17A was also found in serum, thus Th17 cells might also play a role in general immunity of IBD patients.

In conclusion, Th17 cells may exist in locally infiltrated lymphocytes in colon of IBD patients. In addition, IL-17A is detected in serum of IBD patients, thus we presume that Th17 cells may also have effects on general immunity of IBD patients. Further research and discoveries regarding the effects of immune cells and cell factors on IBD can inspire scholars to reevaluate the effects of Th1 cells and Th2 cells on autoimmune diseases.

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LETTER TO THE EDITOR

COMPARISON OF LAPAROSCOPY AND OPEN SURGERY IN TREATING SEVERE ACUTE PANCREATITIS AND ITS RELATIVE AFTERCAREJ. ZHANG¹, MX. JIANG², Y. ZHENG³, M. SHU⁴ and SB. SUN¹

¹Department of General Surgery, The 2nd Affiliated Hospital of Harbin Medical University, Harbin, China; ²Department of Radiotherapy, Harbin Medical University Cancer Hospital, Harbin, China; ³Department of Ear-Nose-Throat, The 2nd Affiliated Hospital of Harbin Medical University, Harbin, China; ⁴Department of Orthopaedics, The 2nd Affiliated Hospital of Harbin Medical University, Harbin, China

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The first two authors contributed equally to this work

This study was designed to determine the effects and compare laparoscopic intervention and open surgery in treating severe acute pancreatitis (SAP) and its relative aftercare, to improve the overall treatment of SAP. Ninety patients with SAP were enrolled from the 2nd Affiliated Hospital of Harbin Medical University from 2008 to 2014 and divided into a laparoscopic intervention group (25 cases) and an open surgery group (65 cases). Patients were asked for clinical symptoms, general hospital information, laboratory inspection, imageological examination, local and systemic complications, treatment and outcome. SAP patients' relevant clinical indicators were compared between the two groups before and after the operation. Results revealed that there was no statistical significance in lesion range and main scoring indexes for reflecting the severity of the disease. For both groups statistical significance was found in blood loss (285.3 ± 79.8 mL vs 362 ± 91.6 mL), intensive care unit (ICU) monitoring time (9.04 ± 6.35 d vs 12.48 ± 8.34 d) and service time of breathing machine (9.47 ± 6.24 d vs 12.98 ± 8.25 d), and the laparoscopic operation group was superior to the open surgery group ($p < 0.05$). Besides, the laparoscopic operation group was also superior to the open surgery group in demand for main analgesics one week after the operation, as well as for recovery rate and incidence of complications ($p < 0.05$). Thus, it can be concluded that patients undergoing laparoscopic intervention are less likely to develop pulmonary infection and more likely to be cured in comparison with patients who receive open surgery. In addition, laparoscopic intervention results in less damage, lighter pain and fewer complications compared with open surgery.

With the continuous development of China's economy and incessant improvement of people's living standards, incidence of severe acute pancreatitis (SAP), resulting from diet, emotion and decreased immunity, increases constantly, presenting a rising trend year after year. SAP has become a public problem seriously threatening people's health. It also causes a sharp increase in medical expense

and rapid depletion of medical resources. SAP as a critical disease commonly seen in the digestive system is typically characterized by massive fat necrosis inside and around the pancreas as well as lesion and bleeding in pancreatic parenchyma, which is mainly caused by hyperamylaseemia resulting from secondary infection around pancreas tissues (1-3). SAP, which develops rapidly and is accompanied

*Key words: laparoscope, open surgery, severe acute pancreatitis, nursing, effect comparison**Mailing address:*

Dr Shibo Sun,
Department of General Surgery,
The 2nd Affiliated Hospital of Harbin Medical University,
No 246, Xuefu Road, Nangang District,
Harbin 150086, Heilongjiang Province, China
e-mail: shibosun026@163.com

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with many complications, can result in multiple organ failure (MOF) and even endanger a patient's life (4). Under normal circumstances, pancreatic juice produced by the body will not digest tissues. However, when pancreatic juice secretion increases suddenly (5) or pressure in the pancreatic duct increases and the pancreatic gland acinus bursts under stimulation, it leads to pancreatic enzyme explosive release as well as automatic digestion of pancreatic enzyme to pancreas and the surrounding tissues (6).

To date, minimally invasive laparoscopic peritoneal lavage drainage (LPLD), the preferred regimen for SAP, is able to remove the patient's pancreas and the surrounding necrotic tissues, thus effectively reducing pressure in the abdominal cavity. This method is beneficial to the patient's clinical symptoms and recovery after intervention (7-8). In recent years, laparoscopic operation, which not only has the characteristics of traditional open surgery, but also has advantages that cannot be ignored, has received extensive interest in the medical field. Whether SAP can be treated by laparoscopy only or not is worthy of further discussion (9). SAP treatment with laparoscopy is rarely studied and reported at present. Laparoscopy has gradually become more popular with the development of laparoscopic instruments and techniques (10). This study determined different effects of laparoscopic intervention and open surgery on SAP treatment, which is of important scientific value and is of great significance in clinical practice.

MATERIALS AND METHODS

Study subjects

Ninety SAP patients (42 males, 48 females; ages 20-76 years, mean 44.5 ± 15.5 years) who were admitted to the 2nd Affiliated Hospital of Harbin Medical University between 2008 and 2014 were selected as a study group (average length of stay: 57.45 ± 22.23 days). Thirty patients (33.3%) died of SAP. The patients were divided into a laparoscopic intervention group (25 cases) and an open surgery group (65 cases). This study was approved by the ethics committee of our hospital and all the patients signed informed consent.

The patients in both groups conformed to clinical diagnosis standards. The laparoscopy group required no conversions to open surgery; the patients in the open surgery group were treated with traditional laparotomy to remove necrotic tissues, combined with traditional duct drainage or closed peritoneal lavage.

Methods of operations

For the laparoscopy group, after the patients were generally anesthetized, artificial pneumoperitoneum (APP) was set up immediately and the abdominal pressure was controlled between 13.0 and 13.5 mmHg (11-13). An arc incision (1 cm long) was cut under the umbilicus to build APP with a Veres needle and then a laparoscope was placed to guide the placing of trocars. The omentum majus was lifted so that it could be cut along the inferior margin of greater curvature with an ultrasound knife to enter the omental bursa. After that, percolate and necrotic pancreatic tissues were absorbed with an aspirator and the abdominal cavity was washed with a large amount of saline until the washing fluid became clear.

For the open surgery group, the operation was performed according to common surgery methods. A drainage tube was placed as circumstances required and some of the patients received closed peritoneal lavage after the operation, based on drainage condition.

Postoperative care

Changes in the patients' vital signs, daily amount of irrigation solution and drainage liquid's amount and color were monitored, to reduce direct chemical damage to body tissues as well as systemic inflammatory response syndrome (SIRS). Daily volumes of irrigation inflow and drainage liquid were balanced and changes in the patients' conditions were closely observed to make timely adjustment when necessary. In the course of the treatment, balance of water, electrolyte, and acid in the patient's body were monitored.

Statistical analysis

Data were analyzed using SPSS 13.0 statistical software and expressed as mean $\pm$ standard deviation (SD). Measurement data were compared using *t*-test and the ratio of the two groups was compared using χ^2 test. The difference was considered to be statistically significant if $p < 0.05$.

RESULTS

Comparison of SAP patients' severity

The two groups of patients were found without statistical significance in SIRS, acute physiology and chronic health evaluation (APACHE) II, Ranson and CTS I scorings ($p > 0.05$), Table I.

Relevant surgical situations

There was no statistical significance in lesion range (Table II) ($p > 0.05$) while a statistical significance was found in the duration of the operation and blood loss between the two groups ($p < 0.05$). Laparoscopy had a longer operating time but less blood loss during the operation compared with open surgery.

Blood amylase changes

As shown in Table III, there was a statistical significance in blood amylase on the 1st, 3rd, 5th and 7th day after the operation between the two groups ($p < 0.05$), and blood amylase was found to be lower in the laparoscopic intervention group than in the open surgery group, $*p < 0.05$.

Blood calcium concentration

Blood calcium concentration was statistically significant on the day of operation (Table IV) ($p < 0.05$), while there was no statistical significance on the 1st and 3rd day after the operation ($p > 0.05$). $*p < 0.05$, $**p > 0.05$ compared with the open surgery group.

ICU monitoring time, service time of breathing machine and fasting time

ICU monitoring time and service time of a breathing machine were found to be statistically significant between the two groups after the operation ($p < 0.05$), while there was no statistical significance in fasting time ($p > 0.05$), as shown in Table V. $*p < 0.05$, $**p > 0.05$ compared with the open surgery group.

Requirements of main analgesics one week post-operative

As shown in Table VI, total usage amounts of the two analgesics (tramadol and demerol) commonly used within one week after the operation were compared between the two groups and a statistical significance

was found ($p < 0.05$). $*p < 0.05$ compared with the open surgery group.

Incidence of common complications

It can be seen from Table VII that complications which occurred frequently after the operation in the two groups, such as pulmonary infection, multiple organ dysfunction syndrome (MODS) and double infection, were found to have a statistical significance ($p < 0.05$); while there was no statistical significance comparing the incidences of MODS and double infection ($p > 0.05$). $*p < 0.05$, $**p > 0.05$ compared with the open surgery group.

Disease outcome between the two groups

A statistical significance was found comparing recovery rate and death rate between the two groups ($p < 0.05$) (Table VIII). $*p < 0.05$ compared with the open surgery group.

DISCUSSION

SAP, a severe digestive disease commonly seen in clinics, has complicated clinical manifestations and critical conditions. This disease occurs frequently in 20 to 50-year-olds, and females are more likely to suffer from it than males. It was reported that the ratio of male and female is approximately 1:1.7 (14). SAP belongs to a special type of acute pancreatitis, 10 ~ 20% of patients with acute pancreatitis are diagnosed with severe pancreatitis and 70 ~ 80% of patients develop SAP due to biliary tract diseases, alcoholism and gluttony (15-16). Besides treatment, relative aftercare is also very important. Some studies (17-18) indicated that relative aftercare played a crucial role in the treatment of severe pancreatitis after minimally invasive LPLD, improving the prognosis of patients.

In this study, the laparoscopy group and open surgery group showed no statistical significance in main scoring indexes which reflected the severity of the disease and visible lesion range; while there was statistical significances in intra-operative blood loss, recovery of blood amylase, calcium and sugar, ICU monitoring time, and service time of a breathing machine, as well as total and average length of

Table I. Comparison of SAP patients' severity before the operation

Treatment groups	Number of cases	SIRS score	APACHE II score	Ranson score	CTS I score
Laparoscopy group	25	2.96±0.48*	12.49±5.78*	4.86±1.25*	4.72±1.59*
Open surgery group	65	3.22±0.58	12.95±6.31	5.05±0.98	4.87±1.84

Table II. Comparison of relevant conditions between the two groups.

Treatment groups	Number of cases	Duration of operation (min)	Intraoperative blood loss (mL)	Visible lesion range (cm ³)
Laparoscopy group	25	165.8±59.5*	285.3±79.8*	610.5±182.1**
Open surgery group	65	126±43.1	362±91.6	622.5±193.4

Table III. Comparison of changes of blood amylase after intervention between the two groups.

Treatment groups	Number of cases	Blood amylase after the operation (μ/l)		
		The 1st day	The 2nd day	The 5th ~ 7th day
Laparoscopy group	25	0.98236±0.2134*	0.4522±0.887*	0.2919±0.0512*
Open surgery group	65	1.1212±0.1796	0.5341±0.0978	0.3886±0.0615

Table IV. Comparison of changes of blood calcium concentration after surgery between the two groups.

Treatment groups	Number of cases	Blood calcium concentration (mmol/L)		
		0 day	The 1st day	The 3rd day
Laparoscopy group	25	1.68±0.25*	1.96±0.37*	2.03±0.39**
Open surgery group	65	1.61±0.26	1.94±0.39	1.97±0.31

Table V. Comparison of ICU monitoring time, service time of breathing machine and fasting time between the two groups.

Treatment groups	Number of cases	ICU/day	Service time of breathing machine / day	Fasting time /day
Laparoscopy group	25	9.04±6.35*	9.47±6.24*	15.28±6.52**
Open surgery group	65	12.48±8.34	12.98±8.25	15.09±6.78

Table VI. Comparison of requirements of main analgesics one week after surgery.

Treatment groups	Number of cases	Tramadol injection (mg)	Demerol injection (mg)
Laparoscopy group	23	126.6±69.3*	20.2±11.3*
Open surgery group	68	256.1±105.2	40.1±30.2

Table VII. Comparison of incidences of common complications after surgery.

Treatment groups	Number of cases	Pulmonary infection	MODS	Double infection
Laparoscopy group	23	9*	6*	11*
Open surgery group	68	36	20	33

Table VIII. Comparison of disease outcome.

Treatment groups	Number of cases	Recovery rate	Death rate
Laparoscopy group	23	18*	5*
Open surgery group	68	40	28

stay between the two groups; and the laparoscopy group was superior to the open operation group ($p < 0.05$). Furthermore, the two groups had different requirements of tramadol and demerol within one week after the operation and a statistical significance was found. The laparoscopy group was superior to the open surgery group in comparison with recovery rate, incidence of common complications and pulmonary infection ($p < 0.05$).

Open surgery often needs to be performed once again in a short term since incisions heal incompletely in the first operation, which is a realistic question for surgeons (19). However, laparoscopic operation can remove abscesses via posterior approach and retroperitoneal approach, which not only avoids repeat intervention, but is also beneficial to the relative aftercare and patient post-operative activities as well as reducing damage to the body caused by multiple operations.

This research showed that blood amylase was lower in the laparoscopic intervention group in the first three days post-operative compared with the open surgery group. Patients who underwent laparoscopy were found to have lighter inflammatory stress response, less breathing machine use, and were less likely to develop pulmonary infection in comparison with patients undergoing open surgery. In addition, laparoscopy can avoid retractor traction damage to incisions and the surrounding tissues as well as intense postoperative pain, and avoids excessive muscle and nerve involvement. This kind of pain makes breathing difficult and even results in pulmonary atelectasis, thus increasing the incidence of pulmonary infection after the operation (20). Hence, this study further proves that the patients undergoing laparoscopic intervention were less likely to suffer from pulmonary infection, had shorter average length of stay and required fewer analgesics during one week after the operation than the patients undergoing open surgery. However, the operation time was longer, which might be influenced by the learning curve in addition to the nature of different lesions (21).

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LETTER TO THE EDITOR

**EXPRESSION OF CD4+CD25+ REGULATORY T CELLS AND FOXP3
IN PERIPHERAL BLOOD OF PATIENTS WITH GASTRIC CARCINOMA**

S.L. CHEN, S.R. CAI, X.H. ZHANG, J.J. PENG, W.F. LI, E.T. ZHAI,
J.H. CHEN, H. WU, C.Q. CHEN, J.P. MA and Y.L. HE

*Gastrointestinal Surgery Center, The First Affiliated Hospital of Sun Yat-sen University,
Guangzhou city, Guangdong Province, China*

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The first two authors contributed equally to this work

The aim of this research was to study the clinical significance and expression of CD4+CD25+ regulatory T cells (Tregs) and p3Forkhead transcription factor-3 (Foxp3) in peripheral blood of patients with gastric carcinoma (GC) and to investigate the effects in the occurrence and development process of GC, to further comprehend their clinical values, and provide a theoretical basis for the early diagnosis and immunotherapy of GC. The expression levels of CD4+CD25+Foxp3+Tregs in GC patients, at TNM staging, differentiated degree, lymphatic metastasis, cancer sites and cancer diameter of GC, were analyzed within the groups. The comparison of the expression levels of CD4+CD25+Foxp3+Tregs in peripheral blood between the GC group and the healthy control group showed a statistically significant difference. At TNM staging within the groups, pairwise comparisons of the expression levels of CD4+CD25+Foxp3+Tregs indicated that differences among the stage I+II group, stage III group and stage IV group were statistically significant. The expression levels of CD4+CD25+Foxp3+Tregs are closely relative to the occurrence and development of GC, providing theoretical bases and evidence for the early diagnosis, prognosis evaluation and immunotherapy of GC.

Gastric carcinoma (GC) is a life-threatening disease. Although various treatment methods have achieved some improvement, more effective treatment methods are badly needed, therefore it is necessary to discover the pathogenesis of the disease. GC is one of the commonest gastrointestinal malignant tumors and is characterized by hidden onset, high morbidity, easy transference, and high mortality. It has a multi-stage progressive process and unstated mechanism. The present study is to further discuss the molecular mechanism of the occurrence and development of GC in order to find more effective treatments. Because the clinical manifestation

of early GC is short of specificity, with only one symptom or only with the symptoms for peptic ulcer, the main basis of currently definite diagnosis is on the biopsy for tissues obtained by gastroscopy, assisted with barium meal X-ray. As gastroscopy, as the general survey for GC, has not been launched in China, the diagnostic rate of early GC is smaller, and usually most patients are at the middle or advanced stage of disease when they are definitely diagnosed. The main treatment for early cancer is surgical removal, however the survival time for patients with advanced cancer is short even though chemical treatment and adjuvant radiotherapy are carried out

Key words: GC, CD4+CD25+ Tregs, Foxp3

Mailing address:

Dr Yulong He,
Department of Gastrointestinal Surgery,
The First Affiliated Hospital, Sun Yat-sen University,
58, Zhongshan 2nd Rd, Yuexiu District,
Guangzhou City, 510080, PR China
e-mail: yulonghe163@126.com

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as well as surgical removal. Studies at home and abroad have shown that CD4⁺CD25⁺Foxp3⁺Tregs are highly expressed in peripheral blood and tumor microenvironment, suppressing effector T cells, further suppressing tumor immunity. Therefore, the early diagnosis for GC and the possible therapeutic targets for its occurrence and development have been the hot points of research.

CD4⁺CD25⁺Foxp3⁺Tregs are a class of CD4⁺Treg subgroups, regulating immune tolerance and immunosuppression (1, 2), and playing a crucial role in tumor immunity. CD4⁺CD25⁺Tregs, accounting for 5%-10% in peripheral blood (3), present a high expression in tumor infiltration lymphocyte and partial lymph nodes, directly deciding the severity and therapeutic effect of diseases (4, 5). Forkhead box (FOX), a genetic term of forkhead transcription factors of vertebrates, is a large family of transcription factors with multiple functions, which is generally related to the growth of cells and the regulation of development (6). Foxp3 is a member of transcription factor P subfamily, with its gene positioned on X chromosome. Foxp3 encoding product is a 48-kDa protein, named scurfin. The deficiency of scurfin protein can enhance the occurrence rate of cancer (7). Foxp has a close relationship with the development, peripheral expression and maintaining function of CD4⁺CD25⁺Tregs (8, 9). This study, through detection of the expression levels of CD4⁺CD25⁺Foxp3 Tregs in peripheral blood for 38 GC patients by adopting FCM, discusses the clinical significance of CD4⁺CD25⁺Foxp3Tregs on GC patients, providing a theoretical basis for early diagnosis and immunotherapy of GC.

MATERIALS AND METHODS

Preoperative peripheral blood of 39 patients, 24 males, 15 females, age range 36-78 years (median 56 years), confirmed as GC (adenomatous carcinoma) by postoperative histopathology, hospitalized in the gastrointestinal surgery of The First Affiliated Hospital of Sun Yat-sen University between May and December 2013, was selected as GC group. All the subjects were in the initial treatment, without having received radiotherapy or chemotherapy. Peripheral blood of 24 healthy subjects

(13 males, 11 females; aged from 27-71 yrs - median 48 yrs) in the physical examination center were selected as the healthy control group. Disease of vital organs, such as heart, liver, lungs and stomach, were not present and normal hepatorenal function was shown in the medical examination report. The study was approved by the Ethics Committee of Sun Yat-Sen University and all participants signed an informed consent, according to the Declaration of Helsinki

Human whole blood Tregs detection kit (anti-human CD4-FIFC, anti-human CD25-APC, anti-human Foxp3-PE, red blood cell lysis buffer, flow cytometry buffer solution (FBS), fixation/permeabilization concentrate and Foxp3 fixation/permeabilization dilution) were purchased from eBioscience, Inc. (America); Foxp3 immunohistochemical primary antibody was purchased from Beijing Biosynthesis Biotechnology Co., Ltd.; CD4 immunohistochemical primary antibody was purchased from ZSGB-BIO; CD25 immunohistochemical primary antibody was purchased from ZSGB-BIO; SP-9001 immunohistochemical staining kit (containing 3% H2O2 deionized water, reagent A; sealed with normal goat serum working solution, reagent B; Biotin labeling IgG/Biotin IgG, Reagent C: horseradish peroxidase labeling streptavidin-peroxidase working solution) was purchased from ZSGB-BIO ORIGIN Your Gene Company; DAB color developing agent was purchased from ZSGB-BIO.

Samples

Peripheral venous blood samples of the patients in GC group were taken, preoperatively in the morning. Also 2 ml peripheral blood samples were taken in the morning for examination of the subjects in the healthy control group. Peripheral blood conventional EDTA anticoagulation between the GC group and the healthy control group was stored under normal temperature, and FCM detection was conducted within 4 hours.

FCM detection in the expression of CD4⁺CD25⁺Foxp3⁺Tregs in peripheral blood of GC patients

This study labeled the T-cell surface antigen CD4 and CD25 as well as the antigen Foxp3 in cell nucleus to calculate the ratio between cell count of CD4⁺CD25⁺Foxp3⁺Tregs and CD4⁺Tregs. All the operations of FCM were carried out in accordance with the instructions of human whole blood

Tregs detection kit and flow cytometry. After mixing each blood sample, 100 μ l mixture was prepared and collected for use; 20 μ l anti-human CD4-FITC and 20 μ l anti-human CD25-APC were added to the blood sample and incubated for 30 min at room temperature; 2 ml red blood cell lysis buffer was immediately added to the above mixture and incubated for 10 min at room temperature, then the prepared blood sample was centrifuged for 5 min at 1,500 rpm (about 400 g), and the supernatant was discarded; 2 ml FBS was added to dissolve the sample pellet. After centrifugation for 5 min at 1,500 rpm, the supernatant was discarded. 2 ml FBS was added to dissolve the sample pellet. After centrifugation for 5 min at 1,500 rpm, the supernatant was discarded. Foxp3 fixation/permeabilization working solution was prepared. The fixation/permeabilization concentrate and Foxp3 fixation/permeabilization dilution was at the ratio of 1:3; 1 ml previously prepared Foxp3 fixation/permeabilization working solution was used for mixing and incubation for 40 min at 4°C in the dark; 2 ml FBS was then added to dissolve the above sample. After centrifugation for 5 min at 1,500 rpm, the supernatant was discarded; 2 ml FBS was then added to dissolve the above sample. After centrifugation for 5 min at 1,500 rpm, the supernatant was discarded; 100 μ l anti-human Foxp3-PE was added to the above sample for incubation for 30 min at 4°C in the dark; 2 ml FBS was added to dissolve the above sample. After centrifugation for 5 min at 1,500 rpm, the supernatant was discarded; 0.5 ml FBS was applied for resuspension for the cells and Flow cytometry was used to separate the CD4⁺ Tregs and CD25 positive and Foxp3 positive Tregs in CD4⁺ Tregs. The obtained ratio of CD4⁺CD25⁺Foxp3⁺Tregs/CD4⁺ Tregs was regarded as the expression levels of the CD4⁺CD25⁺Foxp3⁺Tregs.

Statistical analysis

SPAA 16.0 statistical software was used for data statistical analysis. Two-sided test was applied for all the statistical data. $P < 0.05$ was considered as statistically significant difference. For quantitative data, $m \pm s$ was adopted for data analysis, and t -test was employed for information comparison between two samples. Variance analysis was used for the comparisons among three or more samples. LSD method was used for pairwise comparisons among groups, and Pearson's correlation was applied for the correlation analysis of two variances and χ^2 test was used for the qualitative data.

RESULTS

Comparison of basic profiles between GC group and control group

Thirty-nine cases (24 males and 15 females) of the GC group, and twenty cases of the healthy control group (12 males and 8 females) showed no statistically significant difference ($\chi^2 = 0.187$, $P = 0.634$). In the GC group, 16 subjects were under 55 years of age and 23 were 55 years or over, while in the healthy control group, 8 subjects were aged under 55 years and 16 were 55 years or over, indicating no statistically significant difference ($\chi^2 = 0.067$, $P = 0.815$), Table I.

Expression levels of CD4⁺CD25⁺Foxp3⁺Tregs in peripheral blood between GC group and healthy control group

The expression of CD4⁺CD25⁺Foxp3⁺Tregs in peripheral blood of GC patients was $6.87 \pm 1.21\%$, while that in healthy control group was $3.85 \pm 0.91\%$, showing statistically significant difference ($t = 9.845$, $P < 0.001$), Fig. 1.

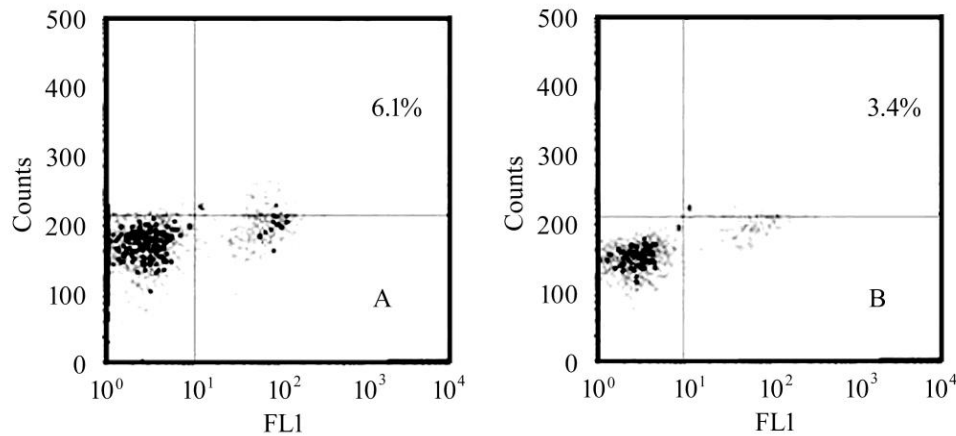
The relationship between expression levels of CD4⁺CD25⁺Foxp3⁺Tregs in peripheral blood and GC relevant parameters for GC patients

Comparing the expression levels of CD4⁺CD25⁺Foxp3⁺Tregs in peripheral blood of GC patients in demographic characteristics, showed that both genders ($t = 0.324$, $P = 0.739$) and ages ($t = 0.976$, $P = 0.329$) were of no statistical significance, Table II.

Statistical analysis was carried out on the expression levels of CD4⁺CD25⁺Foxp3⁺Tregs in peripheral blood of GC patients and the GC relevant parameters, such as TNM staging, differentiated degree, lymphatic metastasis, cancer sites and cancer diameter of GC. The results showed that the expression levels of CD4⁺CD25⁺Foxp3⁺Tregs in GC patients at GC TNM staging and lymphatic metastasis groups had a statistically significant difference ($P < 0.001$), while no statistical significance was shown in the parameters such differentiated degree, cancer sites and cancer diameter, Table III. Pairwise comparisons of TNM staging within the groups showed that the comparison between the stage I+II group and stage III group had

Table I. Demographic characteristics between GC group and healthy control group.

Demographic characteristics	GC group (cases)	Healthy control group (cases)	χ^2 value	<i>P</i> value
<u>Gender</u>				
Male	24	13	0.187	0.634
Female	15	11		
<u>Age (years)</u>				
<55	16	8	0.067	0.815
≥55	23	16		

**Fig. 1.** The expression levels of CD4⁺CD25⁺Foxp3⁺Tregs in the peripheral blood from gastric cancer group (A: GC group; B: Healthy control group).

a statistically significant difference ($P = 0.001$), so did that between the stage I+II group and stage IV group ($P < 0.01$) as well as that between the stage III group and stage IV group ($P < 0.01$).

DISCUSSION

In the early 1970s, Gershon et al. (10, 11), for the first time, put forward the existence of a class of particular lymph cells: suppressor T cells which had the

ability to suppress other T cells in order to achieve the immunoregulation effect, such as immune tolerance and immunosuppression. In the mid-1990s, some scholars (12) proposed the existence of expressing CD4 and new suppressor T cell which was defined as Tregs. Recently, CD4⁺ T cells were divided into two categories: Tregs and traditional helper T cells (Th). Th cells, through activating CD8⁺ cytotoxic T cell, B cells, macrophages and other effector cells, play the control role in adaptive immunity which is specific to

Table II. Comparisons of expression levels of CD4⁺CD25⁺Foxp3⁺Tregs from GC group in the peripheral blood in demographic characteristics.

Demographic characteristics	GC group (cases)	CD4 ⁺ CD25 ⁺ Foxp3 ⁺ Tregs /CD4 ⁺ Tregs (%)	<i>t</i> value	<i>P</i> value
<u>Gender</u>				
Male	24	7.12±1.23	0.324	0.739
Female	15	6.89±1.24		
<u>Age (years)</u>				
<55	16	7.21±1.43	0.976	0.329
≥55	23	6.79±1.03		

Table III. Relationship between the expression levels of CD4⁺CD25⁺Foxp3⁺Tregs in the peripheral blood and correlative parameters of GC from GC group.

Items	Number (cases)	CD4 ⁺ CD25 ⁺ Foxp3 ⁺ Tregs /CD4+Tregs (%)	<i>t/F</i> value	<i>P</i> value
TNM staging				
I+II	12	5.79±0.79	22.739	<0.001
III	18	7.11±0.58		
IV	9	8.43±1.21		
Differentiated				
High differentiation	7	6.09±1.32	2.498	0.087
Moderate	12	7.12±1.54		
Poorly	20	7.19±0.98		
Lymphatic				
Yes	27	7.44±1.21	4.113	<0.001
No	12	6.12±0.76		
Tumor sites				
Cardia and gastric	11	6.41±1.23	2.443	0.105
Gastric body	9	6.72±0.67		
Pylorusand antrum	19	7.37±1.28		
Tumor diameter				
<5	17	6.72±0.89	-0.912	0.365
≥5	22	7.21±1.53		

pathogenic bacteria and cancer, while Tregs show the responsibility for suppressing the harmful activities of Th cells. Tregs extensively express the following markers: CD25 (13), CTLA-4 (14, 15), GITA (16), LAG-3 (17), CD127 (18, 19), Foxp3 (20, 21). CD 25 is the α -chain of the IL-2 receptor and IL-2 is an important factor for the development of T cells, whilst almost all activated Tregs can express CD25. Therefore, CD4⁺CD25⁺Tregs are the most important class of CD4⁺ Tregs. CD4⁺CD25⁺Tregs have the following functions: through establishing and maintaining their own immune tolerance, they can not only prevent autoimmune disease (22, 23), suppress allergic effects and asthma and induce the immune tolerance of parent body on fetus, but also suppress the over immune reaction induced by pathogenic bacteria (24, 25), control the intensity of immune reaction (26, 27) and suppress the overactivity of T cells caused by weak stimulation (28). Moreover, through the feedback of effector Th cells they are able to control the intensity of immune reaction (29, 30), protect the normal flora from elimination by the immune system and suppress the immune response of tumors. Foxp3 is a regulatory factor for the development of CD4⁺ CD25⁺Tregs. Study on tissue distribution has indicated that Foxp3 is mainly distributed in lymph nodes.

This study has taken almost one year, selecting the peripheral blood of 38 GC patients and of 20 subjects in a healthy control group as samples. Detection by the FCM method was carried out to study the relationships between CD4⁺ CD25⁺Tregs and Foxp3 and GC.

The general data regarding gender and age of the two groups showed no statistically significant difference, suggesting that the GC group and healthy control group were from the same ensemble, which was the basis for statistical comparison.

The expression levels of CD4⁺CD25⁺Tregs were detected in peripheral blood of both groups by adopting FCM and were found to be significantly higher in the patients in the GC group than the subjects in the healthy control group, indicating that CD4⁺CD25⁺Foxp3⁺Tregs may have a certain role in the occurrence of GC. Correlation analysis between the expression levels of CD4⁺CD25⁺Foxp3⁺Tregs in the peripheral blood of the patients in the GC group and GC TNM staging, differentiated degree, lymphatic

metastasis, cancer sites and cancer diameter and GC was conducted. The results indicated that there were close correlations between the expression quantity of CD4⁺CD25⁺Foxp3⁺Tregs in the peripheral blood and GC TNM staging and lymphatic metastasis. Pairwise comparisons of expression levels of CD4⁺CD25⁺Foxp3⁺Tregs in TNM staging within the groups showed that the difference among the stage I+II group, stage III and stage IV group was statistically significant. The expression levels of CD4⁺CD25⁺Foxp3⁺Tregs were closely relevant to GC TNM staging, and represented an increasing tendency in TNM staging ($5.79 \pm 0.79\%$), ($7.11 \pm 0.58\%$), ($8.43 \pm 1.21\%$), respectively), which was the basis to indirectly judge GC staging through expression levels of CD4⁺CD25⁺Foxp3⁺Tregs in peripheral blood. This result suggested that the expression quantity of CD4⁺CD25⁺Foxp3⁺Tregs was relative to the local infiltration, lymphatic metastasis and distant organ metastasis. With regard to the expression of CD4⁺CD25⁺Foxp3⁺Tregs, a high level was shown in the local infiltration, lymphatic metastasis and distant organ metastasis, which indicated a large tumor invasiveness. Through the detection of CD4⁺CD25⁺Foxp3⁺Tregs in the expression levels in peripheral blood of patients in the GC group, the tumor TNM staging was able to be indirectly judged in order to guide surgery and prognosis, moreover, to a certain extent, early discovery and early treatment could be achieved.

This study indicated that the expression levels of CD4⁺CD25⁺Foxp3⁺Tregs in both peripheral blood and tissues were related to tumor TNM staging and lymph node metastasis, that is, identically relative to the patients' potential survival. The expression level of CD4⁺CD25⁺Foxp3⁺Tregs in tissues was related to the differentiated degree of GC, hence, the sensitivity of CD4⁺CD25⁺Foxp3⁺Tregs in tissues was larger and the expression levels of CD4⁺CD25⁺Foxp3⁺Tregs in carcinoma tissues could more correctly express the overall condition of GC. It was more convenient for the detection in the CD4⁺CD25⁺Foxp3⁺Tregs in peripheral blood. The detection of the CD4⁺CD25⁺Foxp3⁺Tregs in peripheral blood was conducive to indirectly judge the GC staging in order to achieve early diagnosis, early treatment and prognosis evaluation. At the same

time, this study also helped to provide a theoretical basis for CD4⁺CD25⁺Foxp3⁺Tregs as the target spot of immunotherapy.

In conclusion, the expression levels of CD4⁺CD25⁺Foxp3⁺Tregs was closely related to the occurrence and development of GC, which was able to provide a theoretical basis for early diagnosis, prognosis evaluation and targeted therapy of GC.

Continuous collections

The above-mentioned peripheral blood of GC patients at months 1, 2, 3, 4, 5 and 6 were continuously collected. The expression levels of CD4⁺CD25⁺Foxp3⁺Tregs was performed FCM detection, thus evaluating the relationship between CD4⁺CD25⁺Foxp3⁺Treg and prognosis. The follow-up was continuously conducted to collect relative information, thus evaluating the relationship between CD4⁺CD25⁺Foxp3⁺Treg and prognosis through survival analysis.

Further studies are needed with larger samples to conclude the warning line of the expression level of CD4⁺CD25⁺Foxp3⁺Treg, thus the expression levels of early CD4⁺CD25⁺Foxp3⁺Tregs of GC.

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LETTER TO THE EDITOR

CLINICAL EFFECT OF INTRAVITREOUS INJECTION OF TRIAMCINOLONE ACETONIDE IN TREATING CYSTOID MACULAR EDEMA

PS. YAN, C. QIAN, GM. WAN, WZ. WANG, Y. DONG and FZ. LI

*Department of Ophthalmology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China**Received August 10, 2015 – Accepted February 15, 2016*

Cystoid macular edema (CME), a commonly seen sign for multiple fundus diseases, is able to induce visual deterioration. The incidence rate of CME is constantly increasing; however, the existing clinical treatments cannot achieve satisfactory curative effects. To explore the curative effect of intravitreal injection of triamcinolone acetonide (TA) in treating CME, this study carried out a clinical test on 39 patients (42 eyes) from The First Affiliated Hospital of Zhengzhou University who developed CME induced by central retinal vein occlusion (CRVO). All 42 eyes received intravitreal injection of 40 mg/ml TA (0.1 ml) and then were followed up for 11-23.5 months. Eyes were examined by slit-lamp microscope, fundus fluorescein angiography (FFA) and optical coherence tomography (OCT) and best corrected visual acuity (BCVA), and intraocular pressure (IOP) of those eyes were detected before and after treatment. Average vision of eyes was 0.1 before treatment, and the vision improved in one month (vision ≥ 0.2 : 100%; vision ≥ 0.5 : 42.9%) and three months (vision ≥ 0.2 : 64.3%; vision ≥ 0.5 : 21.4%) after treatment; but as time went on, the vision of some patients declined; at the last follow-up, patients with vision ≥ 0.2 accounted for 28.6% and those with vision ≥ 0.5 accounted for 7.1%; compared to before treatment, 71.4% patients had improved vision and the remaining 28.6% had declined vision. Some patients were observed with high IOP during treatment, and 7 eyes were found with secondary cataract in posterior capsule of lens at the last follow-up. Intravitreal injection of triamcinolone acetonide proved to have significant short-term curative effect on CEM which is non-sensitive to conventional therapies, but it is likely to induce high IPO and posterior capsular opacification.

Cystoid macular edema (CME) is a sign of multiple fundus diseases in the macular region. Diseases which can cause CME mainly include retinal vein obstruction, diabetic retinopathy, retinal vasculitis (1). CME severely threatens the central vision of patients. Treatment for CME has been dominated by grid pattern laser photocoagulation in the past, but its clinical application is limited due to indication choice, recurrence, non-repeatability and complications (2, 3). Thus treating CME with drugs has been frequently discussed in recent years (4), especially intravitreal injection of triamcinolone

acetonide (TA) (5, 6). However, there has been a lack of evidence on the effectiveness and safety of this treatment for CME, therefore to analyse its curative effect, in this study drug treatment was carried out on CME patients who were then followed-up with the aim to provide relatively comprehensive evidence for clinical application of TA.

MATERIALS AND METHODS

General materials

Thirty-nine patients (20 males, 19 females; age

Key words: triamcinolone acetonide, vitreous body, cystoid macular edema, curative effect analysis

Mailing address:

Dr Panshi Yan,
Department of Ophthalmology, The First Affiliated Hospital
of Zhengzhou University, No.1, Jianshe East Road,
Zhengzhou, 450052, China
Tel.: +86 15393702252
e-mail: yanpsyps@163.com

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ranging from 21 ~ 80 years: average 57.1 years) with central retinal vein occlusion (CRVO)-induced CME who received diagnosis and treatment in The First Affiliated Hospital of Zhengzhou University from November 2012 to December 2013 were selected for this study. All patients went to the hospital due to sudden painless loss of vision. Of 42 eyes, 22 were right eyes and 20 were left eyes. The time from the occurrence of disease to triamcinolone acetonide treatment was 16 days to 10 months (average 4.4 months). Patients who were confirmed as having CRVO and macular oedema by fundus fluorescein angiography (FFA) and optical coherence tomography (OCT), had best corrected visual acuity (BCVA) no higher than 0.6 and central macular thickness (CMT) no lower than 250 μ m, had not undergone oral administration of drugs, intravenous medication and fundus laser treatment before admission were included. Those who had cataract, glaucoma, obvious turbid refractive media, poor fixation visions or other eye diseases that can influence FFA and OCT were excluded. All patients received vision examination, FFA, OCT, intraocular pressure (IOP) examination and slit-lamp microscopic examination. All patients signed informed consent before the treatment and this study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Zhengzhou University, Henen, China.

Vision examination

Vision was examined under the same lighting conditions using international standard visual chart and the measurement results were converted into logarithmic vision for statistical analysis (7). FFA was performed using CF-60UD fundus camera (Canon, Japan), and EYECAP image processing system was used to confirm CRVO as well as CME based on petal-like fluorescein accumulated in the macular area. OCT was carried out using Humphrey-Zeiss OCT scanner (Carl Zeiss, Germany). CMT was measured using the automatic scanning function of OCT machine; scanning lines (6 mm) were fixed at 0° and 90°; the average value was taken as the final result. For those whose macular area was difficultly confirmed, height of the highest point was considered as CMT value. In addition, Tonometer TX-10 non-contact tonometer (Canon, Japan) was used for measuring IOP twice, and the average value was taken as the final result. Opacity of lens was tested with SL-1E slit-

lamp microscope (Topcon, Japan). Opacity of posterior capsule, core and cortex of lens was graded according to LOCS III lens opacity grading method, and opacity levels before and after treatment were compared.

Experimental method

Patients were first anaesthetised by benoxil or 1% dicaine, followed by disinfection. After the settlement of eye speculum, 0.2 ml of TA (40 mg/ml) (Bristol-Myers Squibb, Italy) was injected into the site 4 mm behind the infratemporal limbus corneae; then the needle was vertically inserted into pars plana which was 4 mm behind the infratemporal limbus corneae (8). TA was injected into the vitreous cavity; afterwards, a sterile swab was used to oppress the injection site for three to five min to prevent drug reflow and vitreous incarceration. IOP was immediately measured after drug injection and remeasured two hours later. Anterior chamber puncture was needed if IOP was higher than the normal level. All patients received IOP reexamination on the next day and were asked to take a lateral position to prevent impact of drug accumulated in the macular area. Due to the recurrence of CME, 12 eyes received the second injection and 3 eyes received the injection a third time. All patients went to the hospital for follow-up one day, one week, one month, three months and six months after treatment, respectively. After six months they were followed up irregularly. During follow-up, vision, lens, eye ground, IOP, OCT and FFA were all examined. Patients with 26-mm Hg IOP were given IOP-lowering drugs for treatment. The follow-up lasted for 10 ~ 22.4 months (average 15.9 months).

Data processing

SPSS 19.0 software was applied to process data. Measurement data were expressed by Mean $\pm$ SD. CMT values measured before treatment, one month after treatment and at the last follow-up were compared by paired *t*-test. Difference was considered to have statistical significance if $P < 0.05$.

RESULTS

Comparison of measurement results before and after treatment

Vision of all eyes was 0.1 averagely before treatment. Of 42 eyes, 6 had a vision higher than 0.2

(14.3%). All 42 eyes were detected to have petal like fluorescence accumulation in the macular region by FFA (Fig. 1). OCT results suggested that 42 eyes showed CME with different levels (Fig. 2). Average value of CMT was $(801.68 \pm 248.75) \mu\text{m}$. Six eyes (14.2%) were found with CMT less than $600 \mu\text{m}$, 18 eyes (42.9%) had CMT between 600 and $900 \mu\text{m}$, and the remaining 18 eyes (42.90%) had CMT larger than $900 \mu\text{m}$. IOP of eyes ranged from 11 to 16 mm Hg ($1 \text{ mm Hg} = 0.133 \text{ kPa}$) (average $13.6 \pm 1.5 \text{ mm Hg}$). One month after treatment, significantly relieved fluorescence accumulation in the macular region and complete disappearance of CME were found in 30 eyes (Figs. 3 and 4), accounting for 71.4%; and the remaining 12 eyes (28.6%) had obviously relieved edema; average value of CMT resulted as $(309.64 \pm 232.44) \mu\text{m}$, with a decrease of $(492.04 \pm 151.5) \mu\text{m}$ compared to before treatment ($t = -12.15$, $P = 0.000$); 33 eyes were found with CMT larger than $600 \mu\text{m}$ (78.6%) and the remaining 9 eyes had $600 \sim 900 \mu\text{m}$ CMT (21.4%). At the last follow-up, average CMT was $(479.89 \pm 298.6) \mu\text{m}$, with a decline of $(321.79 \pm 275.11) \mu\text{m}$ compared to before treatment ($t = -4.38$, $P = 0.001$); 21 eyes (50%) were observed with $600 \mu\text{m}$ over CMT, OCT confirmed complete disappearance of CMT in 12 eyes (28.6%); 3 eyes had CMT between 600 and $900 \mu\text{m}$ (7.10%);

6 eyes (14.3%) had CMR higher than $900 \mu\text{m}$;; relieved edema in 27 eyes (64.30%) and more severe condition in 3 eyes (7.1%); 30 eyes (71.4%) had recurred CME. Eighteen eyes out of thirty eyes which recovered three month after treatment recurred and the other 12 eyes had different levels of recurred edema.

Improvement of vision after treatment

All patients had different degrees of vision improvement at different time points after injection and the improvement was more obvious in short-term, especially in the first month after treatment. In the third and sixth month, vision of patients declined to some extent (Table I). In October 2014, i.e., the last follow-up, the average vision of patients was 0.05; twelve eyes had at least 0.02 vision (28.6%) and three eyes had a vision no less than 0.05 (7.1%). Compared to before treatment, 30 eyes had improved vision (71.4%) and 28.6% (12 eyes) had decreased vision.

As shown in Table I, the vision one and three months after treatment was significantly different from the vision before treatment ($p = 0.028$ and 0.008); but the vision six months later had no remarkable difference compared to before treatment ($p = 0.128$). Intravitreal injection can give full play

Table I. *Improvement of vision of patients during six months after treatment.*

Time point	No.	Vision [number of eyes and proportion]			Value	Average value of vision
		>0.5	0.2~0.5	<0.2		
Before	42	0	6(14.30%)	36 (85.70%)	0.2	0.1
1 st day	42	0	18(42.90%)	24 (57.10%)	0.5	0.15
1 st week	42	12(28.60%)	30(71.40%)	0	0.6	0.3
1 st month	42	18(42.90%)	24(57.10%)	0	1.0	0.5
3 rd month	42	9(21.40%)	18(42.90%)	15 (35.70%)	1.5	0.2
6 th month	42	6(14.20%)	12(28.60%)	24(57.20%)	1.0	0.08

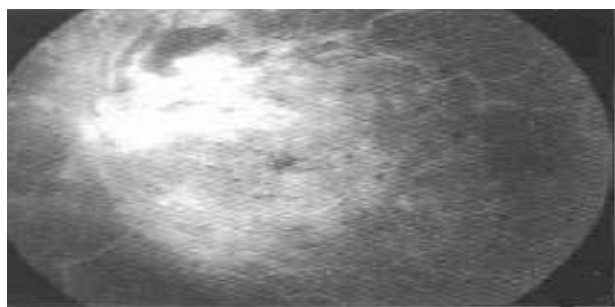


Fig. 1. FFA image of CME eyes before treatment.

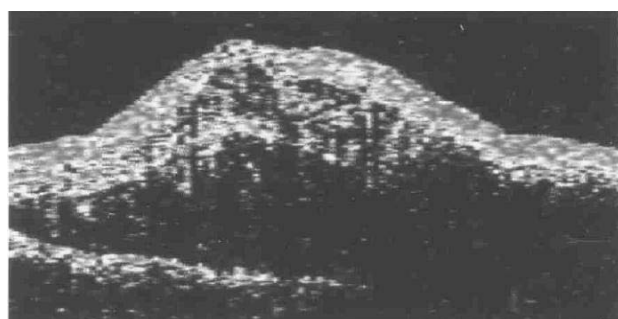


Fig. 2. OCT image of RVO eyes before treatment (obvious macular edema).

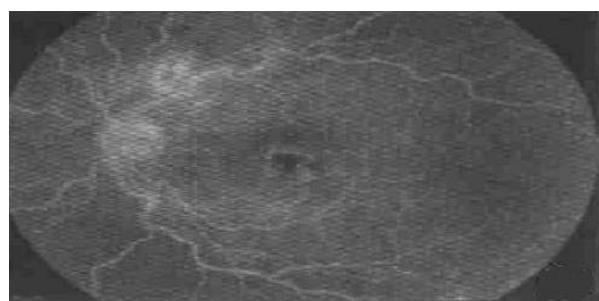


Fig. 3. FFA image of CME eyes 18 months after treatment.



Fig. 4. OCT image of CME eyes 18 months after treatment (macular edema disappeared).

of the bioavailability of drugs. TA usually remains in the vitreous body for 21 to 41 days, but it can still be observed even three months later. The half-life period of TA in the vitreous body is 1.6 days and TA can still be detected in aqueous fluid 24 months later (9, 10). TA can have a long-term effect, and macular oedema gradually disappears in the drug action period (11). The patients in this study had significantly improved vision three months after treatment, suggesting that this was the period that TA achieved its highest effect.

Complications

First was increased IOP. IOP of patients before treatment ranged from 11 ~ 16 mm Hg (average (13.6 ± 1.5) mm Hg). One month, three months and six months after treatment, IOP was (17.93 ± 4.3) mm Hg, (16.53 ± 5.17) mm Hg and (15.73 ± 7.38) mm Hg, respectively, with an increase of 32%, 23% and 13%, respectively, and the highest IOP reached 34 mm Hg. In the period from the 1st week to the 6th month after treatment, 7 eyes had high IOP (22.3 ~ 26.3 mmHg, 27.8 mmHg averagely). Four eyes with IOP ranging from 22.3 ~ 26.3 mmHg recovered without the intervention of drugs, and IOP of 2 eyes recovered after one-month and five-month local medication. One eye without subjective symptom and with lightless feeling, which was given no drugs for lowering IOP, had 34.0 mmHg below IOP. IOP at different time points after treatment had no remarkable difference with that before treatment ($P > 0.05$). No glaucoma was found during follow-up.

At the last follow-up, 7 eyes (16.7%) developed cataract, seen as opacity or aggravated opacity under posterior lens capsule. Of 7 eyes, 4 were observed with yellow or tan opacity, 1 was with mutton fat-like dotted opacity and 2 were with local aggravated opacity. Degree of posterior lens capsular opacification, nucleus and cortex before and after treatment were compared; and the results showed that none of patients had aggravated opacity in lens nucleus. Besides, one eye (2.4 %) developed vitreous hemorrhage; one eye (2.4 %) had pseudo endophthalmitis; one eye developed infectious endophthalmitis, but the disease was

under control after anti-infection treatment; three eyes from two cases had retinal detachment and relieved after resection of vitreous body and silicone oil tamponade.

These findings demonstrate that the complications induced by TA included increased IOP and complicated cataract under posterior lens capsule. We observed no severe cataract during the 6-month follow-up; however, there was incidence of complicated cataract in the final follow-up (average: 15.9 months). The occurrence of cataract appears to be correlated to the dose of drug injected into the vitreous body and action time. All patients who had increased IOP (over 50 mmHg) had no obvious symptoms (including ophthalmodynia, headache and nausea) except for mild eye distension and most patients recovered after one or two hours. However one study found that some patients developed cortico-dependent high IOP one to six months after treatment and most of them had no subjective symptom (12). Hence, regular measurement of IOP is necessary. None of the patients with high IOP needed systemic medication; and generally local medication of IOP-lowering drugs can recover IOP to the normal scope (13). In this study, one case of IOP was not timely measured due to the eruption of atypical pneumonia, leading to the long-term increase of IOP and absence of subjective symptom; besides, IOP of that case became 33 mmHg in reexamination, eventually resulting in optic atrophy and feeling of lightless. Therefore, it can be concluded that increased IOP induced by intravitreal injection of TA is a potential risk factor and needs to be taken into consideration by doctors and patients. But increased IOP seems to be in no obvious correlation to repeated injection of drugs, i.e., the concentration and action time of drugs in eyes, but may correlate to target gene corresponding to glucocorticoid induced glaucoma.

DISCUSSION

CME is a major cause leading to the decreased vision. It has been proved that intravitreal injection can overcome blood-eye barrier and act directly on the lesion, avoiding the side effects caused by the application of glucocorticoids on the whole body (14,

15). Currently, TA is the preferred drug in treating CME (16). TA is a long-acting glucocorticoid with hydrophobic property. An animal experiment (17) suggested the application of TA in eyes was applicable and intravitreal injection of TA had no impact on electroretinogram and retinal tissues of rabbits. A dose of 25 mg is safe for the eyes, and drugs injected into eyes can be maintained for three months (18). This is consistent with the recurrence of macular edema after one to three months that we observed clinically. It is worth noting that repeated injection of drugs seemed to be ineffective in inhibiting the recurrence of macular edema, suggesting the recurrence of macular edema was in no obvious correlation to the dose and action time of drugs.

Intravitreal injection of TA is invasive in treating macular edema, and the complications induced include increased IOP, eye infection, vitreous hemorrhage and phacoscotasmus. Technical operation and toxic and side effects are two main factors resulting in complications. The results of this study suggested that the increase of IOP and posterior lens capsular opacification are the major complications in the treatment process. Intravitreal injection of TA had no obvious toxic or side effects and did not induce severe complications.

To sum up, intravitreal injection of TA has a remarkable short-term curative effect in the treatment of CME. However, how to reduce the recurrence of CME and avoid complications remains to be further explored as the sample size of the study was small.

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LETTER TO THE EDITOR

ULTRASOUND DIAGNOSIS IN GYNECOLOGICAL ACUTE ABDOMEN

J. WU¹, SH. CUI², HZ. LI¹, QH. LI¹, R. YUAN¹, YP. ZHANG¹ AND TW. ZHAO³

¹Department of ultrasound, the Third Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China; ²Department of Obstetrics and Gynecology, the Third Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China; ³GE Company, Zhengzhou, Henan, China

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As ultrasound diagnosis is applied more frequently in gynecological acute abdomen, we carried out this study to discuss the diagnosis of endometriosis with ultrasound imaging and analyze the efficacy of microRNA used for diagnosing endometriosis and evaluating prognosis by comparing differentially expressed microRNA in subjects with or without endometriosis. Ultrasound examination results and clinical pathological examination results of 60 cases of gynecological acute abdomen were compared. Blood samples were collected from patients with endometriosis. Of 60 cases, 38 cases recurred in 20 months after surgery and the remaining 22 cases had no recurrence in the 30-month follow-up. Additionally, blood was collected from 40 patients without endometriosis as control. Then total RNA was extracted from these blood samples to determine the difference of expression of microRNA (miR-17-5p, miR-20a, miR-199a and miR-141). Compared to healthy subjects, the endometriosis patients showed significantly increased expression of miR-199a, but the expression of miR-17-5p, miR-20a and miR-141 had an obvious decrease; the differences were statistically significant ($p < 0.01$). For recurred cases, miR-199 showed a remarkably high expression and miR-17-5p and miR-20a expressed significantly low.

Gynecological acute abdomen mainly occurs as abdominal pain (1) and is characterized by sudden onset, severe symptoms and high risk. Endometriosis, one category of gynecological acute abdomen, is an estrogen-dependent inflammatory gynecological benign disease and also the leading cause for female infertility. Symptoms of endometriosis includes dysmenorrheal, chronic pelvic pain and secondary infertility (2).

Many scholars have studied the application of ultrasound diagnosis in gynecological acute abdomen. Li et al. (3) proposed in 2011 that the accuracy rate of ultrasound diagnosis was 94.17%,

and they recognized its important role in diagnosing gynecological acute abdomen. In 2014, Saddala et al. (4) declared that ultrasound examination was convenient and accurate. Demidov et al. (5) once made an analysis on ultrasound diagnosis. Schmid-Tannwald et al. (6) put forward in 2012 that the resemblance and intersection in ultrasonogram still required being identified and analyzed in combination with clinical results, though ultrasound diagnosis was convenient and highly accurate.

We performed ultrasound diagnosis on 60 cases of endometriosis and compared the results obtained with clinical pathological results. Moreover,

Key words: ultrasound diagnosis, microRNA, gynecological acute abdomen, endometriosis

Mailing address:

Ms Shihong Cui,
Third Affiliated Hospital of Zhengzhou University,
No. 7, before street, the Two Seven district,
Zhengzhou, Henan, 450052, China
e-mail: cuishihongsh@163.com

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microRNA in blood collected from subjects with or without endometriosis was compared.

MATERIALS AND METHODS

Experimental materials

Instruments used in the study included VOLUSON E8 color Doppler ultrasound machine (GE, USA), ACUSON X700 high-end four-dimensional color Doppler ultrasound machine (GE, USA), Allegra X-15R desktop refrigerated centrifuge (Bio-RAD Company, USA), Revco ultra cold storage freezer (EXF series, Bio-RAD Company, USA), ABI PRISM 7000 Sequence Detection System (Bio-RAD Company, USA), real-time quantitative Polymerase Chain Reaction (PCR) amplifier (Bio-RAD Company, USA) and NanoDrop 2000c spectrophotometer (Thermo Company, USA).

Reagents used included RNA extraction reagent (Invitrogen Company, USA), ReverTra Ace qPCR RT Kit (TaKaRa Company, Japan), SYBR Green PCR master Mix (Applied Biosystems Company, USA), miRNA reverse transcription primer and test primer (Ribo Bio Company, China) and RNeasy Plus Micro Kit (Qiagen Company, Germany).

Study subjects

Patients who were diagnosed with endometriosis by ultrasound and surgical pathology, had regular menstrual cycle, received non-radical operation (with at least one side of ovary reserved), had not undergone surgery for endometriosis, aged no more than 45 years, had not taken hormone drugs within 6 months before surgery and had no reproductive malformation, malignant tumor or chronic internal disease were enrolled in the study.

Subjects who were diagnosed without endometriosis by ultrasound and surgical pathology, aged under 45 years, had regular menstrual cycle, had no uterine adenomyosis, had not taken hormone drugs within the previous 6 months and had no reproductive malformation, gestation-associated diseases or complex chronic medical diseases were selected as controls.

Whole blood sample (6 ml) was extracted from each patient before treatment and preserved in an anticoagulative tube that had been processed by ethylenediamine tetraacetic acid (EDTA-K); afterwards, the tube was preserved at 4°C. Within 24 h, white blood

cell and plasma were isolated by centrifuging at 5000 bpm. Then the white blood cells and blood samples were preserved at -80°C.

In the control group, subjects were proved without endometrium, uterine adenomyosis or uterine myoma by ultrasound and surgical pathology. The second group was a non-recurrence group. Patients were followed-up every three months in the first year after treatment and once a year from the second year. During follow-up, patients were allowed to go to hospital at any time if they felt uncomfortable. Ultrasound examination was performed during follow-up. Typical ovarian ectopic cyst was not observed in those patients and patients suffered from no discomfort, such as chronic pelvic pain. Those who did not have a recurrence in the period from the end of operation to the last follow-up (> 30 months), totally 22 cases, were recruited into the non-recurrence group. The last group was the recurrence group. Patients who were observed with typical image of ovarian ectopic cyst (single or multiple round or oval cysts with thick wall, homogeneous low-echo liquid mass scattered on tiny bright light point/band, no solid or papillary structure in cyst or rich blood flow in focus) and who recurred less than 20 months after operation, totally 38 cases, were recruited into the recurrence group. Informed consent was obtained from all subjects and the study was approved by the medical ethics committee of the Third Affiliated Hospital of Zhengzhou University and reviewed by pathologists.

Extraction of microRNA from different blood samples

Plasma preserved at -80°C was dissolved in 37°C water and then put on ice. 200 µl of plasma was blended with 1000 µl of Qiazol Lysis using oscillator or pipette. Then it was placed at 15~25°C. Six minutes later, 3 µl of marinas' Serun/Plasma Spike in control was added, mixed using a pipette and centrifuged. Then chloroform (200 µl) was added. After 15 s of shaking, the mixture was placed at room temperature. 3~4 min later, it was put into an Eppendorf (EP) tube and centrifuged at 12000 g for 15 min. The supernate obtained was transferred into a new EP tube (1.5 ml). Then absolute ethyl alcohol whose volume was 1.5 times of the supernate was added. The liquid in the EP tube was then transferred into RNeasy MinElute spin column. 70 µl liquid was taken from the column each time and put into a clean collecting tube (2 ml), followed

by centrifuge at 8000 g for 15 s at 15~25°C. When the volume was more than 700 µl, the liquid in the EP tube was transferred into RNeasy MinElute spin column. 700 µl of Buffer RWT was added into the RNeasy MinElute spin column, followed by centrifuge at 8000 g for 15 s at 15~25°C. Next, 500 µl of Buffer RPE was added into the RNeasy MinElute spin column, followed by centrifuge at 8500 g for 15 s at 15~25°C. Then ethyl alcohol (80%, ratio of absolute ethyl alcohol to RNase free water: 4:1) was added to the RNeasy MinElute spin column, followed by centrifuge at 8500 g for 2 min at 15°C ~ 25 °C. After the cover of RNeasy MinElute spin column was opened, the liquid was centrifuged at 12000 g for 5 min at 15~25 °C. Then 16 µl of RNase-free water was added onto the center of filter membrane of the RNeasy MinElute spin column. One minute later, the liquid was centrifuged at 12000 g at 15°C ~ 25°C for 1 min to elute RNA. RNA collected was finally placed on ice and detected for the concentration with NanoDrop 2000c spectrophotometer. Then it was preserved at -80°C.

Reverse transcription of RNA of plasma sample

NanoDrop 2000c spectrophotometer was used to test the concentration of total RNA in the plasma extracted; 50 ng of RNA was taken for subsequent reverse transcription. When the extracted total RNA was less than 50 ng, the maximum loading quantity of sample was taken for reverse transcription.

Reverse transcription primer mixture (80 µl) was prepared using 1 µl of miRNA RT-primer and diethyl pyrocarbonate (DEPC) water. Reverse transcription primer mixture (19 µl) composing of 50 ng of RNA template, DEPC water and 4 µl of primer mixture was preheated at 70°C for 10 min and then processed by ice bath for 2 min. Besides reverse transcription system was set up based on a reaction system composing of 15 µl of DEPC water, 10 µl of RT-butter, 4 µl of deoxy-ribonucleoside triphosphate (dNTP), 1 µl of RNase inhibitor and 1 µl of RT Aase. The prepared system (31 µl) was added into the preheated reverse transcription primer mixture. The product of reverse transcription was finally preserved at -80°C.

Statistical analysis

Statistical Package for Social Sciences version 17.0 for Windows (SPSS, Chicago, IL, USA) was used for data analysis. Corresponding scatter diagram was made using

GraphPadPrisni (version 17.0). Continuous variable was expressed as mean $\pm$ SD, and classified variable was expressed as frequency and percentage. *t*-test was used to compare continuous variables between groups. Fisher's exact test or *chi*-squared test was applied to compare classified variables between groups (7).

RESULTS

A total of 49 cases were diagnosed with endometriosis, mostly on the right side, 3 cases of which were misdiagnosed as cystic teratoma and 6 cases which actually had endometriosis were misdiagnosed as ovarian tumor (8). In ultrasound image, endometriosis presented as round or oval cystic mass with thick and rough wall and unclear edge behind uterus; the diameter of mass was 3 ~ 6 cm; the largest and smallest size of mass was 8.6 cm $\times$ 7.6cm $\times$ 5.3cm and 2.3 cm $\times$ 1.9 cm $\times$ 2.2 cm respectively. We found fluid sonolucent area within cyst showed two types of light spot, which were concentrated and even spot (41 cases) and sparse spot (8 cases). Cases with concentrated and even spot tended to have larger and high-tensile mass during menstrual period. Of the cases with sparse spot, 3 were found with separation light band within cysts (9). Fig. 1 shows the ultrasound image of one case of endometriosis.

Clinical data of subjects

General data of subjects in the recurrence group, the non-recurrence group and the non-endometriosis group are shown in Table I. The ages of all subjects were analyzed and no significant difference was found. Factors such as Revisal-American Fertility Score (RAFS) and stage, postoperative pregnancy rate and the maximum diameter of ovarian cyst were observed with no remarkable difference between the recurrence group and the non-recurrence group.

Results of Real-time Quantitative PCR

Expression of four candidate miRNA in cases of endometriosis and controls was detected with real-time quantitative PCR. Compared to the non-endometriosis group, miRNA-20a, miRNA-141 and miRNA-17-5p showed a declined expression and miRNA-199a increased in patients with

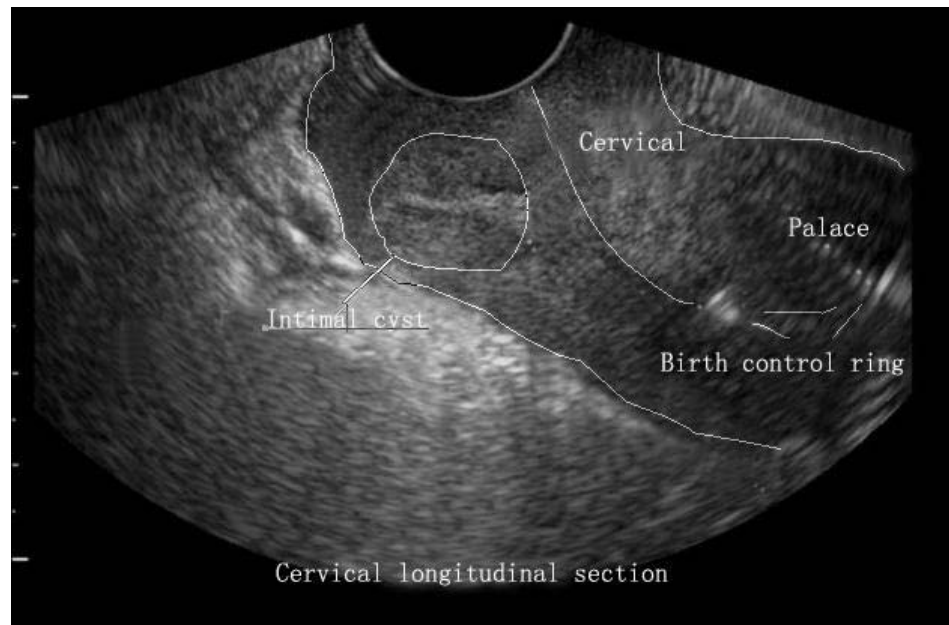


Fig. 1. Ultrasound image or endometriosis.

Table I. General data of cases.

Variable name		Recurrence group	Non-recurrence group	Normal control group	p value
Age at surgery		31.29±6.55	31.90±7.27	30.59±6.87	NS
RAFS score		42.53±25.04	41.48±22.55	—	NS
Pregnancy		9 (28.21%)	7(30.01%)	—	NS
RAFS stage	III	17(54.03%)	13 (51.86%)	—	NS
	IV	15(47.11%)	11 (45.44%)		
Body mass index (BMI)		21.28±3.07	23.66±4.20	—	NS
Complaint of Infertility	Yes	11(34.67%)	8 (33.43%)	—	NS
	No	21(65.55%)	16 (66.89%)		
Complaint of Dysmenorrheal	Yes	23(71.79%)	17 (70.26%)	—	NS
	No	9(28.32%)	7 (29.19%)		
Size of cyst		5.87±2.76	6.66±2.53	—	NS

NS: not significant

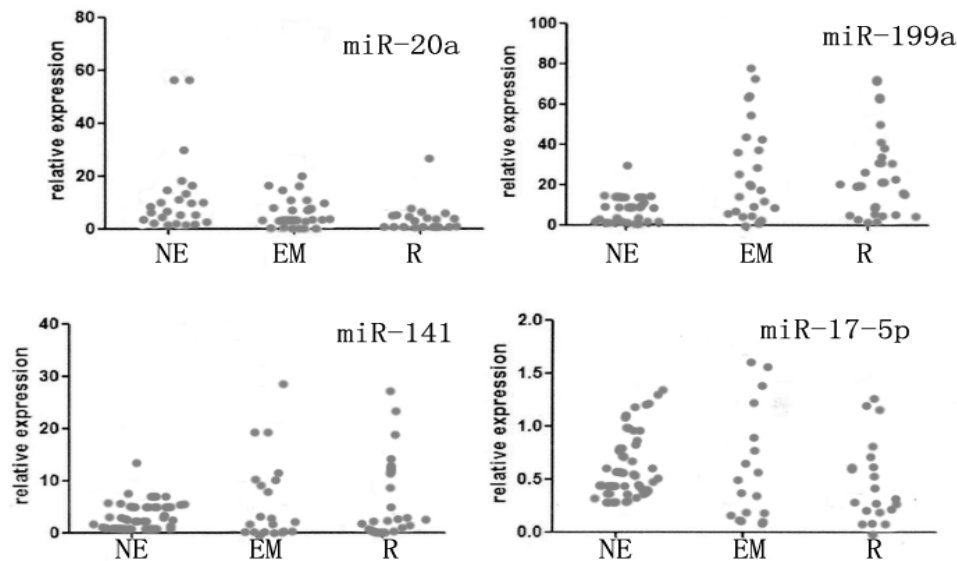


Fig. 2. *miRNA-20a, miRNA-141, miRNA-199a and miRNA-17-5p expression levels in normal case, recurrent case and non-recurrent case.*

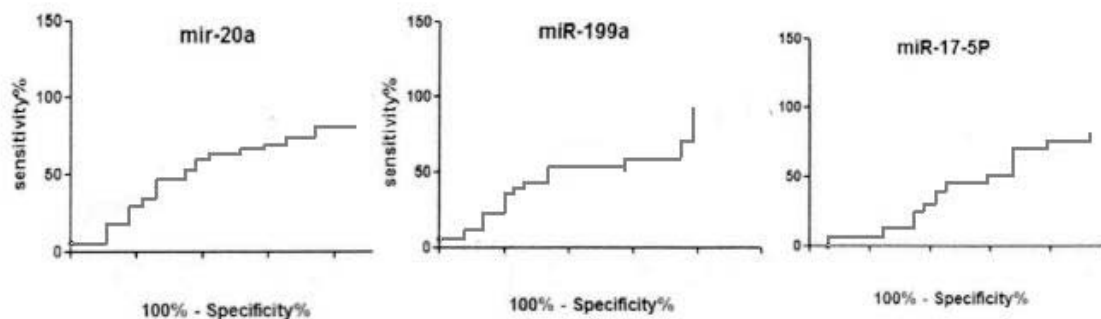


Fig. 3. *Analysis of detection efficacy of miRNA-20a, miRNA-27-5p and miRNA-199a with ROC curve.*

endometriosis ($p < 0.05$). Expression of miRNA in the recurrence group and the non-recurrence group was compared. We found miRNA-20a and miRNA-17-5p in the recurrence groups expressed lower compared to the non-recurrence group, and the difference was remarkable ($p < 0.05$); miRNA-199a expressed higher in the recurrence group and the difference was statistically significant ($p < 0.05$); miR-141 in the recurrence group and the non-recurrence group had no significant difference.

Analysis of detection efficacy of various indexes with receiver operator characteristic (ROC) curve

Through analyzing ROC curve, we found that, miRNA-20a, miRNA-17-5p and miRNA-199a all could be regarded as effective biological indexes for distinguishing recurrence and non-recurrence cases. Area under the Curve (AUC) of miRNA-20a, miRNA-17-5p and miRNA-199a was 0.83 (95% CI=0.74-0.99), 0.76 (95% CI=0.64-0.89) and 0.71 (95% CI=0.58-0.83), respectively.

DISCUSSION

Endometriosis usually reflects different severity of clinical performance; mild cases of endometriosis often present no clinical symptoms (10). We found miRNA-20a, miRNA-17-5p and miRNA-199a in plasma could be taken as the biological markers for identifying the recurrence of endometriosis (11). Real-time quantitative PCR detection results suggested that the expression of miRNA-20a, miRNA-141 and miRNA-17-5p reduced and miRNA-199a increased remarkably in endometriosis patients compared to the non-endometriosis group (12); the expression of miRNA-20a and miRNA-17-5a in the recurrence group showed an obvious decrease compared to the non-recurrence group. While analyzing the efficacy of the recurrence and non-recurrence group with ROC curve, we found the AUC of miRNA-20a, miRNA-199a and miRNA-17-5p was 0.83 (95% CI = 0.74 – 0.99), 0.76 (95% CI = 0.64-0.89) and 0.71 (95% CI = 0.58 -0.83), respectively. This work provides a new detection means for diagnosis of endometriosis and evaluation of prognosis (13).

Currently we know little about the effect of miR-200 family in endometriosis. Some members in miR-200 family showed reduced expression in tissue from patients with endometriosis (14), which is consistent with the detection results we obtained. However, this study did not find the significant expression difference between the recurrence group and the non-recurrence group. In addition, it has been reported that, miR-200c showed abnormal expression in patients with uterine leiomyoma (15).

Diagnosis with ultrasound imaging is subject to some limitations. Hence medical staff cannot treat patients only relying on the diagnostic results of ultrasound imaging, but should also consider disease history and clinical data.

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LETTER TO THE EDITOR

CORRELATION BETWEEN MOLYBDENUM TARGET MAMMOGRAPHY SIGNS AND PATHOLOGICAL PROGNOSTIC FACTORS OF BREAST CANCER

Y. ZHANG, AD. MA and HX. JIA

*X-ray Department, Shandong Jining No.1 People's Hospital, Jining, Shandong, China**Received November 12, 2015 – Accepted March 7, 2016*

This study explores the correlation between molybdenum target (mo-target) mammography signs and pathological prognostic factors of breast cancer. We selected 320 breast cancer patients who were treated between January 2014 and January 2015; using single-factor and multiple-factor logistic regression method, we made correlation analysis on their clinical features, pathological features and mo-target mammography signs. Among mo-target mammography signs, lumps accompanied with calcification and blurry edge were associated with high histologic grades; lumps accompanied with calcification and clear edge were associated with Ki-67 positive; compared with the patients who had lumps with non-stellate edges, positive rates of estrogen receptor (ER) and progesterone receptor (PR) were significantly higher for the patients who had lumps with stellate edges ($p < 0.01$), while positive rate of human epidermal growth factor receptor-2 (HER-2) and tumor proliferative activity were significantly lower ($p < 0.05$, $p < 0.01$). According to the study, we can conclude that mo-target mammography signs mainly include lumps and calcification. Mo-target mammography can improve the accuracy of diagnosis and reduce misdiagnosis or missed diagnosis. Part of mo-target mammography signs are associated with clinical pathology prognostic factors; by grasping the relation, breast cancer patient conditions are expected to be relieved.

Breast cancer is one of the malignant tumors with high incidence among women worldwide, ranking the fourth in mortality of female cancers, and its incidence is rising continuously (1). As known, early detection and early diagnosis are the keys to improve curative effects of breast cancer (2).

Ductalcarcinoma *in situ* (DCIS), an early stage of breast cancer (3), is generally regarded as palpable breast masses by physical examination; with the wide use of mo-target radiography, DCIS is recognized as impalpable lesions (mostly dense clusters of tiny calcification shadows) by X-ray. Due to the poor transfer ability and favourable prognosis of DCIS, early diagnosis and discovery are of important clinical

significance to the treatment and prognosis. Mo-target mammography is a non-invasive examination method for diagnosis of breast cancer; it is easy to operate mo-target mammography with little pain and high resolution. Moreover, mo-target mammography is of high sensitivity for diagnosis of breast cancer (4). Especially for carcinoma *in situ* with relatively small lesions, mammography is of important diagnostic value (5).

Prognostic indicator of breast cancer is a traditional research subject. In recent years, a lot of attention has been drawn to the value of breast cancer biology factors in prognosis and curative effect prediction (6-8). Biological factors can reflect the

Key words: breast cancer, pathological prognostic factors, molybdenum target mammography

Mailing address:

Dr Huanxiang Jia,
No.6, Jiankang Road, X-ray department,
Shandong Jining No.1 People's Hospital,
Jining, Shandong, 272100, China
e-mail: jhx054@163.com

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biological characteristics of breast cancer and relate to the etiology and development of tumor; it is of vital significance to the prognosis of breast cancer. This study mainly investigated the correlation between mo-target mammography signs and pathological prognostic factors in cases of female breast cancer.

MATERIALS AND METHODS

Research subjects

Between January 2014 and January 2015, 368 cases were verified with breast cancer in Jining No.1 People's Hospital of Shandong province. All of them had preoperative mo-target mammography and postoperative pathology detection. Forty-eight patients who had preoperative neoadjuvant chemotherapy were excluded because neoadjuvant chemotherapy could affect the evaluation of lump size. This study included 320 cases of women (aged from 25 to 80 yrs, average 52 yrs), including 13 cases of carcinoma *in situ* (4.06%), 12 cases of carcinoma *in situ* accompanied with tiny infiltration (3.75%); among the non-special type invasive carcinoma, there were 270 cases of invasive ductal carcinoma (84.38%), 5 cases of invasive lobular carcinoma (1.56%), 8 cases of mixed ductal carcinoma (2.5%); among the special type invasive carcinoma, there were 6 cases of mucous adenocarcinoma (1.88%), 4 cases of medullary carcinoma (1.25%), one case of tubular carcinoma (< 1%), and one case of metaplastic carcinoma (< 1%).

Before the cases were selected, all the patients and their families were particularly informed of the potential risks during the research process; family members of the patients voluntarily signed written informed consent which was preserved with the medical records. After discussion, the hospital's medical ethics committee unanimously agreed that the patients could receive clinical tests in Jining No.1 People's Hospital of Shandong province.

Research methods

All-digital mo-target mammography GE Senographe 2000 D was applied; low energy (20-30 kVp) X-ray and small focus (0.3 mm) radioactive source were used for molybdenum target examination. During breast photography, breast tissue was pressed flat so that rays could penetrate evenly and density difference of image was minimized.

The evaluation criteria of photography are as follows. For cephalopodium axial photography, tube was in 0° horizontal position, breast was in the middle of the photograph, nipples were in tangent position, part of pectoralis major was visible; for medial-lateral oblique photography, tube was swiveled by 45°, nipples were in tangent position, parenchyma of mammary gland was fully unfolded, part of the abdominal wall was included in the photograph yet separate from the lower part of breast, pectoralis major was visible and relatively loose with its lower edge reaching the level of nipples.

Pathological examination

Surgery or excision biopsy was regarded as the gold standard. Prognostic parameters included: histological grade, ER, PR, HER-2, lympho vascular invasion (LVI), tumor proliferation index (Ki-67) and tumor suppressor gene P53. Tumor histological grading covered the evaluation on growth pattern of invasive carcinoma and characteristics of cells; Nottingham breast cancer grading method was adopted for evaluation of the formative proportion of small and medium-sized tubes, nuclear shape and mitotic cell population. Every parameter was evaluated with three levels. Histological grading was divided into three levels: the score of 3 to 5 referred to superior differentiation (grade I); the score 6 to 7 it referred to moderate differentiation (grade II); the score 8 to 9, referred to inferior differentiation (grade III). In this study, grade III was high histological grade, while grade I or grade II were lower grades. In addition, lympho vascular invasion (LVI) was the general terms for tumor lymphatic infiltration and vascular invasion.

In this study, the detection of ER and PR was performed with immunostaining of paraffin sections. When more than 10% of the tumor cells presented nuclear staining, the result was determined as ER and PR positive; when 1% to 10% of the tumor cells presented nuclear staining, the result was determined as low positive; when there was no nuclear staining of tumor cells, the result was determined as ER and PR negative (normal control was necessary). The conditions of HER-2 were detected by using monoclonal antibody with immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH); detection result of IHC was determined as negative for 1+ and positive for 3+. For the breast cancer patients whose HER-2 protein was moderate positive (2+), if HER-2 gene was amplified as positive,

the result was eventually determined as HER-2 positive; if HER-2 gene was amplified as negative, the result was eventually determined as negative. Tumor proliferation index (Ki-67) was determined with IHC technology, the expression of Ki-67 was divided into low expression ($\leq 15\%$, negative) and high expression ($> 15\%$, positive). P53 gene mutation was detected with IHC method; expression of P53 included: - (negative), 1+, 4+ (positive). Table I shows the specific evaluation table.

Analysis of mo-target mammography signs

Signs of lump edges were described as follows: clear edges, partially clear edges or fuzzy edges; lobule edges; sentus edges. There was a distinct boundary between the lumps with clear-cut edges and the surrounding tissue, or at least 75% of the edge was clear-cut, the rest may be unclear due to overlapping tissue. Fuzzy edge means the edges of lumps could not be determined clearly or part of the edges

had infiltration, in which case the possibility of overlapping of normal breast tissue should be excluded.

Statistical analysis

Software SPSS 17.0 was used for statistical analysis. The method of single factor and unconditioned logistic regression analysis was adopted to analyze correlation between mo-target mammography and pathological prognostic factors; entry criterion was 0.05; odds ratio (OR) and its confidence interval (CI) were calculated; correlation between mo-target mammography signs and pathological prognostic factors was analyzed with stepwise method through multi-factor unconditioned logistic regression model, the entry criterion was 0.05; OR value and confidence interval were calculated. In each group of mo-target mammography signs, the most benign signs were used as control for other signs. *Chi-squared* test was used to evaluate the differences between image signs.

Table I. Evaluation table of the variables.

Variables	Evaluation values
Histological grade	1 (Grade I); 2 (Grade II); 3 (Grade III)
Neoadjuvant therapy	0 (none); 1 (exist)
Vessel invasion	0 (none); 1 (exist)
PR positive	0 (none); 1 (exist)
ER positive	0 (none); 1 (exist)
Ki-67 positive	0 (none); 1 (exist)
HER-2 positive	0 (none); 1 (exist)
Pathological types	1 (Invasive ductal carcinoma); 2 (Invasive lobular carcinoma); 3 (DCIS); 4 (DCIS with slight invasion); 5 (Lobular carcinoma in situ); 6 (Mucous adenocarcinoma); 7 (Medullary carcinoma); 8 (Tubular carcinoma); 9 (Metaplastic carcinoma); 10 (Mixed type ductal carcinoma); 11 (Adenoid cystic carcinoma)
X-ray types	0 (None); 1 (Sentus lumps without calcification); 2 (Sentus lumps with calcification); 3 (Lumps without calcification or sentus) 4 (Calcification lumps without sentus); 5 (Single calcification); 6 (Other changes)

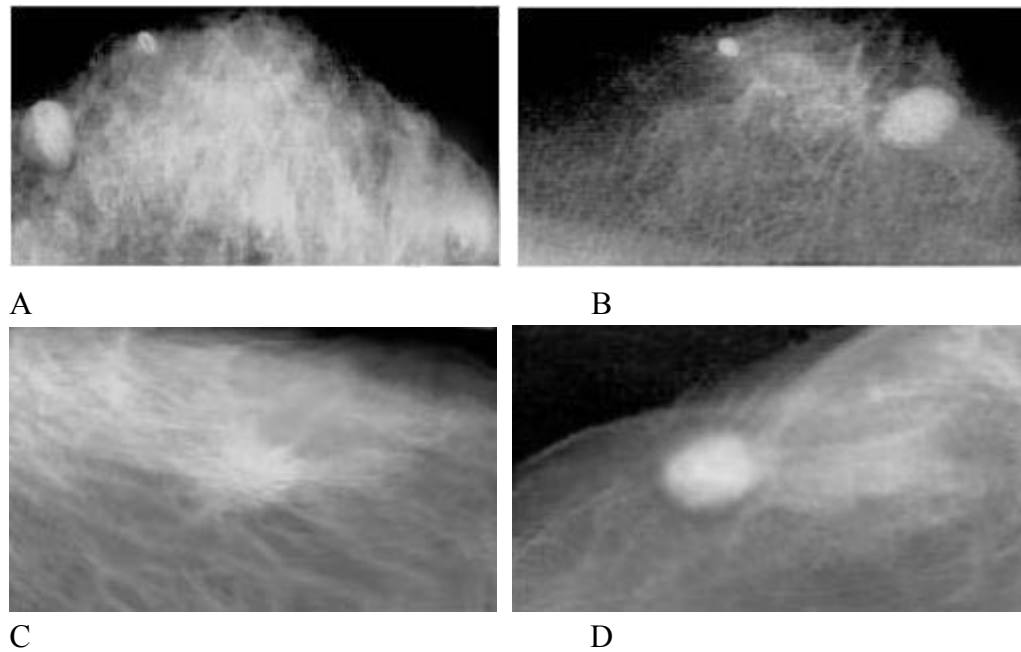


Fig. 1. Breast lesion forms based on single factor analysis of mo-target mammography. *A,B*) Breast fibroadenoma with regular lesion form and clear boundary. *C,D*) Breast cancer with irregular lesion form and fuzzy boundary.



Fig. 2. Patient condition analysis.

RESULTS

Analysis of general data

Based on single factor analysis, high histological grade was related to the lumps without calcification or sentus, lumps with calcification and sentus, calcification lumps without sentus; HER-2 positive was related to the lumps with calcification and sentus, calcification lumps without sentus, single calcification and other changes; Ki-67 was related

to the lumps without calcification or sentus, calcification lumps without sentus. Fig. 1, A and B show images of breast fibroadenoma with regular lesion form and clear boundary; C and D are the images of breast cancer with irregular lesion form and fuzzy boundary.

Correlation between mo-target mammography signs and pathological prognostic factors

Based on multi-factor analysis of mo-target

Table II. Correlation between *mo-target* mammography signs and pathological prognostic factors.

Signs and factors		Subgroup cases	Positive rate (%)	OR	95% CI	P value
High histological grade						
Lump edge	Sentus	70	40.0% (28/70)	Control	—	—
	Fuzzy	70	50.0% (35/70)	2.479	1.478-4.163	0.001
	Clear	70	10.0% (7/70)	1.521	0.632-3.638	0.352
Lumps with calcification	None	70	20.0% (14/70)	Control	—	—
	Exist	70	80.0% (56/70)	1.415	1.012-2.696	0.045
Ki-67 positive						
Lump edge	Sentus	115	56.5% (65/115)	Control	—	—
	Fuzzy	115	33.0% (38/115)	0.792	0.528-1.196	0.068
	Clear	115	10.5% (12/115)	0.686	0.334-1.406	0.012
Lumps with calcification	None	115	39.1% (45/115)	Control	—	0.005
	Exist	115	60.9% (70/115)	1.264	1.204-2.771	—
HER-2 positive 0.005						
Lump size	≤ 2cm	68	36.8%(25/68)	Control	—	—
	2.1-5cm	68	51.5%(35/68)	1.336	0.756-2.357	0.319
	> 5cm	68	11.8% (8/68)	3.822	1.325-11.022	0.012
Lumps with calcification	None	68	26.5%(18/68)	Control	—	—
	Exist	68	73.5% (50/68)	2.405	1.477-3.915	< 0.001

mammography signs, high histological grade was related to calcification and the lumps with fuzzy edges; calcification and large lumps (> 5 cm) were related to HER-2 positive; lumps with clear edges were related to Ki-67 positive; the cases without image signs were related to ER positive, P53 and LVI.

As shown in Table II, it was analyzed that high histological grade was related to the signs of lumps without calcification or sentus, lumps

with calcification and sentus, calcification lumps without sentus; single calcification and the other signs had little correlation with high histological grade; in molybdenum target X-ray signs, blurred edges of lumps and the lumps accompanied with calcification were correlated with high histological grade.

Case analysis

A 51-year-old patient was randomly selected

for examination; according to mo-target mammography, there was patchy calcification in her breast; according to B ultrasound examination, there was duct expansion near areola, and highlight spots were seen in lumen. Postoperative pathology was high-grade intraductal carcinoma. The corresponding images are shown in Fig. 2.

DISCUSSION

In this study, certain mo-target mammography signs (lumps without calcification or sentus, lumps with calcification and sentus, calcification lumps without sentus) were considered to be related to high histological grade, which was proved by several previous studies.

Overexpression of HER-2 is an independent recurrent risk as well as a predictive factor for patients' survival rate (9, 10). In this study, it was found that the overexpression of HER-2 was closely to calcification. Wang et al. (11) reported that spiculated lump edges and calcification were related to HER-2 in ER/PR negative breast cancer. A few studies revealed that survival of the patients with breast cancer changed with lump size: the larger the lumps, the shorter the survival period (12-14).

Ki-67 is an index of tumor proliferation rate (15). It was found that the expression of Ki-67 was related with a series of clinical factors; whether there was lymph node metastasis or not, high expression of Ki-67 could predict the shortening of overall survival and disease-free survival. In this paper, based on mo-target X-ray signs, clear edge lumps and the lumps with calcification were positively related with Ki-67. In general, clear and smooth tumor edge is an important sign of benign tumor in mo-target examination (16-18).

To sum up, mo-target mammography signs of breast cancer are correlated with some of the clinical pathological prognostic factors. Calcification signs are associated with HER-2. With high sensitivity, mo-target mammography signs can improve diagnostic accuracy of breast intraductal carcinoma and reduce the occurrence of misdiagnosis. Undoubtedly, mo-target mammography signs are of high clinical significance.

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LETTER TO THE EDITOR

EARLY ENTERAL AND PARENTERAL NUTRITION ON IMMUNE FUNCTIONS OF NEUROCRITICALLY ILL PATIENTS

SY. QI, WT. WANG, CY. CHEN, ZD. CHU, XJ. LIU and XJ. LIU

Department of Critical Care Medicine, Second Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan, China

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This study was designed to investigate the influence of early enteral and parenteral nutrition on immune functions of neurocritically ill patients. Patients who were admitted to the neurological intensive care unit (ICU) of The Second Affiliated Hospital of Zhengzhou University between May 2014 and January 2015 were selected. They had been hospitalized for more than one week and received enteral nutrition (EN) via nasogastric tube, with a gross energy of 25 kcal/(Kg · d). Patients were divided into EN group, EN + early PN (EPN) group and EN + supplemental PN (SPN) group according to the time of PN support. Differences in patients' general information and changes in serum protein and immune indexes were compared between the three groups. On admission, patients' Glasgow coma scale (GCS), age, immune functions and protein indexes had no obvious differences between the three groups. After nutritional support, serum protein level reduced in the EN group while prealbumin (PALB) and retinol binding protein (RBP) increased in the EN + EPN group and EN + SPN group after one week of admission to hospital, and the differences were statistically significant ($p < 0.05$). Total protein (TP), albumin (ALB), PALB and transferrin (TRF) increased significantly in the EN + EPN group and EN + SPN group compared with the EN group ($p < 0.05$); before and after treatment, an increase was found in ALB in the EN + EPN group in comparison with EN + SPN group, with a notable difference ($p < 0.05$); C3, C4, immunoglobulin M (IgM) and immunoglobulin A (IgA) increased in the EN + SPN group after nutritional support compared with before treatment, and the difference was statistically significant ($p < 0.05$). Moreover, immunoglobulin G (IgG) and IgA in the EN + EPN group increased after nutritional support comparing to prior to nutritional support, and the difference was statistically significant ($p < 0.05$). After nutritional treatment, IgA and IgG increased markedly in the EN + EPN group, and there was a statistical significance between the groups ($p < 0.05$); the EN + EPN group and EN + SPN group exceeded the EN group in total lymphocyte count (TLC), and the difference had a statistical significance ($p < 0.05$). These results demonstrate that neurocritically ill patients achieving the target energy can avoid malnutrition and immunodeficiency; serum protein decrease can cause malnutrition after one week of EN support; and enteral and parenteral nutrition can improve nutritional and immune indicators of neurocritically ill patients in the acute phase. In addition, EPN is more likely to improve malnutrition and immune functions of critical patients than SPN.

Nutritional support refers to maintaining or supplementing a variety of nutrients when the body has abnormal nutritional status or patients' nutrition

is in short supply (1). To date, there are two major nutritional supports, i.e., parenteral nutrition (PN) and enteral nutrition (EN), which can be subdivided

Key words: neurology, enteral nutrition, parenteral nutrition, immune functions

Mailing address:

Dr Xiaojun Liu,
No. 2 Jingba Road, Jingshui District,
Department of Critical Care Medicine,
The Second Affiliated Hospital of Zhengzhou University,
Zhengzhou, Henan, 450014, China
e-mail: xiaojunliuxj@163.com

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into early EN (EEN) and supplemental PN (SPN) according to the time of nutritional support (2-4). A lot of shortcomings exist in PN, which can result in gastrointestinal dysfunction and intestinal mucosa atrophy (5-7). Nowadays, EN is superior to PN (8). The biggest advantage of EN lies in promoting gastrointestinal peristalsis, improving the permeability of intestinal mucosa and reducing intestinal flora as well as endotoxin translocation (9). In an early stage, heat is mainly supplied by PN and then PN intake decreases gradually until heat is completely supplied by EN. This kind of nutritional supply can satisfy people's demand for energy, guarantee the completeness of gastrointestinal tract structures and functions as well as reducing the incidence of complications (10). Yang et al. (11) suggested critically ill patients using PN + EN nutritional support achieve a complementary effect. Hence, taking critically ill patients who were admitted to ICU as research subjects, this study evaluated their nutritional status, adjusted acceptable daily intakes (ADI) of EN and PN based on patients' conditions and analyzed the influences of various nutritional supports on patients' early nutrition as well as immune functions, so as to formulate a reasonable nutrition support regimen and improve patients' nutritional effect.

MATERIALS AND METHODS

A total of 71 (48 males, 23 females, ages 25-82 yrs) critically ill patients who were admitted to neurological ICU in The Second Affiliated Hospital of Zhengzhou University between May 2014 and January 2015 were selected for this study. All subjects were unable to eat by themselves, needed EN treatment via nasogastric tube and had been in hospital for more than one week.

Patients who were diagnosed with critical neurological diseases, scored ≥ 3 points in NRS2002, were unable to eat by themselves and needed EN or PN via nasogastric tube and were aged over 18 years were included in the study.

Patients were excluded from this study if they suffered from digestive system dysfunctions, heart, liver or kidney dysfunctions as well as endocrine diseases or were hospitalized in one week.

Patients and their family members were informed

of their rights and possible risks in detail before case collection, and voluntarily signed the informed consent.

Grouping and treatment methods

The patients were divided into three groups, i.e., EN group, EN + EPN group and EN + SPN group. For EN group, patients were dripped with EN suspension uniformly by a feeding pump within 24 h after admission to hospital. The daily amount of nutrient solution and drip velocity were adjusted according to patients' digestion and absorption conditions, which increased gradually until total heat reached to 25 kcal/(kg · d).

For EN + EPN group, patients were given PN via peripheral veins or central veins after admission to hospital, followed by EN based on the recovery of gastrointestinal tract. The proportion of parenteral and enteral nutrient substances was adjusted appropriately. PN intake reduced little by little while EN intake increased gradually. Total heat was required to reach a target quantity in the 4th day, i.e., 25 kcal/ (kg · d).

For the EN + SPN group, patients were treated with EN suspension within 24 h after admission to hospital. Total heat was required to reach a target quantity in the 4th day, i.e., 25 kcal/ (kg · d). PN intake reduced little by little while EN intake increased gradually.

Serum protein indexes

Patients in the three groups received different nutritional supports. Patients' serum protein was compared before and after one week of nutritional support, including changes in total protein (TP), albumin (ALB), prealbumin (PALB), transferrin (TRF), retinol binding protein (RBP) and haemoglobin B (HB). After nutritional support, serum protein level was compared between the three groups.

Observation of immune indexes

Changes in total lymphocyte count (TLC), IgG, IgA, IgM, C3 and C4 were compared between the three groups before and after one week of different nutritional supports. After nutritional support, immune indexes were compared between the three groups.

Statistical analysis

All data were processed using SPSS 17.0 software and expressed as mean $\pm$ standard deviation (SD). Patients' indexes were compared within the group and between

groups before and after the treatment. Nutritional and immune indexes were compared using *t*-test and three groups of data were compared by variance analysis. The difference was considered to be statistically significant if $p < 0.05$.

RESULTS

Comparison of basic information and relevant indexes on admission

Age, gender, Glasgow coma scale (GCS) scoring, serum protein level and immune indexes were compared between the three groups on admission. The difference was not statistically significant ($p > 0.05$), suggesting that the three groups were comparable.

Comparison of serum protein before and after nutritional support in the three groups

For the EN group, patients' TP, PALB, ALB, HB, TRF and RBP reduced one week after admission to hospital compared with on admission, in which, TP, ALB, HB and TRF decreased significantly ($p < 0.05$); PALB and RBP decreased to some extent, but had no statistical significance ($p > 0.05$). Furthermore, comparing after one week of admission to hospital with on admission, PALB and RBP in the EN + EPN group and EN + SPN group increased and the difference was significant ($p < 0.05$); the rest of serum nutrition indexes all increased, but there was no statistical difference ($p > 0.05$) (Table I).

Table I. Comparison of nutritional indexes before and after nutritional therapy.

Groups	Number of cases		TP(g/L)	ALB(g/L)	HB(g/L)
EN group	24	Before treatment	67.83±9.36	37.89±5.26	143.10±12.95
		After treatment	60.04±6.72	32.75±4.05	135.02±12.85
		<i>P</i>	<0.05	<0.05	<0.05
EN+EPN group	27	Before treatment	64.52±6.23	64.52±6.23	141.81±23.38
		After treatment	65.77±5.87	65.77±5.89	142.02±19.07
		<i>P</i>	>0.05	>0.05	>0.05
EN+SPN group	20	Before treatment	65.45±5.67	36.86±5.67	130.26±16.3
		After treatment	65.85±8.31	37.72±7.06	134.04±11.52
		<i>P</i>	>0.05	>0.05	>0.05
Groups	Number of cases		PALB(g/L)	Transferrin	RBP
EN group	24	Before treatment	0.18±0.48	2.11±0.55	33.24±12.05
		After treatment	0.17±0.05	1.76±0.58	39.07±12.21
		<i>P</i>	<0.05	>0.05	>0.05
EN+EPN group	27	Before treatment	0.24±0.15	2.22±0.53	30.92±13.88
		After treatment	0.26±0.68	2.41±0.72	38.41±12.71
		<i>P</i>	<0.05	>0.05	<0.05
EN+SPN group	20	Before treatment	0.21±0.6	2.25±0.6	30.58±14.55
		After treatment	0.23±0.54	2.32±0.63	40.59±13.24
		<i>P</i>	<0.05	>0.05	<0.05

(mean ± SD)

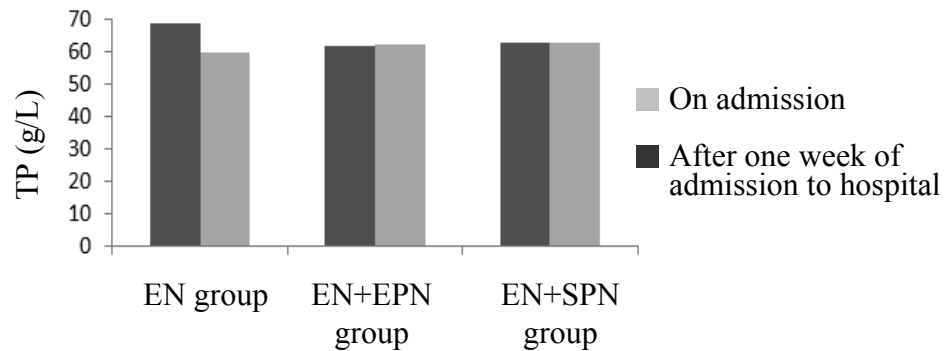


Fig. 1. Comparison of TP between the three groups before and one week after admission to hospital.

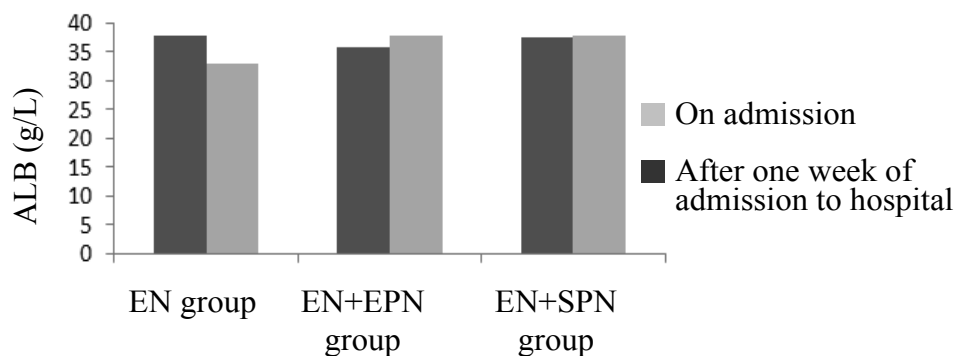


Fig. 2. Comparison of ALB between the three groups before and one week after admission to hospital.

Comparison of immune functional indexes before and after nutritional therapy in the three groups

Compared with before treatment, the EN + SPN group was found with a rise in C3 and C4 after nutritional therapy, and the difference had a statistical significance ($p < 0.05$). The EN group and EN + EPN group increased in C3 and C4 in comparison with prior to treatment, but the difference was not statistically significant ($p > 0.05$). TLC increased in the three groups before and after treatment, in which, the EN + EPN group and EN + SPN group were found with an obvious increase in TLC after treatment, and there was a statistical difference ($p < 0.05$).

After nutritional support, IgA, IgG and IgM in patients in the EN group increased, but no statistical difference was found ($p > 0.05$). Compared with before nutritional support, the EN + SPN group was found with increases in IgA and IgM and increases in IgG and IgA for the EN + EPN group, and the differences were statistically significant ($p < 0.05$).

Comparison of serum proteins and immune-related indexes between the three groups after nutritional support

Through comparing various nutritional indexes between the three groups after treatment, TP, ALB, PALB and TRF in the EN+EPN group and EN+SPN

group went up in comparison with the EN group, and the differences were significant ($p < 0.05$). After nutritional treatment, the EN+EPN group was found with an increase in ALB compared with the EN+SPN group, and the differences were significant ($p < 0.05$); IgA and IgG improved obviously in the EN+EPN group after EN support, and the comparison among groups had a statistical significance ($p < 0.05$). Moreover, TLC was notably higher in the EN+EPN group and EN+SPN group than in the EN group after treatment, and the differences were statistically significant ($p < 0.05$); there was no statistical significance in the differences of C3 and C4 between the three groups after nutritional treatment ($p > 0.05$).

DISCUSSION

For patients with critical neurological diseases, a reasonable and effective nutritional support is able to correct malnutrition which has occurred or will occur, improve nutritional status as well as enhance patients' resistance to diseases (12-13). Total PN (TPN) or PN is given to patients in the early nutritional support, followed by decreased intake of PN and increased intake of EN. In this way, the amount of PN and EN can be adjusted dynamically to ensure energy and nitrogen supplies that are required by patients. Some studies (14-15) revealed that patients could receive PN to supplement corresponding energies if EN failed to cover 60% of the target nutrition. In this study, the EN group was found with a decrease in serum protein after nutritional treatment. Considering patients' high catabolism state, EN could not provide enough energy and protein and intestinal intolerance occurred frequently, which affected nutrient uptake of the intestinal mucosa cells. EEN usually leads to insufficient nutrient intake, thus requiring EN and EN + PN to improve critically ill patients' immune functions, which conforms to the results obtained from this experiment.

In conclusion, this study draws the following conclusions: first of all, patients with critical neurological diseases can avoid malnutrition and immunodeficiency by achieving the target energy supply in an early stage; secondly, a decrease in serum protein can result in malnutrition after

EN support; thirdly, EN and EN + PN therapies can improve nutritional indexes in patients with critical neurological diseases compared with EN therapy only; finally, EPN is more able to improve malnutrition and immune functions of neurocritically ill patients than SPN.

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LETTER TO THE EDITOR

CHANGES OF HMGB1 EXPRESSION ON ANGIOGENESIS OF OVARIAN CANCER
AND ITS MECHANISM

LY. ZHOU, LY. SHI and Y. XIAO

*Yantai Hospital of Traditional Chinese Medicine, Zhifu Zone, Yantai, China**Received January 18, 2016 – Accepted March 4, 2016*

This study was designed to investigate the changes of high-mobility group box-1 (HMGB1) expression and its effects on regulating the angiogenesis of ovarian cancer. HMGB1 eukaryotic expression plasmid and artificially synthesized small interfering ribose nucleic acid (siRNA) were constructed to transfer SKOV3 cell, respectively. Western blot was adopted to investigate the changes of HMGB1, CXCL12 and vascular endothelial growth factor (VEGF) before and after the transfection and flow cytometry (FCM) was applied to detect SKOV3 apoptosis. Results revealed that the apoptosis rates of SKOV3 cell were $32.8 \pm 2.2\%$, $33.9 \pm 1.9\%$ and $11.7 \pm 1\%$, respectively, in the control group, no-load group and transfection group after 2-d cisplatin treatment ($10 \mu\text{g/mL}$). The apoptosis rate in the transfection group was obviously lower than that in the control group and no-load group ($p = 0.00$) while no significant difference was found in the apoptosis rate in the other two groups ($p = 0.75$). Furthermore, the apoptosis rates of SKOV3 cell in the SKOV3 group, negative control group, SKOV3-ribose nucleic acid interfere (RNAi) group were $7.9 \pm 0.5\%$, $8.3 \pm 0.8\%$ and $29.5 \pm 1.3\%$ respectively. The apoptosis rate was notably higher in SKOV3-RNAi group than in the SKOV3 group and negative control group ($p < 0.001$) while no significant difference was found in the apoptosis rate in the other two groups ($p = 0.89$). Thus, it can be concluded that HMGB1 interference can reduce VEGF and CXCL12 expression in ovarian cancer cells, but increase the apoptosis of ovarian cancer cells. Moreover, HMGB1 is highly expressed in cytoplasm and karyon.

Ovarian cancer is one of the malignant tumors commonly seen in gynecological diseases, and its incidence and fatality rate increase year by year and five-year survival rate is only 20-30% (1, 2). It has been proved that HMGB1 is overexpressed in many malignant tumors, which may be closely associated with the inflammatory microenvironment (3-5).

Referring to angiogenesis, vascular endothelial growth factor (VEGF) comes to mind. As known, VEGF, a major positive regulatory factor in tumor angiogenesis, is involved in the development of tumor. HMGB1 has been defined as a pro-angiogenic

factor, which is able to stimulate macrophage and VEGF secretion, so as to promote angiogenesis. It is well-known that HMGB1 combined with receptors for advanced glycation end-products (RAGE) can also promote tumor angiogenesis. However, it has been found that RAGE gene deletion fails to completely inhibit angiogenesis, which indicates that there may be other receptors or pathways promoting tumor angiogenesis in combination with HMGB1. HMGB1 is overexpressed in different types of tumors by activating angiogenesis (6-9). This research investigated the influences of

Key words: ovarian cancer, HMGB1, angiogenesis, SKOV3, vascular endothelial growth factor

Mailing address:

Dr Yan Xiao,
NO.39 Xingfu Road, Yantai Hospital
of Traditional Chinese Medicine,
Zhifu Zone, Yantai, 264000, China
e-mail: xiaoyan3689@163.com

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HMGB1 on VEGF expression and its mechanism as well as preliminarily discussing the mechanism of angiogenesis in ovarian cancer.

MATERIALS AND METHODS

Clinical data

Fifty patients, ranging in age from 35 to 75 years (average: 55 years), who had been confirmed with epithelial ovarian cancer (EOC) and received surgical treatment in Yantai Hospital of Traditional Chinese Medicine between January 2011 and January 2014 were taken as research subjects. According to the International Federation of Gynecology and Obstetrics (FIGO) (2000), patients were divided as follows: clinical stages: I~II stage (24 cases), III~IV stage (26 cases); pathological grades: G1~G2 (20 cases), G3 (20 cases); pathological types: mucinous cystadenocarcinoma (10 cases), ovarian serous cystadenocarcinoma (30 cases), clear cell carcinoma (4 cases), endometrioid adenocarcinoma (6 cases), lymph node and distant metastasis (30 cases), no metastasis (20 cases). Twenty patients, age ranging from 50-60 years (average: 50 years) undergoing normal ovarian tissue excision were selected. Patients were excluded if they suffered from other organ tumors or used no chemotherapy drugs and immunosuppressive drugs before surgery. This experiment was approved by the ethics committee of Yantai Hospital of Traditional Chinese Medicine and all of the patients signed informed consent.

Experimental methods

SKOV3 and human umbilical vein endothelial cell strains (HUVEC) (Xiehe Cell Bank, Beijing, China) were selected.

Cell culture

The first step was cell recovery. The frozen cells which had been conserved at -80°C were put into a water bath (Chenghui instrument factory, Jintan, Jiangsu, China) at 37.2°C. After dissolution, cells were put on an irradiated bench (Sujing Group, Jiangsu, China) and moved to a sterile centrifuge tube containing 4 mL of fresh 1640 medium (10% of fetal bovine serum (FBS, Hyclone)) and centrifuged at 1000 rpm/min for 5 min, then cells were drained. After that, 2 mL of Roswell Park Memorial Institute (RPIM 1640) medium containing 10% of FBS

was added, beaten and blown evenly and then moved into a culture flask with the same medium (3 mL) for cell culture (37°C, 5% CO₂).

The second step was cell passage. After the medium in the culture flask was sucked out with a transfer pipette, 3 mL of phosphate buffer solution (PBS, Dingguo Biotechnology Company, Beijing, China) was added and cells were then washed thrice. Then, the culture flask was added with 1 mL of pancreatic enzyme (0.25%) and shaken slightly to make them mix fully, and then put onto a bench and digested for 2~3 min. Afterwards, 1 mL of culture medium containing 10% of FBS was added to terminate the digestion if the space got larger and cells became rounder under a microscope (Olympus). Cells were blown and beaten evenly with a transfer pipette and then centrifuged at 1000 rpm/min for 3~5 min. Cells were drained into 3 culture flasks and added with 3 mL of 1640 medium containing 10% of FBS, and then blown and beaten. After that, every culture flask was added with 4 mL of culture medium and then cultured.

The third step was cell cryopreservation. Cells were drained and washed 2~3 times with PBS and then added with 1 mL of pancreatic enzymes (SuoLaiBao, Beijing, China). Three minutes later, 1 mL of complete medium was added to terminate the digestion. After resuspending, cells were moved into a sterile centrifuge tube (15 mL) and centrifuged at 1,000 rpm/min for 3-5 min. Cells were then drained and resuspended with 1 mL of frozen stock solution made up of dimethylsulfoxide (DMSO), FBS and medium (1:2:7). The cell suspension was moved into a sterile cryopreserved tube and stored at -4°C for 30 min, -20°C for 1 h, and finally preserved at -80°C.

The fourth step was cell inoculation. Cells were drained and washed three times with PBS and then added with 1 mL of pancreatic enzymes (0.25%). Three minutes later, 1 mL of complete medium was added to terminate the digestion. Cells were blown and beaten to be single cell suspension and centrifuged at 1000 rpm/min for 5 min. After that, cells were drained and added with complete medium and then counted after resuspending. The density of cells was adjusted to 3×10^5 and cultured for one day and plasmid DNA transfection was then performed when cells covered 80% of the wall.

Construction of eukaryotic expression plasmid

Eukaryotic expression plasmid pcDNA3.1

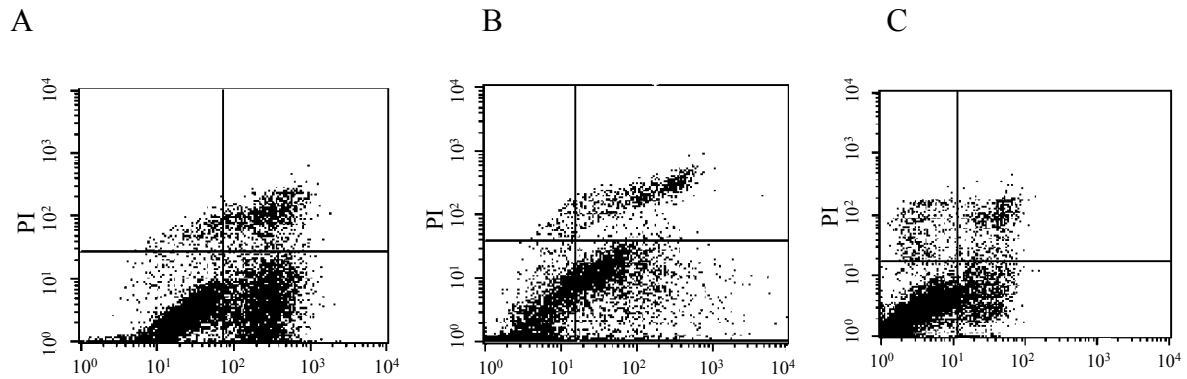


Fig. 1. Annexin V-FITC in (A) control group, (B) no-load group and (C) transfection group.

Table I. Results of Western blot.

Groups	HMGB1/ β -actin	CXCL12/ β -actin	VEGF/ β -actin
SKOV3 group	1.1 $\pm$ 0.04	0.6 $\pm$ 0.02	1.0 $\pm$ 0.02
Negative control group	1.1 $\pm$ 0.02	0.5 $\pm$ 0.01	1.0 $\pm$ 0.03
SKOV3-RNAi group	0.5 $\pm$ 0.03	0.3 $\pm$ 0.03	0.4 $\pm$ 0.20

Note: 1: relative expression quantity of HMGB1 protein in cells; 2: relative expression quantity of VEGF protein in cells; 3: relative expression quantity of CXCL12 protein in cells.

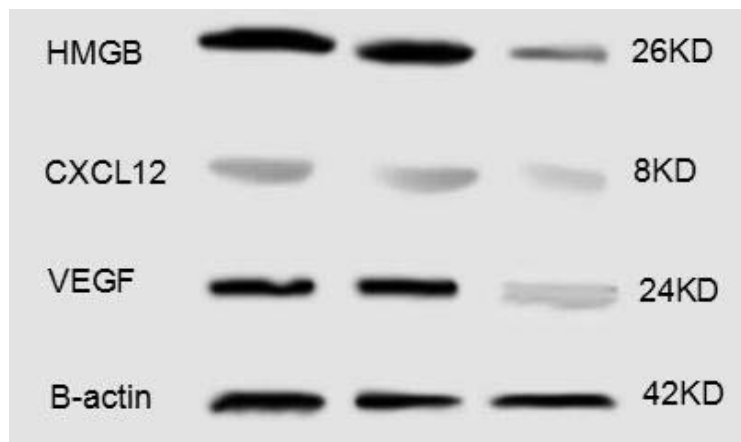


Fig. 2. Relative expression quantities of HMGB1, CXCL12 and VEGF proteins in each group.

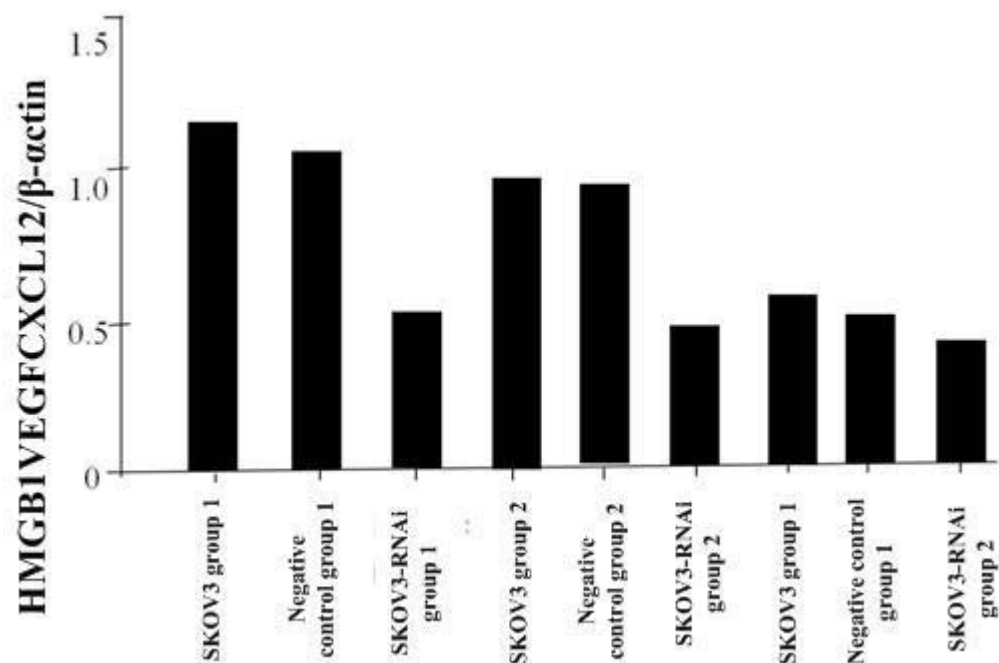


Fig. 3. Expression of HMGB1, CXCL12 and VEGF proteins in each group.

FlagHMGB1 was provided by Addgene Company and pcDNA3.1 no-load carrier was kindly given by the hospital. Plasmid was inserted into puncture bacteria with a disinfected and sterilized inoculating loop to obtain plasmid *Escherichia coli*, which was then inoculated in the pre-prepared LB solid medium (paddle board) and conserved overnight at room temperature. Then, single colony was selected and inoculated into the pre-prepared LB fluid medium (200 mL) and put on a shaking table (250 rpm) overnight (12-18 h) at 37°C. Afterwards, the medium was observed to be muddy, part of which was stored in 25% of glycerinum for bacteria breed conservation preservation and the rest was used for plasmid DNA purification. Plasmid DNA was extracted as per the instructions of Endo Free Plasmid Kit (Omega Company).

In vitro transfection of SKOV3 cells

One day before transfection, SKOV3 cells were inoculated into a petri dish (60 mm) with the density of 3×10^5 and cultured at 37°C for one day (5% CO₂). Plasmid DNA was transfected when cells covered 70%

~ 90% of the wall. After that, 600 µL of RPIM 1640 medium as well as 8 µg of plasmid DNA (27 µL) were added into an Ep tube and mixed evenly. An amount of 12 µL of TuberFect™ transfection reagent was added into the tube and kept at room temperature for 15-20 min and then transferred to a petri dish (60 mm).

Grouping

Three groups were set in this experiment, i.e., PCDNA3.1-HMGB1 transfection group, PCDNA3.1-N1 plasmid transfection group and untransfected SKOV3 cell group.

Cell apoptosis detected with FCM

SKOV3 cells in the logarithmic phase were digested with 0.25% of pancreatic enzymes and then added with RPMI 1640 medium containing 10% of FBS to make a single cell suspension. Cells, with the density of 1×10^4 mL⁻¹, were inoculated into a 96-well plate (100 µL/well) and five replica wells were set in each group. One day after transfection, differently concentrated cisplatin were added in each group and 10 µL of CCK-8 (cholecystokinin

octapeptide-8) was added into each well after 1-d culture. 0.5 ~ 4 h later, optical density (OD) value of each well was measured by enzyme-linked immunosorbent assay (ELISA) at a wavelength of 490 nm. Samples were repeatedly detected for three times within 30 min. Inhibition rate of cell proliferation = (OD in the untransfected group - OD the intransected group) / OD in the untransfected group. SKOV3 cells in the logarithmic phase were added with cisplatin with a terminal concentration of 10 $\mu\text{g/mL}$; at the same time, another three groups of cells were taken and added with the same volume of culture solution as the control. After 2 days of drug action, cells were digested with 0.25% of trypsin containing no ethylene diamine tetraacetic acid (EDTA) and drained and centrifuged at 1000 r/min for 3 min. Afterwards, the density of cells was adjusted to $\geq 1 \times 10^5$ /L and cells were analyzed on a flow cytometer as per the instructions of Annexin-V/PI. This experiment was repeated 3 times.

Protein expression in each group detected by western blot

To investigate the down-regulated expression of HMGB1, cells were cultured and grouped again. Ovarian cancer cell strain SKOV3 was cultured in RPMI 1640 medium containing 10% of FBS. Cells in the logarithmic phase were subcultured and divided into three groups (SKOV3-HMGB1RNAi group, SKOV3-RNAi negative control group and untransfected SKOV3 cell group). One–two days later, SKOV3 cells were detected and transfected *in vitro*.

Statistical analysis

Data were processed with SPSS 20.0 software and expressed as mean $\pm$ standard deviation (SD). Least significant difference-t (LSD-T) was applied to analyze data in the case of equal variance assumed and Dunnett's T3 was used in the case of equal variance not assumed. The groups were compared by analysis of variance and the difference was considered to be statistically significant if $p < 0.05$.

RESULTS

Cell apoptosis detected by FCM

The apoptosis rates of SKOV3 cell in the control group, no-load group and transfection group after 2-d cisplatin treatment (10 $\mu\text{g/mL}$) were (32.8 $\pm$ 2.2)%,

(33.9 $\pm$ 1.9)% and (11.7 $\pm$ 1)% respectively. The apoptosis rate in the transfection group was obviously lower than that in the control group and no-load group ($p = 0.00$) while no significant difference was found in the other two groups ($p = 0.75$) (Fig.1 A, B and C).

Western blot

The apoptosis rates of SKOV3 cell in the SKOV3 group, negative control group and SKOV3-RNAi group were (7.9 $\pm$ 0.5)%, (8.3 $\pm$ 0.8)% and (29.5 $\pm$ 1.3)%, respectively. The apoptosis rate in the SKOV3-RNAi group was obviously higher than that in the SKOV3 group and negative control group ($p < 0.001$) while no significant difference was found in the other three groups ($p = 0.89$) (Fig. 4, 5, Table I).

DISCUSSION

The growth and transfer of ovarian cancer cannot do without the formation of new angiogenesis. Relevant studies have confirmed that VEGF mRNA and protein expression can be found in ovarian cancer cells. VEGF receptors are mainly expressed on the surface of tumor cells and endothelial cellular membrane, which can accelerate angiogenesis and the differentiation and proliferation of tumor according to paracrine and autocrine mechanisms (10). *In vitro* experiment showed that HMGB1 could stimulate VEGF and bFGF secretion, which further confirmed the characteristic of angiogenesis promotion of HMGB1 (11-13). Overexpression of HMGB1 is of significance to the development of tumors, especially in the metastasis of tumor cells and metastasis ability (14). One study (15) verified that, HMGB1 together with RAGE, could promote angiogenesis, but RAGE gene deletion could not completely inhibit angiogenesis resulting from HMGB1.

This experiment demonstrates that the death rates of cells in the transfection group decreased obviously compared with the control group after the addition of cisplatin. Highly concentrated HMGB1 had an effect on protecting SKOV3 cells. Besides, the apoptosis rates of cells in the transfection group were significantly lower than that in the control group

and no-load group and highly concentrated HMGB1 was able to inhibit the apoptosis of SKOV3 cells. In 2009, Mantovani (16) found local inflammatory and anti tumor immune responses in the tumor microenvironment. It is reported that there is a vital relationship between HMGB1 expression and tumor microenvironment as HMGB1 played a significant role in proinflammatory reaction.

Limitations of this study

Defects, for instance, small sample size, non-randomized groups, etc., still exist in this study, therefore follow-up research is required. Differences of various effects should be compared, and factors affecting long-term effects need to be explored. In the meantime, there are several differences between experimental hypotheses, which require further discussions, to better elaborate the changes of HMGB1 expression on angiogenesis of ovarian cancer and its mechanism.

In conclusion, HMGB1 plays a crucial role in the expression of VEGF and CXCL12. This thesis verifies that HMGB1 has an important effect on the formation of ovarian cancer angiogenesis and ascites as well as apoptosis, which is expected to be a new target for the prediction of prognosis and inhibition of cancer reoccurrence and metastasis.

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CORRELATION BETWEEN GENETIC SUSCEPTIBILITY OF TUBERCULOSIS AND MACROPHAGE MIGRATION INHIBITORY FACTOR

YL. WANG, CX. ZHANG, GC. SHI, QY. ZHANG and WG. LIU

Tuberculosis Department of the First Affiliated Hospital of Xinxiang Hospital Henan, Henan, China

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This paper aims to study the relationship between genetic susceptibility of tuberculosis and macrophage migration inhibitory factor (MIF) and provide theoretical basis and foundation for further studies on pathogenesis and treatment of tuberculosis. Enzyme Linked Immuno Sorbent Assay (ELISA) was applied to detect the difference of MIF protein expression level in peripheral serum of the test subjects, and analyze the difference of MIF protein expression levels of different genotypes and alleles at -794CATT locus and -173G/C locus. The results showed that MIF protein expression level in serum of patients in the tuberculosis group was higher than that of the healthy control group ($p < 0.05$). The MIF protein expression level of genotype (5/5+5/6+6/6) and (7/X+8/X) at -794CATT locus of the tuberculosis group was higher than that of the healthy control group ($p < 0.05$), and MIF protein expression level of genotype GG and (GC+CC) at -173G/C locus of the tuberculosis group was higher than that of the healthy control group ($p < 0.05$). Therefore, macrophage migration inhibitory factor is an important cell factor which plays a regulating role in the immune system, as it can inhibit macrophage migration and promote the gathering, infiltration and proliferation of macrophages at inflammatory sites. Furthermore, it can secrete some cell factors which play a central role in immunological regulation.

Tuberculosis (TB) is a chronic infectious disease caused by mycobacterium tuberculosis (MTB) which can affect multiple body organs, and pulmonary tuberculosis is the most common tuberculosis disease (1). Though the susceptible populations of TB are mainly males, infection rate of women has risen in recent years. According to the latest report of the World Health Organization (WHO), in 2012 there was an estimated 410,000 deaths of infected female patients out of 2.9 million TB cases and more than 70,000 deaths of infected children out of 530,000 TB cases (2). The main immune protection mechanism of TB is cellular immunity. After the body is infected with MTB (3), macrophages in

pulmonary alveoli secrete dozens of cell factors of IL-1, IL-6 and TNF- α , which gather lymphocytes and monocytes to the MTB invasion area and form tuberculous granuloma which limit MTB spread and kills MTB (4). The interaction and coordination between T cells and macrophages is very important for the improvement of immune protection effect (5). MTB is an intracellular bacterium which mainly parasitizes in macrophages, so macrophages in the body are the first line of defense of MTB infection, as well the key line of defense (6).

To date, many studies have been undertaken in order to solve this problem; for example, Li et al. (7) in 2012 analyzed the relationship between MIF and

Key words: MIF, tuberculosis, DNA, susceptibility

Mailing address:

Dr Yongliang Wang,
NO.88, Jiankang Road, Tuberculosis Department,
The First Affiliated Hospital of Xinxiang Hospital,
Wei Hui, Xin Xiang, Henan, 453100, China
e-mail: yongliangwangyl@163.com

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susceptibility of tuberculosis by studying the MIF expression level between TB patients and healthy subjects; Benigni et al. (8) found that migration inhibitory factor (MIF) in human could inhibit the growth and reproduction of pathogenic MTB; Lu et al. (9) found in 2014 that MIF had an antagonistic anti-inflammatory effect on glucocorticoids and a strong stimulating effect on production of proinflammatory cytokines. Thus it can be seen that MIF plays an important role in TB disease development process.

MATERIALS AND METHODS

Study subjects

Two hundred TB cases were selected for this study, including 100 cases of initial treatment TB patients (57 males and 43 females; average age of 40.17 ± 19.98 years), and 100 cases of retreatment TB patients (74 males and 26 females; average age of 40.53 ± 15.69 years). For the control group, 100 healthy subjects (38 males and 58 females, 4 unclassified; with an average age of 28.64 ± 9.16 years) were selected. All projects in this study were carried out with informed consent of all subjects and verified by pathologists.

Experimental instruments

-86°C ultra-low temperature freezer, -30°C cryogenic refrigerator, 4°C refrigerator, HF Super NW series ultrapure water system, vertical high-pressure sterilizer, single track manual genex, etc., were applied in this study.

Experimental reagents

Ray Bio Human MIF ELISA Kit and RNase-free water were selected as the experimental reagents.

Specimen collection and processing

Firstly, a sample of 2 ml of venous blood was taken from all research subjects. Then, serum separation was carried out on venous blood after centrifugation and anticoagulation treatment by 2% Ethylene Diamine Tetraacetic Acid (EDTA) and the obtained serum was preserved at -80°C. No cortisol drug or drug which might affect hemorheology was administered to subjects before blood sampling.

DNA Extraction of blood genome

The DNA extraction of blood genome was performed by ELISA. The extracted genome DNA was in large fragments,

with high purity and stable and reliable quality and then it was preserved for standby application.

Detection of MIF protein expression in serum

ELISA was used to establish standard curve: 100 µl of standard substance was added to the blank micro pores on the ELISA plate; sample number was marked and 100 µl of defrosted serum sample was added to the blank micro pores; a control group was set up for both samples and standard substances. Then, after gentle oscillation, the ELISA plate was kept for two and a half hours at room temperature.

Board-washing: after 20 times dilution of concentrated washing liquid with double distilled water, washing was carried out four times on the ELISA plate. The washing liquid was added by 250 µl/pore each time and kept for one minute and then dried on filter paper.

Diluted biotin-antibody was added by 100 µl/pore and was then placed for one hour at room temperature after the liquid was blended by gentle oscillation. The washing was repeated. Then Avidin HRP-SPA was added by 100 µl/pore and was then placed for 45 min at room temperature after the liquid was blended by gentle oscillation.

Washing was repeated, then TMB Substrate was added with 100 µl/pore and was then placed for 30 min of color development at room temperature away from light after the liquid was blended by gentle oscillation. Stop solution was added by 50 µl/pore for termination of the reaction. OD value of each pore was read on the ELISA with a wavelength of 450 nm within 10 min; the standard curve was drawn and corresponding concentration of MIF was calculated.

Statistical analysis

Based on direct counting method and *chi*-squared test, analysis on whether distribution of the experimental group and control group met Hardy-Weinberg was carried out. Genotype frequencies were compared between the two groups and odds ratio and 95% credibility interval were calculated. Difference between the two groups was considered statistically significant if $p < 0.05$.

RESULTS

Genomic DNA identification and observation of tuberculosis cells

The results showed that the extractive genome DNA content was within the range of 0.2-0.4µg/

ml and OD260/OD280 was between 1.61-1.81, in accordance with the requirement of the experiment. Fig. 1 shows the sepharose gel electrophoresis image of genome DNA from a random TB patient and a healthy subject.

Difference analysis on MIF protein expression level between the TB group and healthy control group

Through detection of MIF concentration in serum of the four groups, the results showed that MIF protein expression level of initial treatment TB group (8.21 ± 4.61) ng/ml, retreatment TB group (11.19 ± 8.98) ng/ml and TB group (9.68 ± 7.23) ng/ml was significantly higher than that of the healthy

control group (4.58 ± 1.88) ng/ml. The difference was statistically significant, $p < 0.05$ (Table I).

Difference analysis on MIF protein expression level of genotype (5/5+5/6+6/6) at CATT locus of -794 region between TB group and healthy control group

As shown in Fig. 2, MIF protein expression level of genotype (5/5+5/6+6/6) at CATT locus of -794 region was as follows: initial treatment TB group (8.37 ± 4.26) ng/ml, retreatment TB group (10.86 ± 8.53) ng/ml, TB group (9.61 ± 6.84 ng/ml), all of which were significantly higher than that of the healthy control group (4.51 ± 1.97) ng/ml, $p < 0.05$, and the difference was statistically significant.

Difference Analysis on MIF protein expression level of genotype (7/X+8/X) at CATT locus of -794 region between TB group and healthy control group

MIF protein expression level of genotype (7/X+8/X) at CATT locus of -794 region was as follows: initial treatment TB group (7.85 ± 5.31) ng/ml, retreatment TB group (11.91 ± 9.96 ng/ml), TB group (9.77 ± 8.07) ng/ml, all of which were significantly higher than that of the healthy control group (4.81 ± 1.58) ng/ml, $p < 0.05$, and the difference was statistically significant.

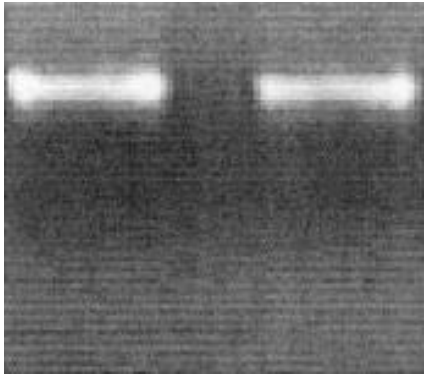


Fig. 1. Genomic DNA electrophoresis image of 2 random samples.

Table I. MIF detection results of four groups (ng/ml).

group	Number of cases	MIF concentration (ng/ml)
Healthy control group	100	4.58 ± 1.88
Initial treatment TB group	100	8.21 ± 4.61
Retreatment TB group	100	11.19 ± 8.98
TB group	200	9.68 ± 7.23

Note: the difference between initial treatment TB group, retreatment TB group, TB group and healthy control group had statistical significance, $p < 0.05$; the difference between initial treatment TB group and retreatment TB group had statistical significance, $p < 0.05$.

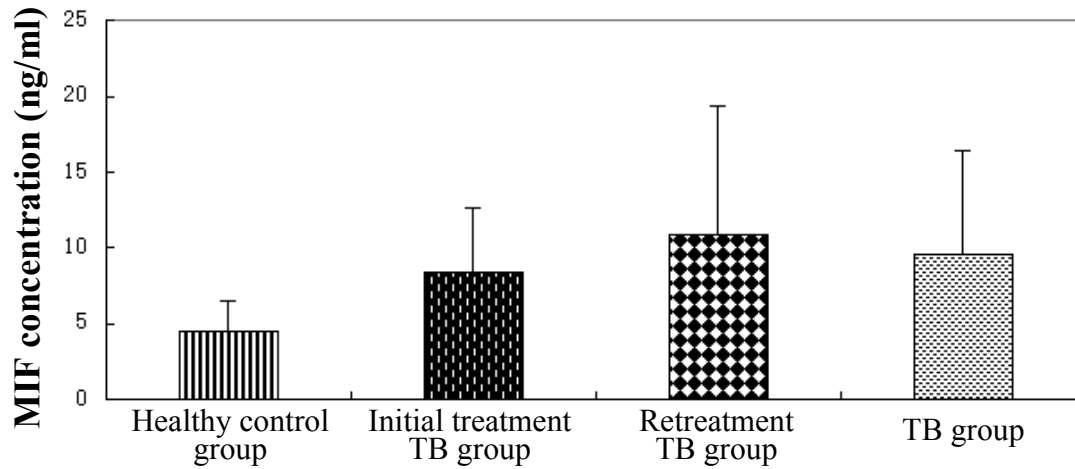


Fig. 2. MIF comparison of genotype (5/5+5/6+6/6) at CATT locus of -794 region.

Table II. MIF detection results of genotype GG at G/C locus of -173 region (ng/ml).

group	Number of cases	MIF concentration (ng/ml)
Healthy control group	57	4.47±1.98
Initial treatment TB group	42	7.51±4.16
Retreatment TB group	38	12.62±7.23
TB group	80	9.81±6.23

Note: the difference between initial treatment TB group, retreatment TB group, TB group and healthy control group had statistical significance, $p < 0.05$; the difference between initial treatment TB group and retreatment TB group had statistical significance, $p < 0.05$.

Difference analysis on MIF protein expression level of genotype GG at G/C locus of -173 Region between TB group and healthy control group

As shown in Table II, MIF protein expression level of genotype GG at G/C locus of -173 region was as follows: initial treatment TB group (7.51±4.16) ng/ml, retreatment TB group (12.62±7.23) ng/ml, TB group (9.81±6.23) ng/ml, all of which were significantly higher than that of the healthy control group (4.47±1.98) ng/ml, $p < 0.05$, and the difference was statistically significant.

Difference analysis on MIF protein expression level of genotype (GC+CC) at G/C locus of -173 region between TB group and healthy control group

The MIF protein expression level of genotype (GC+CC) at G/C locus of -173 region was as follows: initial treatment TB group (8.66±4.92) ng/ml, retreatment TB group (10.46±9.74) ng/ml, TB group (9.58±7.83) ng/ml, all of which were significantly higher than that of the healthy control group (4.62±1.78) ng/ml, $p < 0.05$, and the difference was statistically significant.

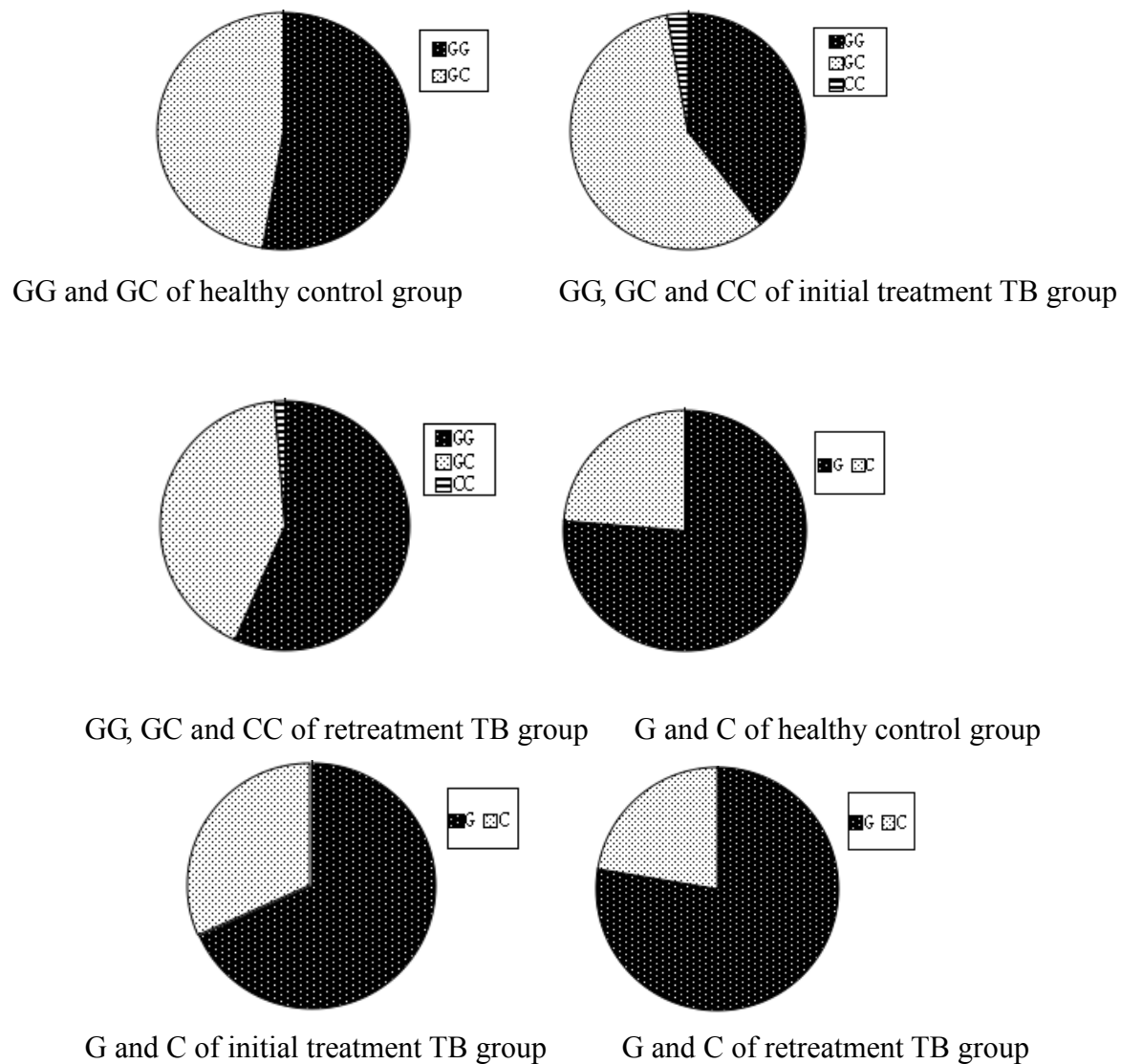


Fig. 3. Each group of MIF - 173 genotype and distribution of alleles.

Three groups of MIF - 173 genotypes and distribution of alleles

The MIF-173 gene polymorphism, GG, GC and CC frequency in healthy control group, initial treatment TB group, retreatment TB group and frequency of allele G and C in healthy control group, initial treatment TB group and retreatment TB group are shown in Fig. 3.

DISCUSSION

TB is an infectious disease caused by MTB infection, with a high mortality due to single pathogenic bacteria infection (10). TB is still a major worldwide public health and social problem which constitutes a serious impediment to social and economic development (11). MIF is an

important cytokine which plays a regulating effect in the immune system, as it can inhibit macrophage migration and promote the gathering, infiltration and proliferation of macrophages at inflammatory site. Furthermore, it can secrete some cell factors which play a central role in immunological regulation (12).

This study detected MIF concentration of patients in initial treatment TB group, retreatment TB group and healthy people and the results showed that MIF concentration of initial treatment TB group and retreatment TB group was significantly higher than that of the healthy control group and the differences were statistically significant ($p < 0.05$), which suggested that MIF was closely related to TB occurrence and development (13). From the difference in analyses of MIF protein expression level of genotype (GC+CC) at G/C locus of -173 region between TB group and healthy control group, it was found that MIF concentration in serum of GG genotype patients of initial treatment TB group and retreatment TB group was higher than that of GC+CC genotype patients. The difference was statistically significant in the retreatment TB group ($p < 0.05$) (14). With the development of genomics and proteomics, there are an increasing number of studies on the relationship between MIF and tuberculosis disease. At the same time, many studies also proved that the relationship between MIF and TB was very close (15). Thus it can be inferred that the gene variation in MIF promoter region may alter some of the inflammatory pathway function of TB (16), which increases the risk of development of TB. However, the specific mechanism needs to be further confirmed through a large number of experimental studies.

In conclusion, as a widely distributed cytokine in the body, MIF plays a regulating role in cell gene expression, cell differentiation and apoptosis and participates in a multitude of disease development. Its gene promoter polymorphism is associated with susceptibility of many diseases.

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LETTER TO THE EDITOR

EFFICACY OF TRADITIONAL CHINESE MEDICINE FOR COOLING BLOOD AND ELIMINATING TOXINS AND STRENGTHENING AND PURIFYING SPLEEN IN THE TREATMENT OF REFRACTORY HEPATITIS CFD. JING¹, ZG. MA², YB.LIU³ and G. YAN¹¹*Traditional Chinese medicine department of Yantaishan Hospital, Yantai, China;*²*Integrated Traditional and Western Medicine Department, Yuhuangding Hospital Affiliated to Qingdao University, Yantai, China;* ³*Department of Rehabilitation, Yantaishan Hospital, Yantai, China**Received September 10, 2015 – Accepted March 11, 2016*

The first two authors contributed equally to this work

This study was designed to observe the comprehensive efficacy of traditional Chinese medicine (TCM) combined with interferon in the treatment of chronic hepatitis C (CHC), compare this combined therapy with interferon therapy alone and investigate its possible mechanism to provide a basis for the development of an integrated traditional Chinese and Western medicine for the treatment of CHC. According to patient contraindications for antiviral treatment, patients who were suitable for interferon therapy and willing to use TCM were enrolled as combined traditional Chinese and Western medicine group, and 21 CHC patients were selected as Western medicine control group; patients who had contraindications for antiviral treatment were included in the TCM group. The three groups of patients were all diagnosed with positive hepatitis C virus - ribonucleic acid (HCV-RNA). The treatment course lasted for one year and the patients were followed up for 12 months. Patients' demographic data, course of disease, chronic liver disease questionnaire (CLDQ), genetic typing, biochemical indexes, HCV-RNA and side effects were compared between the groups. The efficacy, incidence of side effects and improvement in quality of life were analyzed in each group. Results showed that the combination of TCM and interferon could protect liver, reduce side effects and also improve quality of life of the patients, while the antiviral activity of TCM alone was not obvious.

Hepatitis resulting from hepatitis C virus (HCV) is spreading widely throughout the world (1-3). After HCV infection, patients suffer from liver inflammation and then develop chronic hepatitis C (CHC), with no response or low response rate in virology (4-6). To date, an antiviral treatment protocol combining long-acting interferon with ribavirin is mainly adopted to treat CHC. This therapy has reliable efficacy in eliminating viruses, inhibiting viral replication, improving liver damage, preventing the occurrence of liver cirrhosis and liver cancer as well as improving quality of life of patients, but some problems still

exist, such as poor response to some viruses and appearance of refractory CHC. Limited by many contraindications and side effects, high price, poor compliance, the effect of interferon in the treatment of CHC is unsatisfactory (7-9). This study aims to observe the comprehensive efficacy of traditional Chinese medicine (TCM) combined with interferon in the treatment of CHC, compare this combined therapy with interferon therapy alone, and investigate its possible mechanism, to provide a basis for the development of an integrated traditional Chinese and Western medicine for the treatment of CHC.

Key words: refractory hepatitis C, TCM therapy, cooling blood, eliminating toxins, strengthening and purifying spleen, clinical efficacy

Mailing address:

Dr Ge Yan,
Traditional Chinese Medicine Department of
Yantaishan Hospital, No. 91, Jiefang Road,
Zhifu District, Yantai, 264001, China
e-mail: yange4247@163.com

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

MATERIALS AND METHODS

Materials

A total of 63 patients who were admitted to the Hospital of Traditional Chinese Medicine and People's Hospital of Jiangsu (China) from December 2014 to October 2015 were enrolled in the study. According to patients' contraindications for antiviral treatment, patients who were suitable for interferon therapy and willing to use TCM were recruited as the combined traditional Chinese and Western medicine group; also 21 CHC patients were selected and taken as the Western medicine only control group; patients who had contraindications for antiviral treatment were included in the TCM group. The three groups of patients were all diagnosed with positive hepatitis C virus - ribose nucleic acid (HCV-RNA). The treatment course lasted for one year and the patients were followed up for 12 months. Patients' demographic data, course of disease, chronic liver disease questionnaire (CLDQ), genetic typing, biochemical indexes, HCV-RNA and side effects were compared between the groups. The efficacy, incidence of side effects and improvement in quality of life were analyzed in each group. This experiment was approved by the ethics committee of the Hospital of Traditional Chinese Medicine and People's Hospital of Jiangsu and all the patients signed informed consent.

Diagnostic criteria

Referring to the Hepatitis C Prevention Guide revised by the Hepatology Branch and Infectious Diseases and Parasitic Epidemiology Branch of Chinese Medical Association (CMA), diagnostic criteria included over 6-month HCV infection or unknown onset date, no history of hepatitis, liver pathology examination conforming to chronic hepatitis or comprehensive analysis of symptoms, vital signs and imaging technology.

Standards for syndrome differentiation of TCM

Depressed liver and deficient spleen: distending pain in hypochondrium, depressed mood, tastelessness in the mouth, lusterless complexion, pale and furry tongue, and thready and slippery pulse.

Liver depression and qi stagnation: yellowish appearance and skin, limb fatigue, palpitation, shortness of breath, pale and furry tongue, and thin and weak pulse.

Humid heat locking the middle energizer: chest hypochondrium, bitter taste in mouth, chest distress and loss of appetite, nausea, constipation, yellow urine, yellowish furred tongue and slippery and rapid pulse.

Exhausted blood blocking collaterals: dull pain in lateral thorax, dry mouth, dysphoria with smothery sensation, dizziness, reddened tongue and less furring, and thin and rapid pulse.

Deficiency of liver-yin and kidney-yin: dizziness, dry eyes, tinnitus, fatigue, numbness of limbs, dry mouth, insomnia and drowsiness, dull pain in lateral thorax, reddened tongue and less furring, and thin pulse.

Differentiation of syndrome unavailable: patients without any signs and symptoms.

Criteria for inclusion and exclusion

Patients were included in the Western medicine control group if they conformed to the above CHC diagnostic criteria, had positive HCV - RNA, aged between 15 and 75 years and had no interferon and ribavirin contraindications.

Patients were included in the combined traditional Chinese and Western medicine group if they conformed to antiviral treatment standards, were willing to receive a combined treatment and had no TCM allergic history.

Patients were included in the TCM group if they had interferon and ribavirin contraindications, stopped using interferon because of its side effects within a month, failed in interferon or ribavirin therapy, aged between 12 and 18 years and/or 65 and 75 years and had no TCM allergic history.

Patients were excluded from the study if they were combined with human immunodeficiency virus (HIV) or other hepatitis virus infections, ALT $\geq 10 \times$ ULN and/or TBIL $\geq 5 \times$ ULN, were diagnosed as having severe hepatitis (acute, sub-acute and chronic severe hepatitis) and had liver cirrhosis, heart, kidney and lung disease and mental disorder; women who were breastfeeding, pregnant or preparing for a pregnancy.

Methods

The basic prescription of the combined therapy was pegylated interferon + ribavirin + TCM; pegylated interferon + ribavirin for the Western medicine group; and TCM for the TCM group. The dosage of TCM was added or subtracted according to patients' symptoms. The

treatment course lasted for one year and the patients were followed up for 12 months.

TCM regimen

The main prescription was Xijiao Dihuang decoction, which was added or subtracted to cool blood and eliminate toxins and strengthen and purify spleen. The decoction was made up of 15 g of *cornu bubali* slice (decoct first), 30 g of *radix isatidis*, 15 g of *scutellaria barbata*, 15 g of *radix rehmanniae* *recen*, 15 g of *cortex moutan*, 15 g of *salvia miltiorrhiza*, 15 g of *chijie*, 6 g of *radix bupleuri*, 10 g of *radix pseudostellariae*, 15 g of yellow breeds contempt, 10 g of *rhizoma atractylodis macrocephalae*, 15 g of *lycium barbarum*, 5 g of licorice roots of northwest origin, 30 g of *polygonum cuspidatum* and 30 g of *oldenlandia diffusa* and was decocted in water for oral dosing, one dose a day, half in the morning and half in the evening. Herbs were added or subtracted according to symptoms:

Rhizoma atractylodis, *magnolia obavata*, were added if the patients were accompanied with damp evil. *Radix curcumae*, *rhizoma curcumae*, etc. were added if the patients were accompanied with blood weakness. The quantities of Huangshen and *rhizoma atractylodis macrocephalae* increase and codonopsis pilosula, Chinese yam, etc. were added if the patients were accompanied with qi deficiency. *Radix adenophorae*, *radix ophiopogonis*, etc. were added if the patients were accompanied with yin deficiency. Zhudong, huodong, pericarpium arecae, etc. were added if the patients were accompanied with edema due to yang deficiency. *Herba epimedii*, *fructus psoraleae*, etc. were added if the patients were accompanied with kidney-yang deficiency.

Western medicine regimen

The patients in the control group were given Peg-IFN-2a (180 µg) by subcutaneous injection (once a week) and ribavirin (10.6-15.0 mg/kg) by oral administration (1-2 times a day). The patients who had no antiviral indications or (and) interferon contraindication were treated with 150 mg of magnesium isoglycyrrhizinate by intravenous drip (once a day) and (or) diammonium glycyrrhizinate by oral administration to protect liver and reduce enzyme (6).

Data collection and processing

Patient physical examination (hear rate, heart rhythm, blood pressure and weight), routine blood and urine tests,

electrocardiogram, renal function (Cr, BUN) were carried out before and after treatment. Blood routine, blood sugar and thyroid function of the patients with interferon treatment were detected periodically based on hepatitis C prevention guidelines.

Patient liver function, alanine aminotransferase (ALT), aspartate transaminase (AST), gamma glutamyl transaminase (GGT), alkaline phosphatase (AKP), total bilirubin (TBIL), albumin (ALB), globulin (GLB) were examined. Ultrasound was used to measure the size of liver and spleen and the width of portal vein and splenic vein. After grouping, the patients in each group were genotyped. After intervention, the efficacy was evaluated using TCM symptoms integral table and the patients were followed-up for a long term to observe death rate and the incidence of liver cirrhosis, primary liver cancer and complications.

Efficacy evaluation criteria

The patients showed marked response to treatment if the main symptoms basically disappeared, liver function returned to normal and HCV - RNA turned to negative or responded partially. The patients were considered to be effectively treated if the main symptoms were improved or disappeared and an enhancement was found in liver function, but hepatitis C markers barely changed. The treatment was proved to be invalid if the indicators and symptoms barely changed or even worsened.

Statistical analysis

All of the data were tested bilaterally and the difference was considered to be statistically significant if $p < 0.05$. Measurement data were analyzed using *t*-test or variance analysis when conforming to normal distribution and they were analyzed using nonparametric test when nonconforming to normal distribution. Enumeration data were analyzed applying *chi*-squared test.

RESULTS

Comparison of CLDQ before and after treatment between the three groups

CLDQ results indicated that all of the 63 participants completed the questionnaire. There was a significant difference in quality of life before and after treatment ($p < 0.05$) (Table I).

Table I. *Changes of CLDQ.*

Groups	TCM group (n=21)	Combined group (n=21)	Western medicine group (n=21)
Baseline phase	141.74±15.46	141.36±11.21	139.21±13.69
One month after treatment	142.65±16.57	144.65±10.78	146.36±10.47
Three months after treatment	145.21±14.33	149.77±13.78	148.75±14.58
Six months after treatment	161.45±10.28	165.23±13.87	156.78±15.64
Nine months after treatment	171.65±9.56	175.98±15.91	169.36±18.91
Twelve months after treatment	184.41±14.35	189.63±13.65	180.33±13.52

Table II. *Results of ALT and AST during treatment in the three groups.*

Groups		TCM group (n=21)	Combined group (n=21)	Western medicine group (n=21)
ALT(U/L)	Baseline phase	66.52±20.38	70.38±20.47	72.65±16.44
	Three months after treatment	39.32±20.15	46.32±15.63	88.63±10.22
	Twelve months after treatment	22.36±16.32	22.44±15.47	46.96±20.48
AST(U/L)	Baseline phase	48.89±21.45	53.14±23.45	51.67±1.41
	Three months after treatment	29.74±16.39	31.25±22.88	40.89±12.01
	Twelve months after treatment	16.87±10.36	18.45±14.99	32.85±13.74

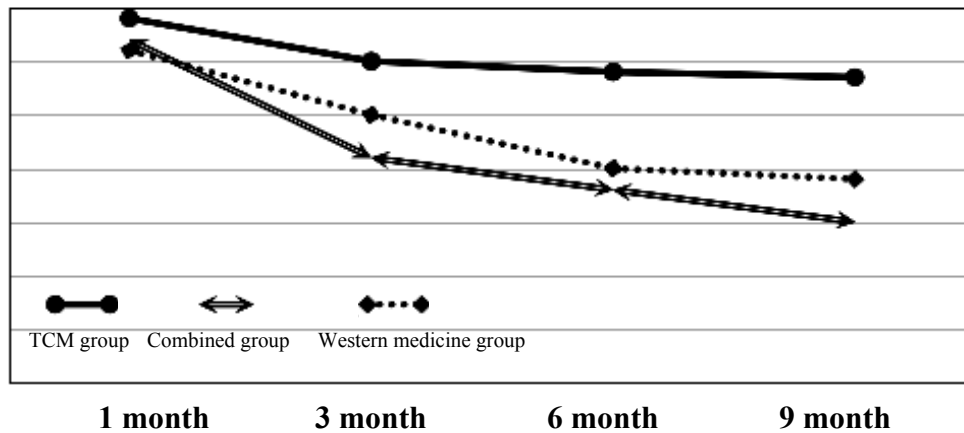


Fig. 1. Comparison on HCV-RNA titer level during treatment between the three groups.

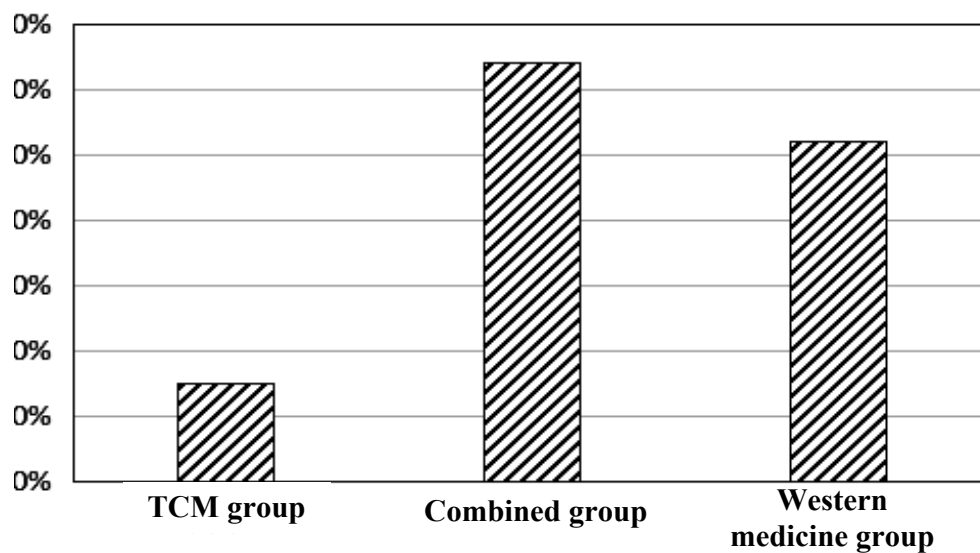


Fig. 2. Comparison on HCV-RNA decrease during treatment between the three groups.

Comparison on ALT and AST during the treatment between the three groups

After 3 and 12 months of treatment, a significant difference was found in ALT and AST between the TCM group and combined traditional Chinese and Western medicine group ($p < 0.05$); after 3 months of treatment, ALT and AST decreased less in the Western medicine group than in the other two groups and they declined not obviously after 12 months of treatment ($p < 0.05$) (Table II).

Comparison on HCV-RNA level during the treatment between the three groups

As shown in Fig. 1, HCV-RNA changed less in the TCM group than in the other groups while the combined traditional Chinese and Western medicine group was found with a higher decrease in the 24th and 36th week than the other two groups. It was hypothesized that TCM might assist the antiviral effect of interferon. However, TCM alone had no obvious antiviral effect, which was in line with previous reports.

As shown in Fig. 2, HCV-RNA titer in the combined traditional Chinese and Western medicine group decreased to below the highest proportion of detection level, which was obviously higher than that in the Western medicine group after treatment, while HCV-RNA titer in the TCM decreased to below the proportion of detection level (10%).

Comparison on side effects

During the treatment, no obvious adverse reaction occurred in the TCM group except for diarrhea, and side effects occurred less after TCM + interferon treatment than after interferon + ribavirin treatment. The combination of TCM and interferon + ribavirin could reduce the side effects of interferon and ribavirin. Diarrhea was the main adverse reaction of TCM and 12 and 8 patients were found it in the TCM group and the combined traditional Chinese and Western medicine group, respectively (Table III).

DISCUSSION

CLDQ designed by Younossii et al. (10) is widely recognized and used for measuring quality of life of patient with chronic liver disease, involving

symptoms of disease, degree of influence on patient's life and emotional changes. The most important reasons for decline in quality of life of CHC patients are physical fatigue, abdominal distension, stress and anxiety resulting from disease as well as social function limitation.

Serum transaminase mainly includes ALT (11-12) and AST (13-15) and their activities are sensitive indicators for liver cell damage. They both change significantly during CHC viral infection. The ratio of AST and ALT is always less than 1 while it is more than 1 in patients with liver cirrhosis. ALB synthesized by liver parenchyma cells is able to maintain plasma colloid osmotic pressure and the transport function of plasma albumin. It has no obvious performance in the early stage of CHC and is usually synthesized insufficiently in patients with liver cirrhosis.

During HCV-RNA reverse transcription-polymerase chain reaction (RT-PCR) (16-17), HCV cannot be detected with general methods due to its very low content in serum. The detection of anti-HCV can neither distinguish previous and present infections nor viremia and serum infections.

It is still difficult to fully present the actual

Table III. *Frequency of cumulative occurrence of side effects in the three groups during treatment.*

Groups	TCM group (n=21)	Combined group (n=21)	Western medicine group (n=21)
Influenza-like symptoms	0	11	10
Bone marrow suppression	0	7	8
Hemachrome decrease	1	8	7
Loss of weight	3	12	7
Hair loss	0	10	8
Formation of autoimmune antibody	0	7	12
Diarrhea	12	8	2

situation due to small sample size and non-randomized groups, although the results have statistical significance. Besides, the patients are followed-up just for one year and virus bounce rate, recurrence rate, patient's compliance, symptoms and improvement in quality of life after drug withdrawal should be further observed to provide an adequate basis for the prevention and cure of CHC in the future.

To sum up, the combination of TCM and interferon in the treatment of hepatitis C is able to protect liver, reduce side effects and improve quality of life of the patients. However, TCM alone has no obvious antiviral effect.

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LETTER TO THE EDITOR

ANCILLARY THERAPY OF INTRANASAL T-LysYal® FOR PATIENTS WITH ALLERGIC, NON-ALLERGIC, AND MIXED RHINITIS

M. GELARDI¹, S. TALIENTE¹, M.L. FIORELLA¹, N. QUARANTA¹, G. CIANCIO¹,
C. RUSSO², P. MOLA³, A. CIOFALO⁴, G. ZAMBETTI⁴, A. CARUSO ARMONE⁵,
E. CANTONE⁶ and G. CIPRANDI⁷

¹Otolaryngology, Department of Basic Medical Science, Neuroscience and Sensory Organs, University of Bari “Aldo Moro”, Bari, Italy; ²U.O.C. di Otorinolaringoiatria - Ospedale Di Venere, Carbonara Di Bari (BA), Italy; ³Division of Otolaryngology, Pinerolo and Rivoli Hospitals, ASL TO3, Pinerolo, Italy; ⁴Rhinology and Immuno-Allergy Unit, Sense Organs Department, Otolaryngology Section, “Umberto I” General Hospital, “La Sapienza” University, Rome, Italy;

⁵AIAS – Afragola (NA), Italy; ⁶Department of Neuroscience, Reproductive and Dentistry Science, ENT Unit; “Federico II” University, Naples, Italy; ⁷Department of Medicine, Istituto di Ricovero e Cura a Carattere Scientifico (I.R.C.C.S.) - Azienda Ospedaliera Universitaria San Martino, Genoa, Italy

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Allergic rhinitis (AR) is caused by an IgE-mediated inflammatory reaction. Non-allergic rhinitis (NAR) is characterized by a non-IgE-mediated pathogenesis. Frequently, patients have the two disorders associated: such as mixed rhinitis (MR). Hyaluronic acid (HA) is a fundamental component of the human connective tissue. HA may exert anti-inflammatory and immune-modulating activities. Recently, an intranasal HA formulation was proposed: a supramolecular system containing lysine hyaluronate, thymine and sodium chloride (T-LysYal®). This randomized study investigated whether intranasal T-LysYal® (rinoLysYal®, Farmigee, Italy) was able to reduce symptom severity, endoscopic features, and nasal cytology in 89 patients (48 males and 41 females, mean age 36.3±7.1 years) with AR, NAR, and MR. Patients were treated with intranasal T-LysYal® or isotonic saline solution as adjunctive therapy to nasal corticosteroid and oral antihistamine for 4 weeks. Patients were visited at baseline, after treatment and after 4-week follow-up. Intranasal T-LysYal® treatment significantly reduced the quote of patients with symptoms, endoscopic features, and inflammatory cells. In conclusion, the present study demonstrates that intranasal T-LysYal® is able, as ancillary therapy, to significantly improve patients with AR, NAR, and MR, and its effect is long lasting.

Allergic rhinitis (AR) is a very common disorder and may affect up to 40% of the population (1, 2). AR is caused by an IgE-mediated inflammatory reaction consequent to causal allergen inhalation. Allergy is sustained by a functional defect of allergen-specific T regulatory cells allowing T helper 2 (Th2) cell polarization. Th2 produce specific interleukins (IL), including IL-4, IL-5, and IL-13, able to drive

allergic inflammation, signed by eosinophil infiltrate. Allergic inflammation causes typical AR symptom occurrence, such as itching, sneezing, rhinorrhoea, and nasal obstruction (3). The same symptoms also characterize non-allergic rhinitis (NAR), which is defined by a non-IgE-mediated pathogenesis. Different types of NAR exist; their classification is based on the predominant cellular infiltrate:

Key words: T-LysYal®, allergic rhinitis, non-allergic rhinitis, mixed rhinitis

Mailing address:
Giorgio Ciprandi, M.D.
Largo R. Benzi 10,
16132 Genoa, Italy
Tel: + 39 10 35338120
e-mail: gio.cip@libero.it

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eosinophil (NARES), neutrophil (NARNE), mast cell (NARMA) or combined pattern, such as NARESMA, which is characterized by predominant eosinophilic and mast cell infiltrate (4). However, a relevant number of patients have both disorders associated, such as allergic and non-allergic rhinitis, so the term is mixed rhinitis (MR). The shared pathogenic basis of MR is nasal hyperreactivity: the exposure to non-specific stimuli (i.e. cool and dry air, vapors, irritants, spicy foods) elicits nasal symptoms.

The main prescribed medications for rhinitis are antihistamines and intranasal corticosteroids, which are usually effective, but may sometimes have adverse events. For this reason, the number is growing of doctors who also prescribe other drugs, including sodium hyaluronate: a salt of hyaluronic acid (HA) (5). HA is a fundamental component of the human connective tissue. It is chemically definable as a glycosaminoglycan. At physiological pH, the carboxyl groups of the glycuronic units are ionized, so HA acquires high polarity and consequently high water solubility. Thanks to this property, it is able to complex with many water molecules, reaching high level of hydration. Recently, a new HA formulation was developed: T-LysYal[®], which is a supramolecular system containing lysine hyaluronate, thymine, and sodium chloride. By its intermolecular powers, the aggregate has its own biological properties, which are different from those of its components and from sodium hyaluronate itself (6-9). There is a body of evidence that renders T-LysYal[®] interesting as a promising treatment for nasal disorders, as it is able to modulate inflammatory response, cellular proliferation, and remodelling of extracellular matrix. In particular, it has been reported that T-LysYal[®] increases fibroblast proliferation and collagen synthesis (promoting tissue repair) and inhibits, in a dose-dependent manner, basophil degranulation induced by antibodies anti-IgE receptors (Gruppo Biolab, data on file). In addition, T-LysYal[®] is more resistant to the lytic enzyme hyaluronidase (from *Streptomyces hyaluronitcus*) than HA, a fact accounts for a superior stability of the product that may exert a repairing activity for a longer period (Gruppo Biolab, data on file). Moreover, T-LysYal[®] inhibits the ICAM-1 expression on HUVEC induced by TNF- α in a

dose-dependent manner: this fact could be clinically relevant as reduced expression of ICAM-1 affects the adhesion of inflammatory cells on endothelial cells, a step of infiltration process (10). Previously, it was reported that Lysine Hyaluronate was better than HA in the healing process of decubitus ulcers in hospitalized patients (11).

More recently, the nasal formulation of T-LysYal[®] (rinoLysYal[®], Farmigea, Italy) was proposed. On the basis of this background, it was hypothesized that rinoLysYal[®], administered intranasally, could favour the process behind the regeneration of the nasal mucosa and create a lubricating, emollient, and protective action. It could aid the repairing process of the nasal mucosa, injured or dry. Therefore, the present randomized study aimed to investigate whether rinoLysYal[®], administered as adjunctive therapy, could improve symptoms, endoscopic features, and cytological parameters in patients with AR, NAR, or MR.

MATERIALS AND METHODS

Globally, 89 patients (48 males and 41 females, mean age 36.3 ± 7.1 years) were enrolled. The inclusion criteria were: i) either gender and adult age, ii) documented diagnosis of AR, NAR or MR, iii) presence of nasal symptoms for at least one month, documented by a run-in period, iv) written informed consent. Exclusion criteria were: i) concomitant comorbidities, including respiratory infections, ii) chronic illness, iii) hypersensitivity to T-LysYal[®].

All patients were treated with a standard therapy: intranasal mometasone furoate (1 puff per nostril twice a day), rupatadine fumarate (1 tablet daily), and nasal lavage with isotonic saline solution (1 puff per nostril twice a day). The treatment lasted for 4 weeks. At baseline, the patients were randomly (1:1 ratio) subdivided into two groups: 48 patients were adjunctively treated with nasal T-LysYal[®], rinoLysYal[®], (Group A), and 41 were considered as control (Group B). Active medication was intranasal cream T-LysYal[®]: 1 cm per nostril in the afternoon. Patients in the control group used only the standard therapy.

Patients were visited at baseline (T0), after treatment (T1), and after a one month follow-up period (T2). During the visits, patients were evaluated by clinical assessment, endoscopic exam, and nasal cytology. Adverse events

were registered as usual. As specified in the technical profile, clinically relevant adverse events are not known for rino-LysYal®; in presence of nasal crust, a burning sensation may occasionally occur, but it disappears soon after.

Clinical parameters

The following parameters were evaluated: nasal obstruction, epistaxis, rhinorrhoea, itching, sneezing, crust, post nasal drip, headache, burning, dryness, and smell impairment, and were assessed if present at visit.

Endoscopy

Nasal endoscopy was carried out by a 3.4 mm diameter flexible fibroscope (Vision-Sciences® ENT-2000), to assess the presence of major abnormalities, such as septal deviation, polyposis, turbinate hypertrophy, or exudation from the ostio-meatal complex. Presence of turbinate hypertrophy and oedema, and the mucosal characteristics were assessed.

Nasal cytology

This procedure was performed by scraping the middle part of the inferior turbinate with a Rhino-Probe® device (Arlington Scientific). The sample was smeared on a slide, air-dried, then stained with the May-Grünwald Giemsa preparation. The type and cell number were examined using microscopy (Nikon® E600). Cell types were identified, and intracellular components were studied at 1000x in oil immersion. The mean number per 50 fields was calculated and reported (12, 13). It was performed only at baseline and after follow-up

Statistical analysis

Statistical analysis of the results was performed using χ^2 and Wilcoxon tests for intergroup and intragroup analysis at the different times. Clinical parameters and endoscopic features were expressed as percentage of patients presenting the specific symptom. Nasal cytology data were expressed as absolute values. The statistical software (Statistica 8.0) was used for analysis. RESULTS All patients completed the study; the treatments were well tolerated and no clinically relevant adverse event was reported.

Clinical parameters

Nasal obstruction: the percentage of patients with

obstruction significantly decreased during the study in Group A (T0= 85.4%; T1= 50%; T2= 16.7%) as well as in Group B (T0= 78%; T1= 63.4%; T2= 51.2%) as shown in Fig. 1. The intragroup significance was for Group A $p=0.003346$ between T0 and T1 and $p=0.000027$ between T0 and T2; for Group B $p=0.027709$ between T0 and T1; $p=0.003346$ between T0 and T2. The intergroup comparison showed that there was a significant ($p=0.000$) difference between groups at T2.

Epistaxis: the percentage of patients with epistaxis diminished in Group A (T0= 12.5%; T1= 2.1%; T2= 2%), but significantly increased in Group B (T0= 17%; T1= 14.6%; T2= 29.7% $p=0.038153$ between T0 and T2). The intergroup comparison showed that there was a significant difference between groups at T1 and T2 ($p=0.0013$ and $p=0.000$, respectively).

Rhinorrhoea: the percentage of patients with rhinorrhoea significantly reduced in Group A (T0= 85.4%; T1= 27.1%; T2= 27%; $p=0.004729$ T0-T1, $p=0.000293$ T0-T2) as well as in Group B (T0= 73.17% T1= 58.54%; T2= 53.65%; $p=0.043115$ T0-T2, $p=0.017961$ T0-T2), as shown in Fig. 1. The intergroup comparison showed that there was a significant difference between groups at T0 ($p=0.0325$), T1 ($p=0.0000$), T2 ($p=0.0001$).

Itching: the percentage of patients with itching significantly decreased in Group A (T0= 60.4%; T1= 16.7%; T2= 2.1%; $p=0.000655$ T0-T1 and $p=0.000293$ T0-T2) as well as in Group B (T0= 65.8%; T1= 29.3%; T2= 31.7%; $p=0.007686$ T0-T1 and $p=0.000655$ T0-T2), as reported in Fig. 1. The intergroup comparison showed that there was a significant difference between groups at T1 and T2 ($p=0.034$ and $p=0.000$, respectively).

Sneezing: the percentage of patients with sneezing significantly diminished in Group A (T0= 68.7%; T1= 25%; T2= 10.4%; $p=0.003779$ T0-T1 and $p=0.000040$ T0-T2) as well as in Group B: (T0= 71.3%; T1= 46.3%; T2= 31.7%; $p=0.003346$ T0-T1 and $p=0.000419$ T0-T2), as shown in Fig. 1. The intergroup comparison showed that there was a significant difference between groups at T1 and T2 ($p=0.0000$ and $p=0.034$, respectively).

Crust: the percentage of patients with crust significantly reduced in Group A (T0= 39.6%; T1= 56.2%; T2= 4.2%; $p=0.017961$ T0-T1 and $p=0.002218$ T0-T2), but not in Group B (T0= 36.6%; T1= 46.3%; T2= 26.8%). The intergroup comparison showed that there was a significant ($p=0.000$) difference between groups at T2.

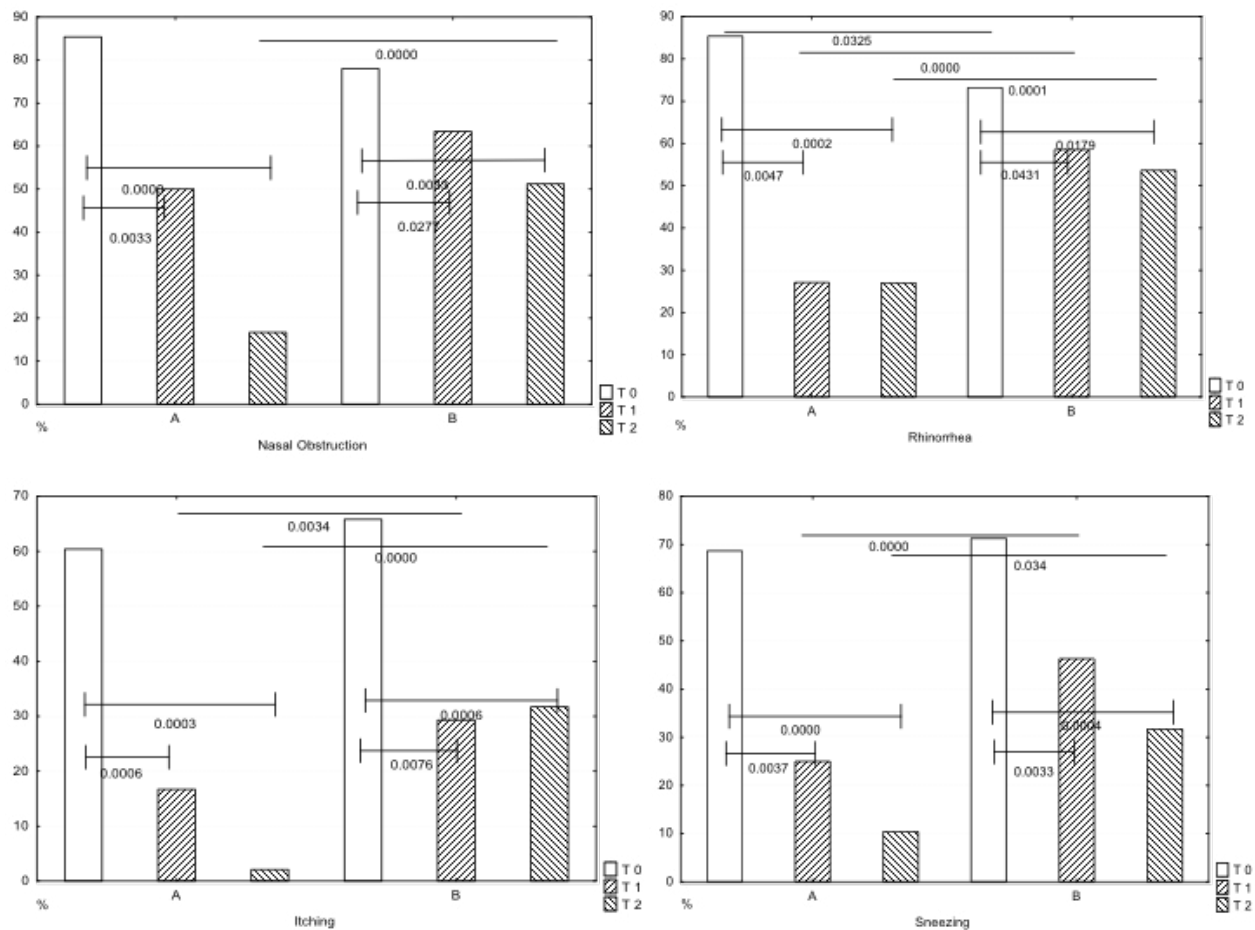


Fig. 1. Upper left quadrant= percentages of patients with obstruction in groups A and B at T0, T1, and T2. Upper right quadrant= percentages of patients with rhinorrhoea in groups A and B at T0, T1, and T2. Lower left quadrant= percentages of patients with itching in groups A and B at T0, T1, and T2. Lower right quadrant= percentages of patients with sneezing in groups A and B at T0, T1, and T2. (*p* values are reported upon bars).

Burning: the percentage of patients with nasal burning significantly diminished in Group A (T0= 52.1%; T1= 12.5%; T2= 8.3%; $p=0.007133$ T0-T1 and $p=0.001474$ T0-T2), but not in Group B (T0= 41.4%; T1= 31.7%; T2= 19.5%). The intergroup comparison showed that there was a significant difference between groups at T1 and T2 ($p=0.001$ and $p=0.022$, respectively).

Nasal dryness: the percentage of patients with nasal dryness significantly reduced in Group A (T0= 27.1%; T1= 2.1%; T2= 2%; $p=0.017961$ T0-T1 and $p=0.017961$ T0-T2), but not in Group B (T0= 17%; T1= 17%; T2= 19.5%). The intergroup comparison showed that there was a significant difference between groups at T1 and T2 ($p=0.0003$ and $p=0.0001$, respectively).

Smell impairment: the percentage of patients with smell impairment significantly reduced in Group A: T0=

52.1%; T1= 25%; T2= 8.3%; $p=0.017961$ T0-T2 and $p=0.007686$ T0-T2), but not in Group 2 (T0= 39%; T1= 26.8%; T2= 26.8%). The intergroup comparison showed that there was a significant ($p=0.0006$) difference between groups at T2.

Endoscopic features

Hypertrophic turbinates: the percentage of patients with hypertrophic turbinates in Group A were 41.67% at T0, 45.83% at T1, 31.25% at T2, and in Group B were 48.78% at T0, 56.1% at T1, 60.98% at T2, as shown in Fig. 2. The intergroup analysis demonstrated that the number of patients with HT significantly ($p=0.0000$) diminished only in Group A at T2.

Oedematous turbinates: the percentage of patients with oedematous turbinates in Group A were 52.08% at

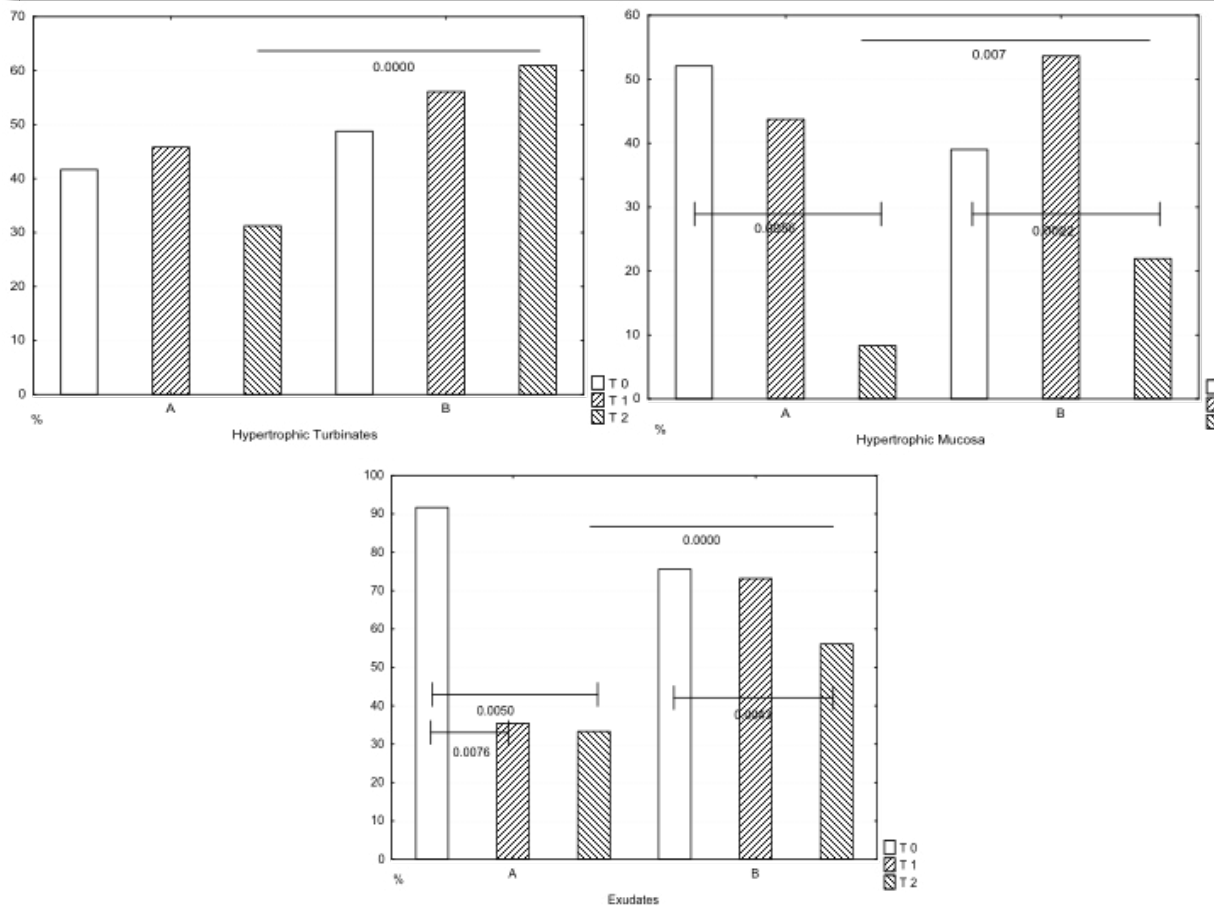


Fig. 2. Upper left quadrant= percentages of patients with hypertrophic turbinates in groups A and B at T0, T1, and T2. Upper right quadrant= percentages of patients with hypertrophic mucosa in groups A and B at T0, T1, and T2. Lower quadrant= percentages of patients with exudates in groups A and B at T0, T1, and T2. (p values are reported upon bars).

T0; 18.75% at T1 and 2.08% at T2, and in Group B were 24.39% at T0 56.09% at T1, and 60.97% at T2. There was a significant reduction in Group A between T0 and T2 ($p=0.007686$), but a significant increase in Group B between T0 and T2 ($p=0.027709$). The intergroup analysis showed a difference at T2 ($p=0.0000$).

Hypertrophic mucosa: the percentage of patients with hypertrophic mucosa significantly decreased in Group A (T0 52.08%, T1 43.75%, T2 8.33%; $p=0.005618$ between T0 and T2) as well as in Group B (T0 39.02%, T1 53.65%, T2 21.95%; $p=0.002218$), as shown in Fig. 2. The intergroup analysis showed a significant difference ($p=0.007$) at T2.

Disepithelized mucosa: in Group A the percentages were 22.92% at T0, 25% at T1, 6.25% at T2, and in Group B were 26.83% at T0, 24.39% at T1, and 36.58% at T2.

The intergroup analysis showed a significant difference ($p=0.0000$) at T2.

Presence of exudates: in Group A the percentages significantly diminished from 91.67% at T0, to 35.42% at T1, and 33.33% at T2 ($p=0.007686$ and $p=0.005062$, respectively), and in group B significantly diminished only at T2 (75.6% at T0, 73.17% at T1, and 56.1% at T2; $p=0.004315$), as shown in Fig. 2. The intergroup analysis showed a significant difference at T2 ($p=0.000$).

Nasal cytology

Neutrophil: patients of Group A had a significant decrease (T0= 208; T2= 97; $p=0.0000$) as did patients of Group B (T0= 222; T2= 156; $p=0.000$), as shown in Fig. 3. There was a significant difference between Groups A and B at T2 ($p=0.0000$).

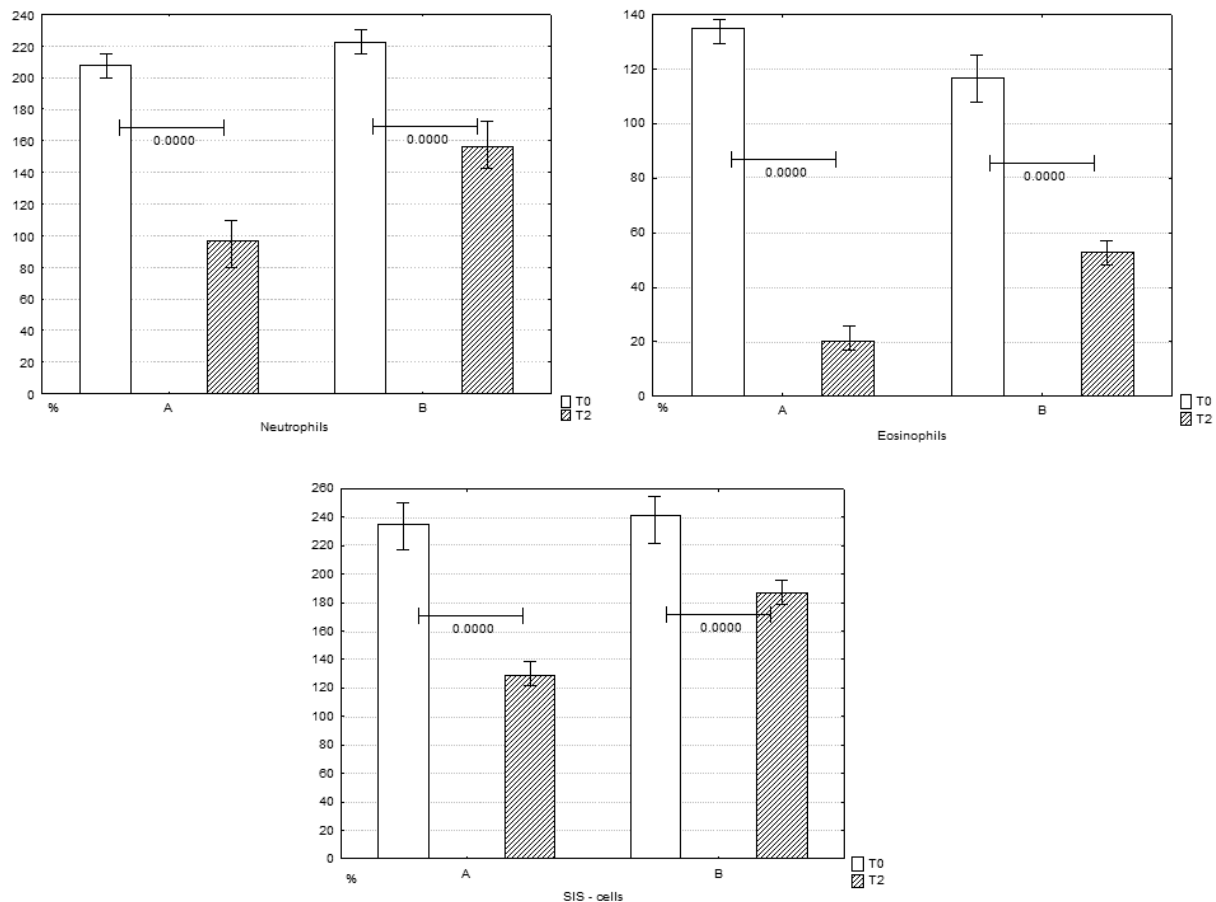


Fig. 3. Upper left quadrant= mean number of neutrophils in groups A and B at T0 and T2. Upper right quadrant= mean number of eosinophils in groups A and B at T0 and T2. Lower quadrant= mean number of SIS negative cells in groups A and B at T0 and T2. (data are expressed as mean $\pm$ S.D.; p values are reported upon bars).

Eosinophil: patients of Group A had a significant decrease (T0= 135; T2= 20; $p=0.0000$) as did patients of Group B (T0= 117; T2= 53; $p=0.0000$), as shown in Fig. 3. There was a significant difference between Groups A and B at T2 ($p=0.0000$).

SIS- cells: patients of Group A had a significant decrease (T0= 235; T2= 129; $p=0.0000$) as did patients of Group B (T0= 241; T2= 187; $p=0.0000$). There was a significant difference between Groups A and B at T2 ($p=0.0000$).

SIS+ cells: patients of Group A had an increase (T0= 100; T2= 216) as did patients of Group B (T0= 76; T2= 112; $p=0.012$).

Mast cell: Group A had at T0= 39; at T2= 12.5. Group B had at T0= 28; at T2= 12. The number significantly diminished in both groups ($p=0.0001$ and $p=0.01$, respectively), without intergroup difference.

Bacteria: Group A had at T0= 47; at T2= 4. Group B had at T0= 48; at T2= 9. The number significantly diminished in both groups ($p=0.0000$ for both), without intergroup difference.

Lymphocyte: Group A had at T0= 35; at T2= 6. Group B had at T0= 14; T2= 2. The number significantly diminished in both groups ($p=0.0001$ and 0.002 , respectively), without intergroup difference.

DISCUSSION

This randomized study provided the first evidence that intranasally-administered T-LysYal[®] was able to significantly affect nasal symptoms, endoscopic features, and cytological data in patients with AR, NAR, and MR. The Authors designed this initial study considering that T-LysYal[®] could be firstly

proposed as ancillary treatment, waiting for further evidence of it as first line therapy.

These findings may be explained by the complex anti-inflammatory and immune-modulating activity exerted by the compound. In fact, hyaluronan is a non-sulfated glycosaminoglycan found in the extracellular matrix of all tissues; it plays a multifunctional role in the reparative process and is essential in the homeostasis of respiratory airways (14-17).

In particular, the number of patients with symptoms and endoscopic features typical of rhinitis significantly decreased after intranasal T-LysYal® treatment. These positive effects are consistent with the parallel effect of nasal inflammation pattern. Indeed, intranasal T-LysYal® therapy is able to significantly reduce the grade of eosinophilic and neutrophilic infiltrate as well as increase the quote of well-being epithelial cells. This local anti-inflammatory activity may explain the relevant effect on symptoms (mainly obstruction) and signs (mainly oedema) of rhinitis. However, it has to be considered that the decrease in count of bacteria and neutrophils might be due to normal immune response against bacterial infection.

In addition, the presence of lysine may exert synergic effects by the specific activities exerted by this aminoacid. Noteworthy, the effectiveness of T-LysYal® lasted also during the follow-up period. This fact is particularly relevant as it demonstrates the profound impact exerted on mucosal repair.

However, the present study has some limitations: there were a restricted number of patients, and a lack of the demonstration of effects on pathophysiologic mechanisms. In this regard, there is an ongoing study exploring the effects of intranasal T-LysYal® on mucociliary clearance that may provide new information on the mechanism of action. On the other hand, this study is randomized and has a follow-up period of observation. However, further studies should be conducted to confirm this initial evidence.

Of course, it has to be noted that intranasal T-LysYal® was prescribed as ancillary therapy in addition to standard treatment of rhinitis, such as intranasal corticosteroid and oral antihistamine.

In conclusion, the present randomized study provides evidence that intranasal T-LysYal® is able,

as ancillary therapy, to significantly affect symptom severity, endoscopic features, and cytological parameters in patients with AR, NAR, and MR, and its effect is long lasting.

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LETTER TO THE EDITOR

8-ISOPROSTANE IN EXHALED BREATH CONDENSATE AFTER EXPERIMENTAL EXPOSURE TO WOOD SMOKE IN HUMANS

N. MURGIA^{1,2}, L. BARREGARD¹, G. SALLSTEN¹, A.C. ALMSTRAND¹, P. MONTUSCHI³,
G. CIABATTONI⁴ and A.C. OLIN¹

¹Department of Occupational and Environmental Medicine, University of Gothenburg, Gothenburg, Sweden; ²Section of Occupational Medicine, Respiratory Diseases and Occupational and Environmental Toxicology, University of Perugia, Perugia, Italy; ³Department of Pharmacology, Faculty of Medicine, Catholic University of the Sacred Heart, Rome, Italy; ⁴Department of Drug Sciences, School of Pharmacy, University of Chieti "G. d'Annunzio", Chieti, Italy

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Wood smoke, a well-known indoor and outdoor air pollutant, may cause adverse health effects through oxidative stress. In this study 8-isoprostane, a biomarker of oxidative stress, was measured in exhaled breath condensate (EBC) and urine before and after experimental exposure to wood smoke. The results were compared with measurements of other biomarkers of oxidative stress and inflammation. Thirteen subjects were exposed first to clean air and then, after 1 week, to wood smoke in an exposure chamber during 4-hour sessions. Exhaled breath condensate, exhaled nitric oxide, blood and urine were sampled before and at various intervals after exposure to wood smoke and clean air. Exhaled breath condensate was examined for 8-isoprostane and malondialdehyde (MDA), while exhaled air was examined for nitric oxide, serum for Clara cell protein (CC16) and urine for 8-isoprostane. 8-isoprostane in EBC did not increase after wood smoke exposure and its net change immediately after exposure was inversely correlated with net changes in MDA ($r_s = -0.57$, $p = 0.041$) and serum CC16 (S-CC16) ($r_p = -0.64$, $p = 0.020$) immediately after the exposure. No correlation was found between 8-isoprostane in urine and 8-isoprostane in EBC. In this study controlled wood smoke exposure in healthy subjects did not increase 8-isoprostane in EBC.

Wood smoke exposure has been associated with indoor and outdoor air pollution and many adverse health outcomes. Fireplaces and wood burning stoves are the source of many health hazards including chemical compounds such as nitrogen oxides, carbon monoxide, particulate matter (PM), benzene and acrolein (1). Some of these substances are well-known inducers of oxidative stress, which is an established mechanism of wood smoke-related lung damage. The association between wood smoke

and oxidative lung damage has been demonstrated at the cellular level (2), in animal studies (3) and also in human experimental settings where experimental exposure in healthy humans, besides other effects, yielded an increase in malondialdehyde (MDA), a marker of oxidative stress, in exhaled breath condensate (EBC) (4). Exhaled breath condensate is one of the most studied techniques to sample airway lining fluid to search for markers of oxidative stress (5). Even if there are still unresolved questions

Key words: oxidative stress, wood smoke, exhaled breath condensate (EBC), 8-isoprostane, malondialdehyde (MDA)

Mailing address:

Dr Nicola Murgia,
Department of Occupational and Environmental Medicine,
University of Gothenburg, Box 414, 405 30 Göteborg, Sweden
Tel.: +4631 786 63 00 - Fax: +463 140 97 28
e-mail: nicola.murgia@unipg.it

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from a theoretical and methodological point of view (6), EBC is one of the more interesting tools for investigating the respiratory system, because it is non-invasive and easy to test. From a clinical and analytical point of view, one of the most reliable biomarkers of oxidative stress in EBC is 8-isoprostane (5). This compound belongs to the family of F2-isoprostanes and was found to be increased in adults with stable asthma (7), patients with chronic obstructive pulmonary disease (COPD) (8) and also subjects with interstitial lung diseases such as sarcoidosis (9).

The aim of this study was to examine whether experimental exposure of healthy humans to wood smoke increases 8-isoprostane in EBC and whether, in the same experimental setting, there is a correlation between 8-isoprostane in EBC and other biomarkers, previously analysed (4), of oxidative stress and inflammation such as exhaled nitric oxide, MDA, 8-isoprostane in urine, and Clara cell protein (CC16) in serum (S-CC16).

MATERIALS AND METHODS

Subjects

Thirteen healthy, non-smokers with normal spirometry and without occupational exposure to irritants or other risk factors for the respiratory tract were enrolled in the study. They were six men and seven women aged 20–56 (mean 34) years and reported to be free from known infections at least 1 week before the experiment and to have not taken drugs in the 2 days prior to the experiment.

The study protocol was approved by the institutional review board of the University of Gothenburg, Gothenburg, Sweden. Written consent was obtained from the subjects.

Study design

All the subjects underwent two sessions in an exposure chamber. First they were exposed to filtered indoor air in an exposure chamber for 4 hours and then, after 1 week, they were exposed to wood smoke in the same chamber for 4 hours. The sessions included two 25-minute periods of light exercise. Sessions started in the morning at 8.30, in two separate rounds on different days with seven and six subjects in the chamber. Prior to exposure,

each session started with urine sampling, followed by fractional exhaled nitric oxide (FeNO) measurements, blood sampling and collection of EBC (a). The sampling was repeated immediately after the exposure (b), and at 3 hours (c) and 20 hours (the morning after) (d) after the exposure. Sampling at (c) did not include urine.

Wood smoke generation and exposure characterization

Generation of wood smoke has been described and the exposure characterized previously in detail (10). Briefly, wood smoke was generated in a cast iron stove outside the chamber and mixed with indoor air, filtered using a high-efficiency particulate air filter, to the target concentration. The exposure to particulate matter (PM) and volatile organic compounds (VOCs) was monitored by personal and stationary measurements. Nitrous oxides, CO₂, CO, temperature and relative humidity were measured with direct-reading instruments and loggers. Details on mass concentrations, particle size distributions, and concentrations of elements and polycyclic aromatic hydrocarbons (PAHs) are reported elsewhere (10). In brief, the median mass concentrations were 279 µg/m³ and 243 µg/m³, respectively, in the two wood smoke rounds. The PM number concentrations were 180000/cm³ and 95 000/cm³, with 65% and 28% ultrafine particles (UFPs) (<100 nm). In the clean air sessions, mass concentrations were low, many below the detection limit. For NO₂, the mean concentration was 0.01 ppm in the clean air experiments and 0.08–0.09 ppm in the two wood smoke sessions. The median concentrations of formaldehyde and acetaldehyde were 114 µg/m³ and 75 µg/m³, respectively, in the first, and 64 µg/m³ and 40 µg/m³ in the second wood smoke round. In the clean air sessions, levels of both substances were ≤10 µg/m³. The median benzene and 1,3-butadiene concentrations were 30 µg/m³ and 6.3 µg/m³ in the first and 20 µg/m³ and 3.9 µg/m³ in the second wood smoke round. The levels in the clean air sessions were at least ten times lower. The exposure to PM and VOCs in the first round of exposure to wood smoke was slightly higher than in the second round of exposure to wood smoke (10).

Symptoms and biomarkers

Symptoms were registered using a self-administered questionnaire. Urinary excretion of free 8-isoprostane (10) was measured. Timed urinary sampling was

incomplete in three subjects; hence these results are based on ten subjects. Clara cell protein in serum was analysed using an enzyme-linked immunosorbent assay (ELISA) (Bio Vendor, Brno, Czech Republic).

A chemiluminescence analyser (NIOX system; Aerocrine AB, Solna, Sweden) was used to measure FeNO at a flow rate of 50 ml/s (FeNO₅₀), 100 ml/s (FeNO₁₀₀) and 270 ml/s (FeNO₂₇₀). A $\pm 10\%$ deviation of the instant flow and $\pm 5\%$ of the mean flow during the plateau phase was considered acceptable. All measurements were performed in duplicate, according to the 2005 American Thoracic Society/ European Respiratory Society (ATS/ERS) recommendations (11), after at least 1 hour of fasting. Exhaled breath condensate samples were collected using an RTube EBC collector (Respiratory Research, Charlottesville, VA, USA). The exhaled volume was measured with an ECoVent spirometer (Jaeger, Würzburg, Germany). Subjects wore a nose clip, rinsed their mouth with distilled water for 30 seconds before sampling, and breathed tidally through the RTube until 80 litres had been exhaled. Samples were frozen at -20°C and then analysed. 8-isoprostane in EBC samples was measured with a specific radioimmunoassay (RIA) validated by reverse-phase high-performance liquid chromatography (HPLC), as described previously (12). The detection limit was 6 pg/mL. Measurement of MDA in EBC was carried out as previously described (13).

Statistics

The levels of FeNO, 8-isoprostane in urine and EBC, and MDA in EBC were not normally distributed, while S-CC16 was. Differences in EBC 8-isoprostane between clean air and wood smoke sessions were tested on every sampling occasion by Wilcoxon's signed-rank test. At exposure to wood smoke, differences in EBC 8-isoprostane before and immediately after the exposure, as well as 3 and 20 hours after the exposure were tested using Friedman test for multiple correlated samples, followed by a pairwise Wilcoxon's signed-rank test with Bonferroni correction. Net changes of 8-isoprostane or other biomarkers were defined as the difference between the levels before and after wood smoke, subtracting the difference between levels before and after clean air. Since net changes in EBC 8-isoprostane were normally distributed, the differences between net changes immediately after the exposure, and again 3 and 20

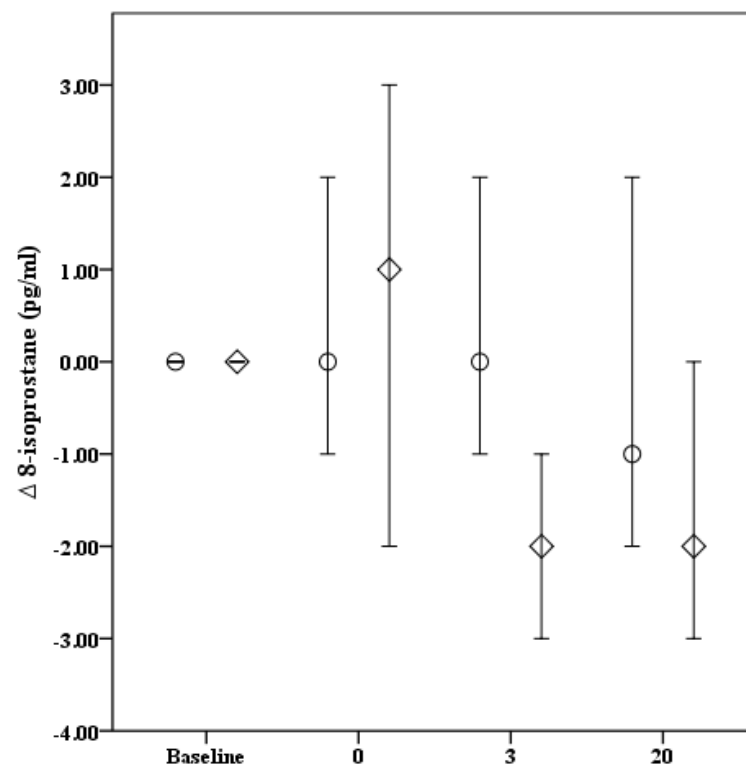
hours after (the day after) the exposure were tested with repeated-measures analysis of variance (RM-ANOVA). For the same reason, the association between net changes of EBC 8-isoprostane and S-CC16 was assessed with Pearson's correlation coefficient (r_p), while the associations between EBC 8-isoprostane and the other biomarkers not normally distributed were assessed using Spearman's rank correlation coefficient (r_s). The analysis was performed with the statistical software package SPSS 18.0 (SPSS IBM, Chicago, IL, USA).

RESULTS

Median values and range of FeNO, MDA and CC16 are already available in a previous publication (4). Medians and ranges of 8-isoprostane in EBC before and after exposure to clean air and wood smoke are given in Table I. Fig. 1 shows the differences between 8-isoprostane in EBC before and 8-isoprostane in EBC after exposure, adjusted for changes after exposure to clean air, setting the baseline at 0, with medians and 90% confidence intervals given. No significant differences were found in median 8-isoprostane concentrations at any of the sampling times, comparing pairwise changes after exposure to clean air and wood smoke. In the wood smoke session an increase was seen in the median EBC 8-isoprostane across the sampling time ($\chi^2 = 8.42$, $p = 0.038$) because 8-isoprostane was higher immediately after wood smoke exposure and lower after 3 hours; however, after Bonferroni correction these differences were both non-significant. No significant differences were found in EBC 8-isoprostane net changes over time, with men and women analysed separately. No correlations were found between EBC 8-isoprostane and 8-isoprostane in urine at any sampling time (Figs. 2 and 3). Net changes in EBC 8-isoprostane immediately after the exposure were inversely correlated with net changes in MDA immediately after the exposure ($r_s = -0.57$, $p = 0.041$) and net changes in S-CC16, 3 hours after the exposure ($r_p = -0.64$, $p = 0.020$). There was a positive correlation between EBC 8-isoprostane and net changes in FeNO₅₀ 3 hours after exposure ($r_s = 0.66$, $p = 0.015$) (Fig. 4).

Table I. Concentration of 8-isoprostane in exhaled breath condensate (EBC) before and after the exposure to clean air and wood smoke.

	Baseline (pg/ml)	8-isoprostane concentration (pg/ml)		
		immediately after exposure	3 hours after exposure	20 hours after exposure
	median (range)	median (range)	median (range)	median (range)
Clean air	16 (12–21)	16 (12–25)	17 (12–23)	14 (12–19)
Wood smoke	15 (13–28)	18 (12–24)	14 (12–24)	15 (12–22)
Net changes	–	0 (–11–5)	–3 (–14–5)	–2 (–9–6)

**Fig. 1.** Changes (Δ) in 8-isoprostane in exhaled breath concentrate (EBC) after exposure to wood smoke (diamonds and lines) and clean air (circles and dashed lines) in 13 subjects. The graph shows the changes (medians and 90% confidence intervals) immediately after exposure (0 h), 3 hours later, and in the morning, 20 hours post-exposure.

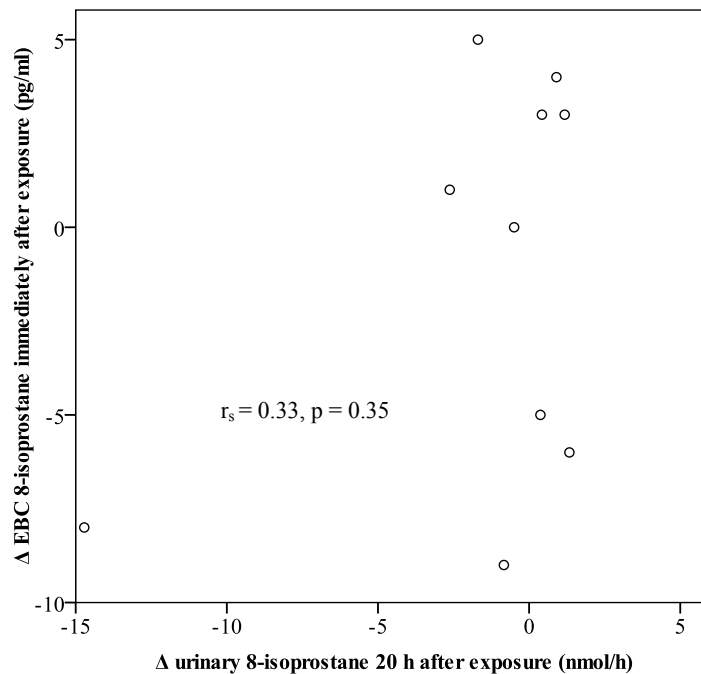


Fig. 2. Correlation between the net change (Δ) in exhaled breath concentrate (EBC) 8-isoprostane immediately after the exposure and urinary 8-isoprostane, 20 hours post-exposure.

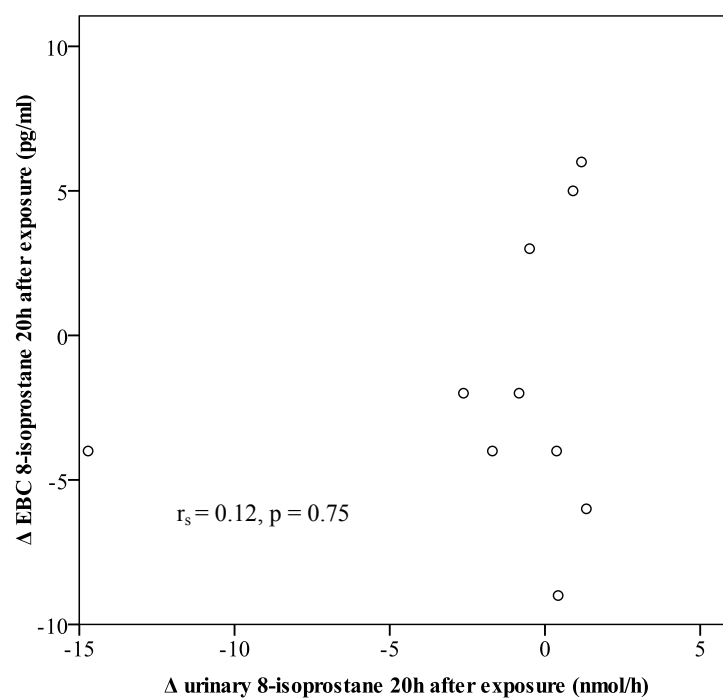


Fig. 3. Correlation between the net change (Δ) in exhaled breath concentrate (EBC) 8-isoprostane and urinary 8-isoprostane, 20 hours post-exposure.

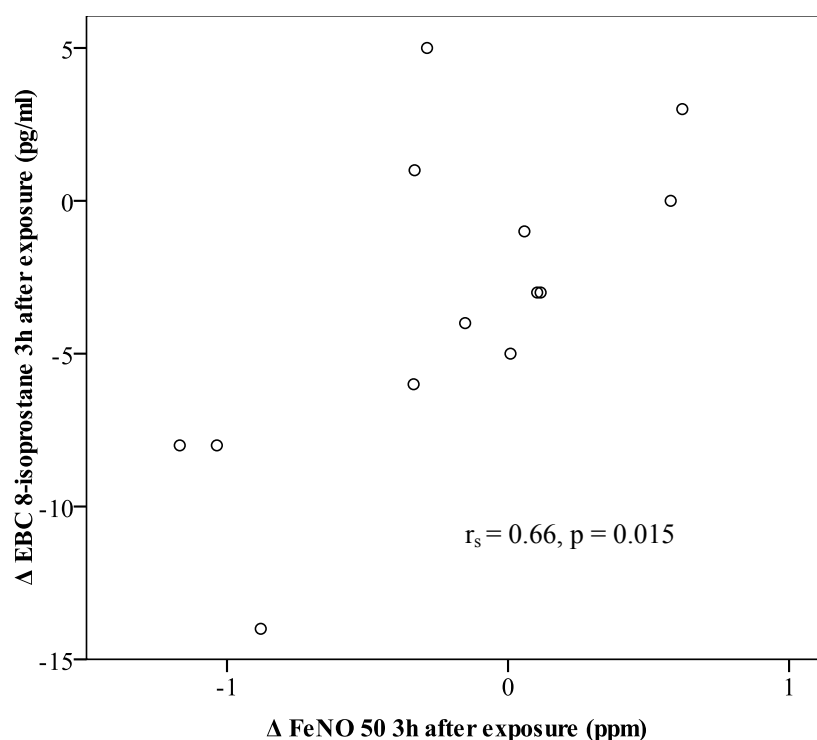


Fig. 4. Association between net changes (Δ) in exhaled breath concentrate (EBC) 8-isoprostane and fractional exhaled nitric oxide at a flow rate of 50 ml/s (FeNO_{50}), 3 hours post-exposure.

DISCUSSION

Exposure to wood smoke, 8-isoprostane and oxidative stress

In this study, experimental exposure to wood smoke in humans did not affect 8-isoprostane in EBC. Comparison between individual changes in EBC 8-isoprostane and changes in other biomarkers yielded inconsistent findings. Some were positive (FeNO_{50}) and some were negative (S-CC16, MDA). As a matter of fact, on the basis of both the current knowledge on EBC 8-isoprostane kinetics after exposure to allergens or cigarette smoke (14, 15) and our previous results in the same experimental setting (4, 10), we could expect a positive correlation between EBC 8-isoprostane immediately after the exposure or 3 hours after the exposure, urinary 8-isoprostane the day after and MDA immediately after the exposure, but there were no such positive associations.

Wood smoke has previously been shown to induce oxidative stress in lung cell lines and in animal

models (16, 17). In the present study, 8-isoprostane in EBC, after an initial, non-significant increase, decreased 3 hours after the exposure.

8-isoprostane is one of the most reliable biomarkers of oxidative stress and inflammation in EBC, where it has been used to study COPD, asthma, cystic fibrosis and other respiratory diseases (5). As mentioned previously, in asthmatics, EBC 8-isoprostane was increased also after allergen challenge (14) and 90 minutes after exposure to mainstream cigarette smoke (15). However, in children with asthma, the exposure to environmental outdoor pollutants, on the day of sampling, was not associated with 8-isoprostane increase (18). Interestingly, in healthy subjects neither exposure to cigarette smoke (15) nor exposure to chlorine during swimming (19) induced a rise in 8-isoprostane in EBC.

These previous findings in healthy individuals are in agreement with the absence, in this study, of an increase in EBC 8-isoprostane after wood smoke exposure. It is possible that in healthy humans, the

increase is either absent or too low to be detected. Surprisingly, we found an inverse correlation between EBC 8-isoprostane and MDA or S-CC16 after exposure to wood smoke, although all of these are relevant indicators of lipid peroxidation during oxidative stress. In fact, MDA is one of the most studied biomarkers of lipid peroxidation (6). Clara cell protein is able to modulate oxidative stress through phospholipase A2, a key enzyme in lipid peroxidation (20). However, the increase in EBC 8-isoprostane, though not significant, was quick and transient (Fig. 1), while the increase in MDA and S-CC16 was slow and was observed the day after the exposure. There was an association between net changes in EBC 8-isoprostane and FeNO₅₀. The changes in EBC 8-isoprostane or FeNO₅₀ were, however, not significant.

Validity issues

The small number of participants can be seen as the main limitation of the present work; on the other hand, this is the first study to use an experimental exposure design to study 8-isoprostane in EBC. The collection of EBC was conducted according to the ERS/ATS task force recommendations, with care taken to avoid saliva contamination, the subjects being invited to rinse and wash the mouth before the sampling. The analysis of 8-isoprostane was conducted using a RIA validated by reverse-phase HPLC (12). All the samples were above the detection limit in both sessions, with values consistent with values in healthy subjects, while for MDA, many samples were below the detection limit (4). Given the fairly homogeneous data, with no undetectable samples, and the procedure used to store and analyse the samples, it is unlikely that the results were influenced by technical issues (e.g. storage, transportation, freezing and thawing), even if the country of sampling (Sweden) differed from the country where the samples were analysed (Italy). The fairly small number of subjects and the 8-isoprostane distribution, not normalized even after log transformation, obliged us to use non-parametric tests to study differences between participants and (in the same subject) at different times and exposures (clean air v wood smoke), as well as the correlation

between biomarkers.

In conclusion, we did not find any significant rise in EBC 8-isoprostane after wood smoke exposure, nor the expected positive correlation with other markers of oxidative stress, apart from a correlation with FeNO 3 hours after exposure. Therefore, 8-isoprostane, a reliable biomarker of lipid peroxidation and oxidative stress, does not appear to be sufficiently sensitive to demonstrate any oxidative stress response after short-term controlled wood smoke exposure at the levels of the present study, at least not in EBC. However, larger exposure studies are needed to fully elucidate the role of lipid peroxidation caused by the exposure to wood smoke in occupational and non-occupational settings.

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LETTER TO THE EDITOR

**HYPERTONIC SALINE MONOTHERAPY IN CHILDREN
WITH PERENNIAL ALLERGIC RHINITIS: A PILOT STUDY**

S. BARBERI¹, E. D'AURIA², L. BERNARDO¹, F. FERRARA¹, B. PIETRA², F. PINTO¹,
F. FERRERO³ and G. CIPRANDI³

¹Department of Pediatrics, A.O. Fatebenefratelli, Milan, Italy; ²Department of Pediatrics, A.O. San Paolo, University of Milan, Italy; ³IRCCS-AOU San Martino, Genoa, Italy

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Perennial allergic rhinitis (PAR) is very common in children and has a relevant impact on their families. House dust mites (HDM) are the most relevant cause of PAR. The present pilot study aimed to evaluate whether hypertonic saline (3%) nasal spray as monotherapy is able to improve: nasal symptom severity and parental perception of rhinitis control, sleep, and school performance in HDM-mono-sensitized children with PAR. Globally, 25 children (13 males and 12 females; mean age 9.5±3.1 years) were treated for 3 weeks. They were visited at baseline, at the end of treatment, and after a 2-week follow-up. Hypertonic saline significantly reduced total symptom score, and improved the perception, according to their parents, of rhinitis control, sleep, and school performance. In conclusion, the present pilot study provided the first evidence that 3% hypertonic saline monotherapy was able to relieve nasal symptoms and parental perception of PAR impact as well as being safe and well tolerated.

Allergic rhinitis (AR) is very common as its prevalence is up to 40% of the paediatric population (1). AR in children has impressive social and economic costs and may significantly affect school attendance and performance, as well as the quality of life and sleep of both children and their parents. For these reasons, AR also has a relevant impact on the families of allergic children.

AR immunopathology is characterized by T-helper-2 (Th2)-driven inflammation and diminished Th1-response. This imbalance is sustained by a functional defect of allergen-specific T regulatory cells, promoting Th2 polarization and Th1 impairment. Allergic inflammation is closely dependent on allergen exposure (2). In this regard, house dust mites (HDM) are ubiquitous and perennially present in the home. Thus, children allergic to HDM have peren-

nial allergic rhinitis (PAR). Moreover, there is evidence that children with PAR have more numerous and severe respiratory infections than non-allergic children (3). Different mechanisms are involved: i) reduced defence by interferon- γ defective production; ii) overexpression of ICAM-1 (the main rhinovirus receptor) induced by allergen exposure; and iii) microbial overgrowth promoted by mucosal function impairment (4). AR is also the main risk factor for asthma onset and worsening (5). Therefore, AR starts a vicious circle characterized by mucosal inflammation, nasal symptoms, respiratory infections, and bronchial involvement in predisposed subjects. Consequently, adequate control of allergic inflammation in AR patients is an important strategy as it induces symptom relief and reduced medication use, and may also prevent infection recurrence, asthma

Key words: hypertonic saline, perennial allergic rhinitis, children

Mailing address:
Giorgio Ciprandi, M.D.
Largo R. Benzi 10,
16132 Genoa, Italy
Tel.: +39 10 35338120
e-mail: gio.cip@libero.it

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worsening, and improve school performance (6).

The treatment of PAR is usually based on medication, such as antihistamines and intranasal corticosteroids, which are often effective, but may have adverse events that may limit their use, including sedation, weight gain, epistaxis, and are rather expensive, mainly with regard to the PAR duration. For these reasons, non-pharmacologic treatments, including nasal irrigation, are commonly used (7, 8). Nasal irrigation is well known and popular, and has been used for nose rinsing for centuries (7-10). However, the term 'nasal irrigation' comprises different ways of administration (nasal irrigation, aerosol, spray) and different saline concentrations (isotonic, such as physiological, or hypertonic with saline content ranging from 1 to 6%) (7, 8).

To the best of our knowledge, only four studies have investigated the effectiveness of hypertonic saline in children with AR, but no study directly considered the children with PAR due to HDM mono-sensitization (11-13). On the basis of this background, the present pilot study aimed to evaluate whether hypertonic saline (3%) nasal spray as monotherapy is able to improve nasal symptom severity and parental perception of rhinitis control, sleep, and school performance in HDM-mono-sensitized children with PAR.

MATERIALS AND METHODS

Twenty-five children were enrolled in the study (13 males and 12 females, mean age 9.5 ± 3.1 years; range 5-15) at the Paediatric Department of San Paolo Hospital, Milan (Italy). The inclusion criteria were: i) age range between 4-17 years; ii) PAR diagnosis based upon the concordance between history of typical allergic symptoms (such as nasal itching, sneezing, watery rhinorrhoea, and congestion) and their immediate occurrence after exposure to house dust; iii) documented HDM mono-sensitization; iv) presence of nasal symptoms (with moderate-severe grade of nasal obstruction and/or rhinorrhoea); v) written informed consent by parents. Exclusion criteria were: i) allergy to other allergens; ii) chronic illness; iii) immune deficiency; iv) continuous use of medications (e.g. antihistamines, corticosteroids) in the previous 4 weeks; v) concomitant use of immune-stimulants; and vi) relevant exposure to passive smoking.

Patients were treated with hypertonic saline solution at 3% (Tonimer Hypertonic Spray: a sterile sea water solution, Istituto Ganassini, Milan, Italy). Patients were instructed to assume 2 sprays per nostril 3 times/day for 3 weeks. Children had to remove nasal secretions, blowing their nose before using the hypertonic spray.

Cetirizine syrup (1 drop/3Kg/bw) was permitted as rescue medication during the study period. Symptomatic use of cetirizine was recorded as days. No specific measure of allergen avoidance was suggested for eliminating possible interfering factors.

Nasal symptoms (itching, sneezing, rhinorrhoea, and obstruction) were scored using a four-point scale (0=no symptoms; 1=mild symptoms; 2=moderate; 3=severe) and assessed by a paediatrician (expert in allergy) recording the severity perception of these complaints. The sum of these symptoms was calculated and expressed as Total Symptom Score (TSS). In addition, a visual analogue scale (VAS) was used for assessing the parental perception of: PAR control (VASa), sleep (VASb), and school performance (VASc). The VAS is a segment of 10 cm where the parents indicated their actual perception of considered issues by marking a point (15). In this study, 0 implied the worst perception, while 10 corresponded to optimal one.

The patients were visited at baseline (T1), after the 3-week treatment (T2), and after the 2-week follow-up (T3); all the above-mentioned parameters were evaluated during each visit. As saline solution is not a pharmacological treatment the treatment period was limited to 3 weeks. Any kind of adverse event had to be registered as usual. The study protocol was approved by the Internal Review Board of the San Paolo Hospital, Milan. The study was performed during the winter 2014-15.

Statistical analysis was performed by Friedman ANOVA and by Wilcoxon post hoc assessment. Data are expressed as median and percentiles, such as inter-quartile ranges (IQR): 25th and 75th percentiles. A p-value ≤ 0.05 was considered statistically significant. All data were analyzed using the Stata statistical package, Release 13.1 Statistical Software (StataCorp, College Station, TX, USA).

RESULTS

All children, suffering from HDM-dependent PAR, completed the study and the treatment was well tolerated without significantly adverse

events. Fig. 1 reports the findings.

TSS significantly changed during the study ($p<0.001$): the basal values (M 6, IQR 4.75-8) significantly diminished ($p<0.0001$) after treatment (M 3, IQR 1-5), but significantly increased ($p<0.0001$) at follow-up (M 6, IQR 4-8), however, a significant difference ($p<0.05$) remained between T1 and T3 values.

VASa significantly changed during the study ($p<0.001$): the basal values (M 2, IQR 2-2) significantly increased ($p<0.0001$) after treatment (M 6, IQR 5-8), but significantly decreased ($p<0.001$) at follow-up (M 4, IQR 3-5), however a significant difference ($p=0.0001$) remained between T1 and T3 values.

VASb significantly changed during the study ($p<0.001$): the basal values (M 2, IQR 2-2) significantly increased ($p<0.0001$) after treatment

(M 8, IQR 6.75-9), but significantly diminished ($p<0.0001$) at follow-up (M 4, IQR 2.75-5), however, a significant difference ($p<0.0001$) remained between T1 and T3 values.

VASc significantly changed during the study ($p<0.001$): the basal values (M 2, IQR 2-2) significantly increased ($p=0.0001$) after treatment (M 5, IQR 3-8), but significantly decreased ($p<0.01$) at follow-up (M 3, IQR 2-5), however, a significant difference ($p=0.0005$) remained between T1 and T3 values.

During the treatment period, 10 children used symptomatic cetirizine (mean days: 4.4); during the follow-up, 5 children (4 of the 10 using cetirizine during the treatment period) used symptomatic cetirizine (mean days: 3.5).

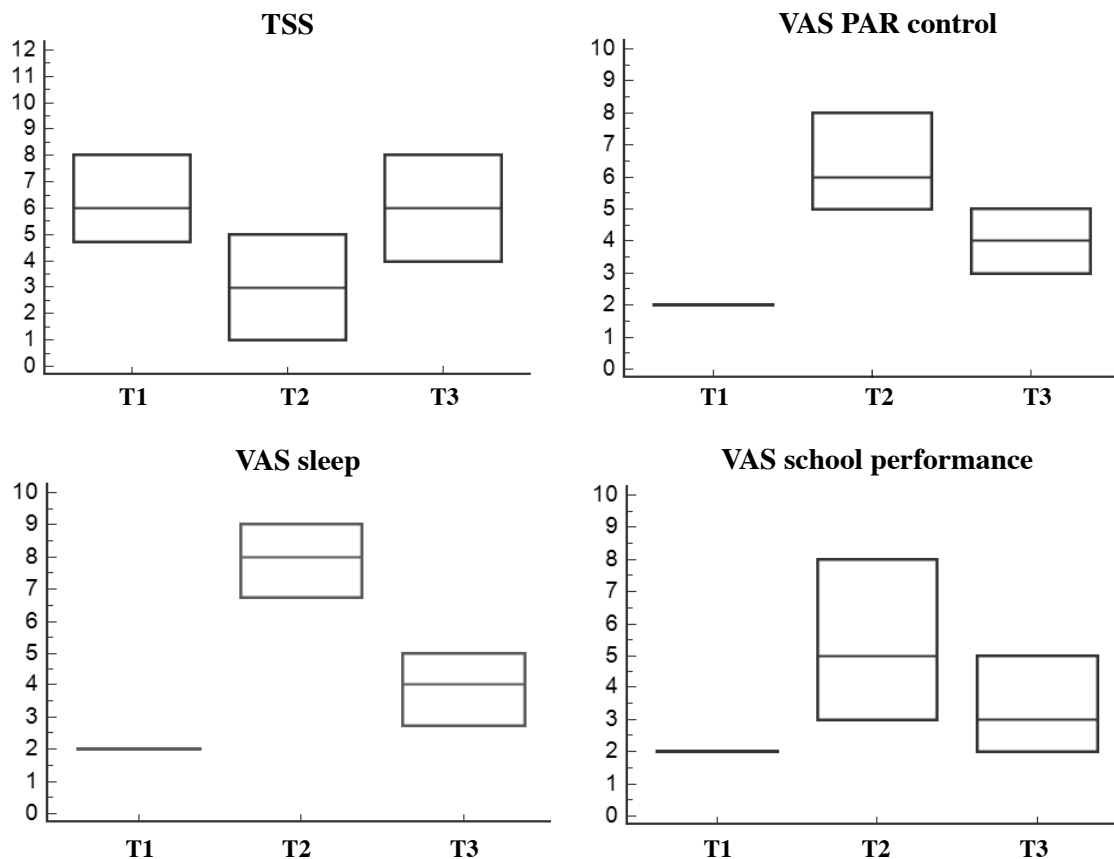


Fig. 1. A= Total Symptom Score (TSS) assessed before (T1) and after treatment (T2) and follow-up (T3). B= VASa assessed before (T1) and after treatment (T2) and follow-up (T3). C= VASb assessed before (T1) and after treatment (T2) and follow-up (T3). D= VASc assessed before (T1) and after treatment (T2) and follow-up (T3).

DISCUSSION

The present pilot study demonstrates that intranasal administration of hypertonic saline at 3% is able to significantly reduce nasal symptoms in children with perennial allergic rhinitis due to HDM mono-sensitization. In addition, this treatment was also able to significantly affect the parental perception of PAR control, sleep, and school performance. These findings support the concept that nasal lavage with hypertonic solution may control allergic reaction, also improving the familiar AR burden. It is to underline that the current treatment was administered as monotherapy: this aspect reinforces the effectiveness of hypertonic saline in PAR. In addition, hypertonic saline was able to limit the use of symptomatic medication: 40% of children used cetirizine for a few days.

Interestingly, the effectiveness persisted after suspension: in fact, there was a significant difference concerning nasal symptom severity, parental perception of efficacy, and symptomatic drug consumption between baseline and the follow-up period. Thus, the positive findings tend to persist over time, i.e. a long lasting effect.

The present study is also consistent with previous studies carried out on children with seasonal allergic rhinitis (11, 12, 14) and another study without clarification about the sensitization pattern (13). In particular, Garavello and colleagues firstly evaluated a group of 20 children with seasonal AR to *Parietaria*: 10 children were randomized to be treated by 3 nasal irrigations with hypertonic saline daily per 6 weeks, the other 10 were considered as the control group without nasal irrigation (11). Nasal symptoms and symptomatic antihistaminic use were scored. Hypertonic saline therapy significantly reduced symptom severity in the 3th, 4th, and 5th weeks as well as antihistaminic use. The same authors, in a second study, enrolled 44 children with seasonal AR to grass pollen - 22 randomly received 3 nasal rinses with hypertonic saline daily for 7 weeks, 22 used as the control group did not receive nasal irrigation (12). The same methodology of assessment was used also in that study. Actively-treated children had a significant reduction of symptoms in weeks 6 and 7

of therapy and of anti-H1 use in 5 out of the 7 weeks. Marchisio and co-workers evaluated 220 children with grass-pollen-dependent seasonal AR: randomly, 80 received isotonic saline, 80 hypertonic saline, and 60 had no treatment for 4 weeks (14). Clinical and endoscopic parameters were significantly reduced by hypertonic saline treatment, whereas normal saline significantly reduced only rhinorrhoea and sneezing. Symptomatic use of antihistamines was less in the hypertonic group than in the other groups. Satdhabudha and colleagues, in a randomized, double-blind placebo-controlled study, compared normal saline with buffered hypertonic saline nasal irrigation in 81 children suffering from symptomatic AR (13). The treatment period lasted for 4 weeks. Nasal saccharine clearance time, symptom score, quality of life, antihistaminic use, and satisfaction were assessed before and after treatments. Buffered hypertonic saline induced better control of nasal congestion, quality of life and reduced the use of antihistamines. The possible explanation of the efficacy of hypertonic saline might depend on some mechanisms, such as removal of mucous containing pro-inflammatory mediators and allergens, reduction of oedema due to osmotic effect, and potential anti-inflammatory activity (9, 10).

The main shortcomings of the present study are: i) the lack of a control group, and ii) the small sample size. However, it has to be considered that isotonic saline is *per se* able to significantly improve nasal symptoms as clearly demonstrated in previous studies (11-14). As the present study was designed as pilot, we believed that it could be correct from a methodological point of view to consider only one active group, as we added a follow-up period. In fact, the follow-up period allowed us to evaluate the long-lasting effects exerted by hypertonic saline. We believe that follow-up might be considered a *per se* "control" of the study. Moreover, the small size of the sample is another relevant limitation, thus much larger studies, including a control group, should be conducted to confirm this preliminary finding. Another point has to be considered: the age range was rather large (i.e. 5-15 years): this issue may affect the reliability of the outcomes. Further studies should enrol children in a smaller age range.

In conclusion, the present pilot study provided the first evidence that hypertonic saline monotherapy at 3% was able to relieve nasal symptoms and parental perception of PAR impact; the treatment was also safe and well tolerated. Thus, this outcome could suggest that one course of this compound could be effective, thus reducing the use of pharmacological therapy.

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LETTER TO THE EDITOR

INTRANASAL T-LysYal® AS ADJUNCTIVE THERAPY FOR PATIENTS AFTER FUNCTIONAL ENDOSCOPIC SINUS SURGERY

M. GELARDI¹, S. TALIENTE¹, M.L. FIORELLA¹, N. QUARANTA¹, N. DE CANDIA¹,
C. RUSSO², P. MOLA³, A. CIOFALO⁴, G. ZAMBETTI⁴, E. CANTONE⁵, F. ARNONE⁷,
A. MACCHI⁸, P. ROSSO⁹ and G. CIPRANDI¹⁰

¹Otolaryngology, Department of Basic Medical Science, Neuroscience and Sensory Organs, University of Bari Aldo Moro, Bari, Italy; ²U.O.C. di Otorinolaringoiatria, Ospedale Di Venere, Carbonara Di Bari (BA), Italy; ³Division of Otolaryngology, Pinerolo and Rivoli Hospitals, ASL TO3, Pinerolo (TO), Italy; ⁴Rhinology and Immuno-Allergy Unit, Sense Organs Department, Otolaryngology Section, Umberto I General Hospital, La Sapienza University, Rome, Italy; ⁵Department of Neuroscience, Reproductive and Dentistry Science, ENT Unit, Federico II University, Naples, Italy; ⁶ASL TO 4 – SC ORL – Ospedale Civico, Chivasso (TO), Italy; ⁷Clinica Otorinolaringoiatrica, Ospedale San Paolo, Milan, Italy; ⁸ORL Clinic University of Insubriae, Varese, Italy; ⁹S.C. Otorinolaringoiatrica, Ospedale Civico di Chivasso (To), Italy; ¹⁰Department of Medicine, Istituto di Ricovero e Cura a Carattere Scientifico (I.R.C.C.S.) - Azienda Ospedaliera Universitaria San Martino, Genoa, Italy

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Functional Endoscopic Sinus Surgery (FESS) is a common day surgery technique for upper airway disorders. Hyaluronic acid (HA) is a fundamental component of the human connective tissue. HA may exert reparative, anti-inflammatory and immune-modulating activities. Recently, a new intranasal HA formulation has been proposed: a supramolecular system containing lysine hyaluronate, thymine and sodium chloride (T-LysYal®). This randomized study investigated whether intranasal T-LysYal® (RinoLysYal®, Farmigee, Italy) was able to reduce symptom severity, endoscopic features, and nasal cytology in 83 patients (49 males and 34 females mean age 45.4±6.2 years) treated with FESS. All patients were treated with isotonic saline solution for 4 weeks, and a sub-group (active group) was also treated with intranasal T-LysYal®. Patients were visited at baseline, after treatment, and after 4-week follow-up. Intranasal T-LysYal® treatment significantly reduced the quote of patients with symptoms, endoscopic features, and inflammatory cells in comparison to isotonic solution. In conclusion, the present study demonstrates that intranasal T-LysYal® is able to significantly improve patients after FESS and its effect is long lasting.

Upper airway surgery is at present based preferentially on slightly invasive methods, such as the use of endoscopic techniques or non-mechanical procedures. In this regard, many upper airway disorders are treated in day surgery, including nasal polyps, rhinosinusitis, septal deviation, and turbinate hypertrophy (1-5). Day surgery is indicated when marked

mechanical obstruction of upper airways or chronic disorders do not respond to intensive medical therapy. Functional endoscopic sinus surgery (FESS) has become a popular treatment for nasal polyps and chronic rhinosinusitis as it is able to restore the physiological function of the nasal structure and to allow a normal drainage from sinuses. Some studies have

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Mailing address:
Giorgio Ciprandi, M.D.
Largo R. Benzi 10,
16132 Genoa, Italy
Tel.: +39 10 35338120
e-mail gio.cip@libero.it

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evaluated the use of topical drugs used for improving symptoms, preventing complications, and aiding *sequelae* after FESS (4-6). In this regard, hyaluronate (a salt of hyaluronic acid) has been prescribed for this issue (7). HA is a fundamental component of the human connective tissue. It is chemically definable as a glycosaminoglycan. At physiological pH, the carboxyl groups of the glycuronic units are ionized, so HA acquires high polarity and consequently high water solubility; thanks to this property, it is able to complex with many water molecules, reaching a high level of hydration. Recently a new HA formulation, T-LysYal[®], was developed. T-LysYal[®] is a supramolecular system containing lysine hyaluronate, thimine, and sodium chloride. By its intermolecular powers, the aggregate has its own biological properties, which are different from those of its components and from sodium hyaluronate itself (8-11). There is a body evidence that renders T-LysYal[®] interesting as a promising treatment for nasal disorders, as it is able to modulate inflammatory response, cellular proliferation, and remodeling of extracellular matrix. In particular, it has been reported that T-LysYal[®] increases fibroblast proliferation and collagen synthesis (promoting tissue repair) and inhibits, in a dose-dependent manner, basophil degranulation induced by antibody anti-IgE receptors (Gruppo Biolab, data on file). In addition, T-LysYal[®] is more resistant to the lytic enzyme hyaluronidase (from *Streptomyces hyaluronitcus*) than HA, a fact which accounts for a superior stability of the product that may exert a repairing activity for a longer period (Gruppo Biolab, data on file). Moreover, T-LysYal[®] inhibits the ICAM-1 expression on HUVEC induced by TNF- α in a dose-dependent manner, which could be clinically relevant as reduced expression of ICAM-1 affects the adhesion of inflammatory cells on endothelial cells, a step of the infiltration process (12). Previously, it was reported that lysine hyaluronate was better than HA in the healing process of decubitus ulcers in hospitalized patients (13).

More recently, the nasal formulation of T-LysYal[®], rinoLysYal[®], has been proposed. On the basis of this background, it was hypothesized that intranasally administered rinoLysYal[®] would be able to favor the process behind the regeneration of the nasal mucosa

and to create a lubricating, emollient, and protective action, which could aid the repairing process of the nasal mucosa, injured or dry. Therefore, the hypothesis to be tested is the effectiveness of intranasal T-LysYal[®] administered to patients after surgery. The present randomized study aimed to investigate whether rinoLysYal[®], administered as adjunctive therapy, could improve symptoms, endoscopic features, and cytological parameters in patients after FESS for nasal polyps, chronic rhinosinusitis, septoplastic, and turbinoplastic.

MATERIALS AND METHODS

Eighty-three patients (49 males and 34 females, mean age 45.4 ± 6.2 years) were enrolled in this study. The inclusion criteria were: i) either gender and adult age, ii) previous FESS for nasal polyps, chronic rhinosinusitis, septoplastic, or turbinoplastic, iii) written informed consent. Exclusion criteria were: i) concomitant comorbidities, including acute respiratory infections, ii) chronic illness, iii) hypersensitivity to T-LysYal[®].

All patients were treated with nasal lavage with isotonic saline solution (1 puff per nostril twice a day). The treatment lasted for 4 weeks. At baseline, the patients were randomly (1:1 ratio) subdivided into two groups: 49 patients were adjunctively treated with intranasal T-LysYal[®] (Group A) and 34 were considered as a control (Group B). Active medication was intranasal cream T-LysYal[®] (rinoLysYal[®]) 1 cm per nostril in the afternoon. Patients in the control group used only the standard therapy.

Patients were evaluated at baseline (T0), after 4 weeks (T1), and after a 4-week follow-up (T2). Clinical parameters were assessed at T1 and T2, whereas endoscopy and nasal cytology were performed at all times. Adverse events were registered as usual.

Clinical parameters

The following parameters were evaluated: nasal obstruction, epistaxis, rhinorrhea, itching, sneezing, crust, post nasal drip, headache, burning, dryness, and smell impairment. They were assessed if present at visit T1 and T2. The presence of symptoms was not considered at T0, as their perception is not reliable immediately after FESS.

Endoscopy

Nasal endoscopy was carried out by a 3.4 mm diameter

flexible fibroscope (Vision-Sciences® ENT-2000), to assess the presence of major abnormalities, such as septal deviation, polyposis, turbinate hypertrophy, or exudation from the ostiomeatal complex. Presence of turbinate hypertrophy and edema, and the mucosal characteristics were assessed.

Nasal cytology

This procedure was performed by scraping the middle part of the inferior turbinate with a Rhino-Probe® device (Arlington Scientific). The sample was smeared onto a slide, air-dried, then stained with the May-Grünwald Giemsa preparation. The type and cell number were examined using microscopy (Nikon® E600). Cell types were identified, and intracellular components were studied at 1000x in oil immersion. The mean number per 50 fields was calculated and reported (14, 15).

Statistical analysis

Statistical analysis of the results was performed using χ^2 and Wilcoxon tests for intergroup and intragroup analysis at the different times. Clinical parameters and endoscopic features were expressed as percentage of patients with the specific symptom. For nasal cytology, an absolute value for quantifying the results was created. In this way, the data obtained for cellularity (e.g. rare cells, some cells, numerous cells, very numerous cells) was expressed by one value and thus compared between the times and the groups. Statistical software (Statistica 8.0) was used for analysis.

RESULTS

All patients completed the study; the treatments were tolerated and no clinically relevant adverse event was reported.

Clinical parameters

Nasal obstruction: the percentage of patients with obstruction significantly decreased in Group A (T1= 32.65%; T2= 2.04%) as well as in Group B (T1= 55.88%; T2=5.88%), as shown in Fig. 1. Both groups showed a significant reduction at T2 (p=0.0000 for both). The intergroup comparison showed that there were significant differences between groups at T1 (p= 0.0009).

Rhinorrhea: the percentage of patients with

rhinorrhea significantly diminished in Group A (T1= 69.4%; T2= 22.4%) as well as in Group B (T1= 79.4%; T2= 55.9%) (p=0.0000 and p=0.0004 respectively), as reported in Fig. 1. The intergroup comparison showed that there was a significant (p=0.0000) difference at T2.

Itching: the percentage of patients with itching did not change in Group A (T1= 10.2%; T2= 10.2%), whereas it significantly decreased in Group B (T1=20.6 %; T2= 2.9%; p=0.0001), as shown in Fig. 1. The intergroup comparison showed that there was a significant difference between groups both at T1 and T2 (p=0.042 and p=0.038, respectively).

Sneezing: the percentage of patients with sneezing significantly diminished in Group A (T1= 30.6%; T2= 16.3%; p=0.017) as well as in Group B (T1= 17.6%; T2= 5.9%; p=0.001). The intergroup comparison showed that there were significant differences between groups at T1 and T2 (p=0.03 and p=0.019, respectively).

Crusts: the percentage of patients with crusts significantly (p=0.0138) diminished in Group A (T1= 40.8%; T2= 24.5%) as well as in Group B (T1= 58.8%; T2= 44.1%; p=0.0035). The intergroup comparison showed that there were significant differences at T1 and T2 (p=0.01 and p=0.003, respectively).

Post nasal drip: the percentage of patients with post nasal drip did not change in Group A (T1= 10.2%; T2= 10.2%), whereas it significantly (p=0.000) decreased in Group B (T1= 38.6%; T2= 5.9%). The intergroup comparison showed that there was a significant (p=0.0000) difference between groups at T1.

Burning: the percentage of patients with nasal burning significantly diminished in Group A (T1= 18.4%; T2= 6.1%; p=0.0082) as well as in Group B (T1= 29.4%; T2= 14.7%; p=0.0121). The intergroup comparison showed that there was a significant difference between groups at T2 (p=0.047).

Endoscopic features

Hypertrophic turbinates: the percentage of patients with hypertrophic turbinates significantly decreased in Group A (T0= 51%; T1= 20.4%; T2= 2%; p<0.05 between T0 and T2) as well as

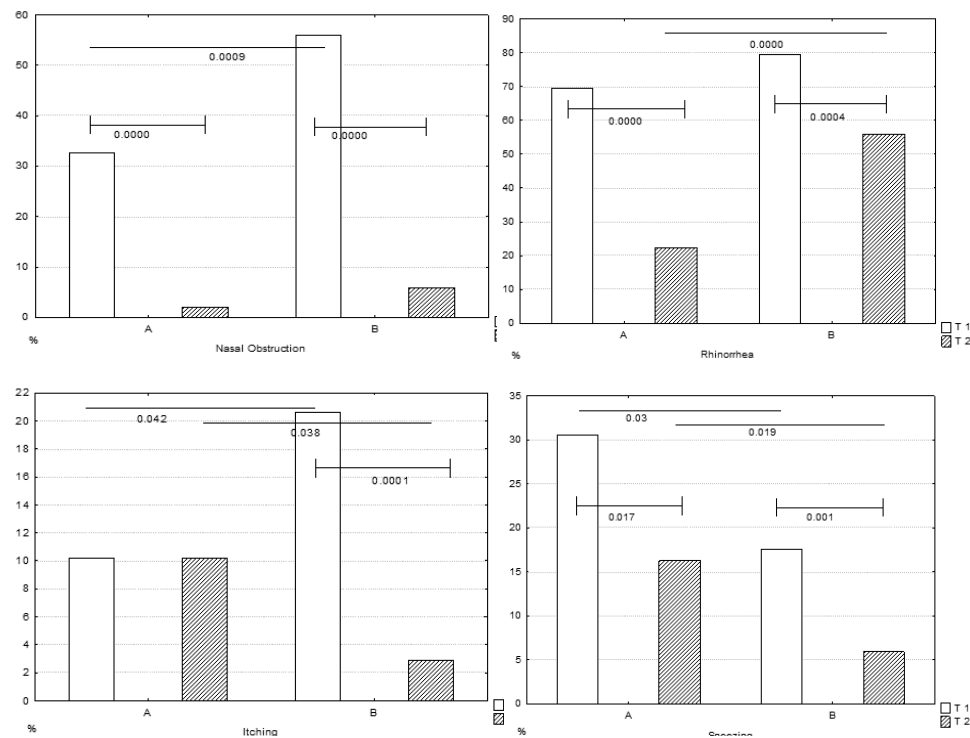


Fig. 1. Upper left quadrant= percentages of patients with obstruction in groups A and B at T1 and T2. Upper right quadrant= percentages of patients with rhinorrhea in groups A and B at T1 and T2. Lower left quadrant= percentages of patients with itching in groups A and B at T1 and T2. Lower right quadrant= percentages of patients with sneezing in groups A and B at T1 and T2.

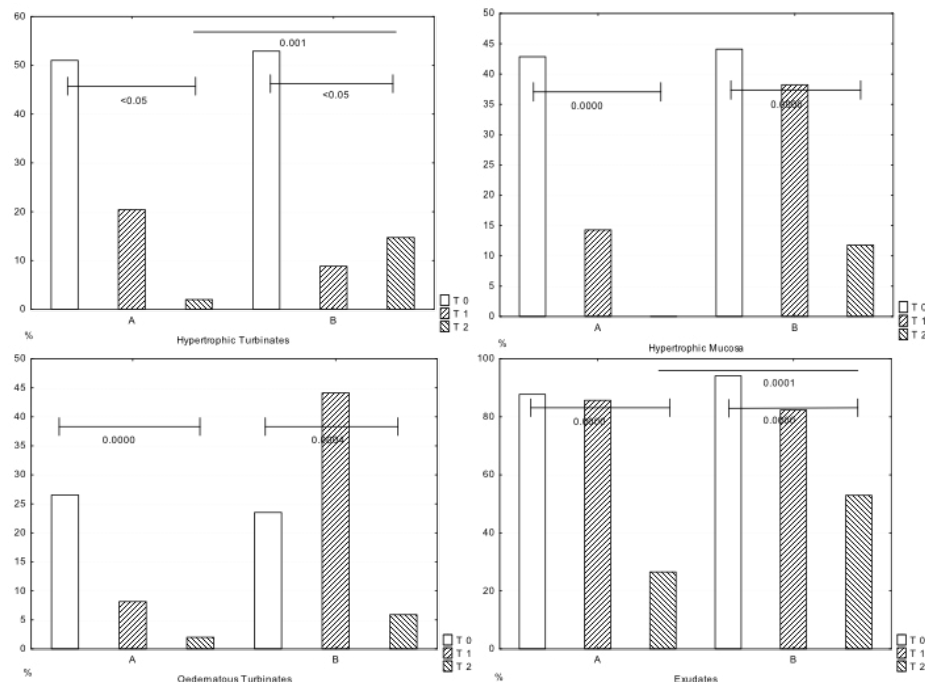


Fig. 2. Upper left quadrant= percentages of patients with hypertrophic turbinates in groups A and B at T0, T1, and T2. Upper right quadrant= percentages of patients with oedematus turbinates in groups A and B at T0, T1, and T2. Lower left quadrant= percentages of patients with hypertrophic mucosa in groups A and B at T0, T1, and T2. Lower right quadrant= percentages of patients with exudates in groups A and B at T0, T1, and T2.

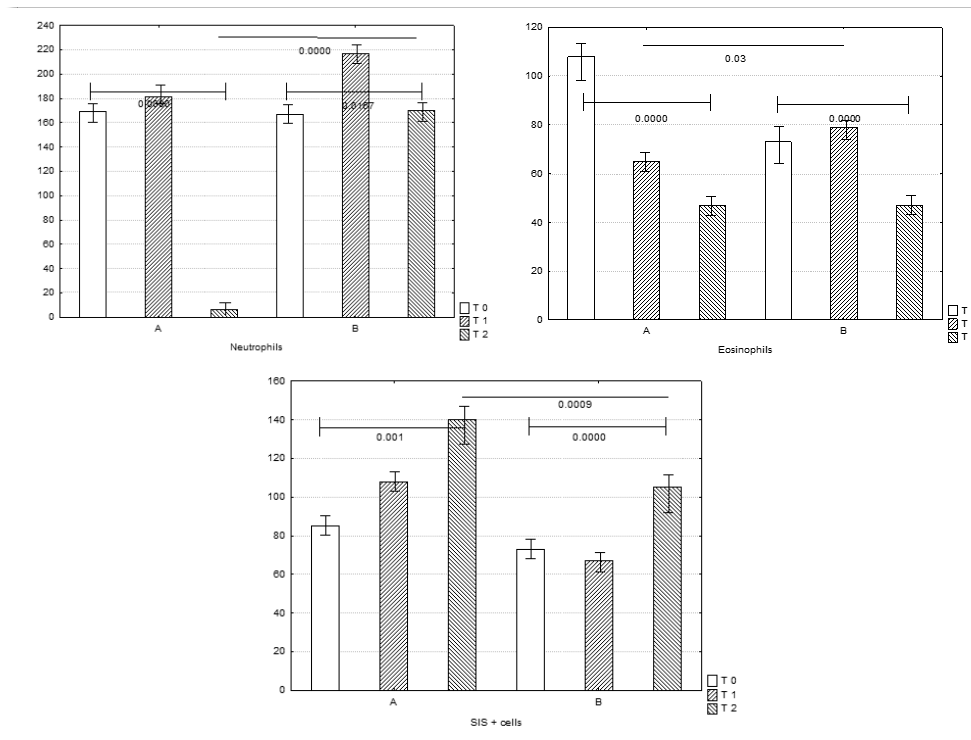


Fig. 3. Upper left quadrant= mean number of neutrophils in groups A and B at T0, T1, and T2. Upper right quadrant= mean number of eosinophils in groups A and B at T0, T1, and T2. Lower quadrant= mean number of SIS positive cells in groups A and B at T0, T1, and T2. Data are expressed as mean $\pm$ S.D.

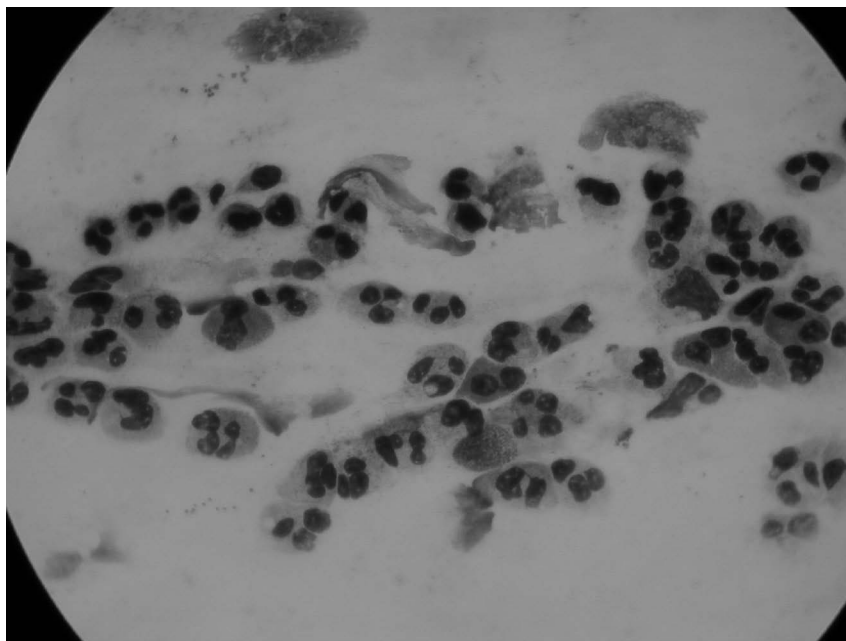


Fig. 4. Cytological picture before treatment: neutrophils and eosinophils plenty infiltrate the microscopic field.

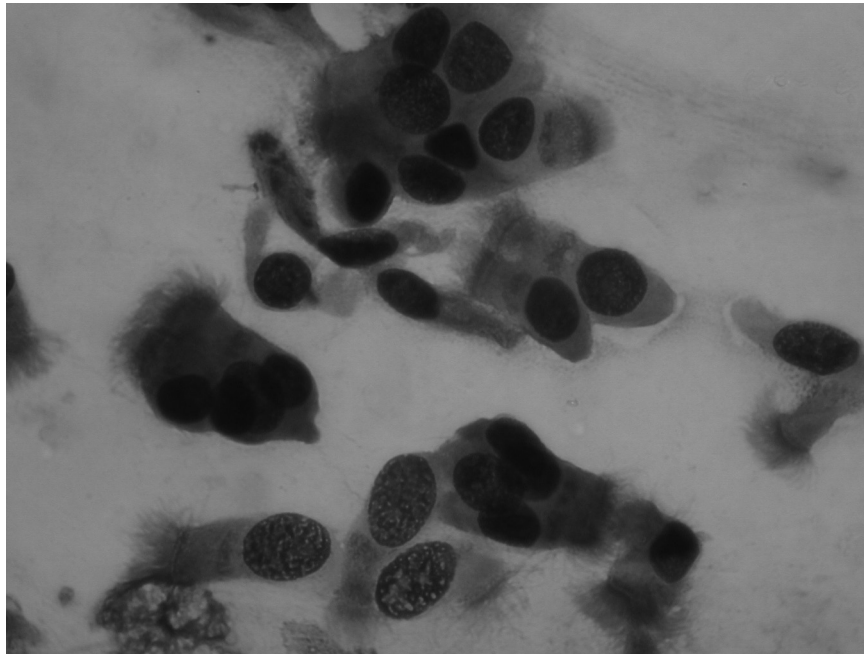


Fig. 5. Cytological pattern after treatment: inflammatory cells are absent. SIS+ cells are clearly visible (a frayed hem is present on the bottom of the syncytium).

in Group B (T0= 52.9%; T1= 8.82%; T2= 14.7%; $p < 0.05$ between T0 and T2), as shown in Fig. 2. The intergroup comparison showed that there was a significant difference between groups at T2 ($p = 0.001$).

Oedematous mucosa: the percentage of patients with edematous mucosa significantly decreased in Group A (T0= 42.85 %; T1= 14.28%; T2= 0%; $p = 0.0000$ between T0 and T2) as well as in Group B (T0= 44.11%; T1= 38.23%; T2= 11.8%; $p = 0.0000$ between T0 and T2), as reported in Fig. 2. The intergroup comparison showed that there was a significant difference between groups at T2 ($p = 0.007$).

Oedematous turbinates: the percentage of patients with edematous turbinates significantly diminished in Group A (T0= 26.53%; T1= 8.16%; T2= 2%; $p = 0.0000$ between T0 and T2) as well as in Group B but only after the follow-up (T0= 23.52%; T1= 44.12%; T2= 5.9%; $p = 0.0004$ between T0 and T2). The intergroup analysis did not show any difference.

Presence of exudates: the percentage of patients with exudates significantly diminished in Group A (T0= 87.75 %; T1= 85.71%; T2= 26.53%; $p = 0.0000$

between T0 and T2) as well as in Group B (T0=94.12%; T1= 82.35%; T2= 52.94%; $p = 0.0000$ between T0 and T2), as shown in Fig. 2. There was a significant difference between groups at T2 ($p = 0.0001$).

Disepithelized mucosa: The number of patients with denuded mucosa significantly ($p = 0.002$) diminished only in Group A at T2 (Group A: T0= 24.48%; T1= 28.57%; T2= 8.16%. Group B: T0= 20.59%; T1= 44.11%; T2= 32.35%). There was a significant difference between groups at T2 ($p < 0.0000$).

Cytology

Neutrophil: the number of neutrophils significantly ($p = 0.0000$) diminished only in Group A at T2 (Group A: T0= 169; T1=181; T2=6. Group B: T0=167; T1=217; T2=170), as shown in Fig. 3. There was a significant intergroup difference at T2 ($p = 0.0000$). Fig. 4 shows the cytological pattern before the treatment, Fig. 5 after treatment.

Eosinophil: the number of eosinophils significantly ($p = 0.0000$) diminished only in Group A at T2 (Group A: T0= 108; T1=65; T2= 47. Group

B: T0=73; T1=79; T2= 47), as shown in Fig. 3. The intergroup analysis showed a significant difference ($p=0.03$) at T1 only.

SIS+ cells: the number of SIS positive cells significantly ($p=0.001$ and $p=0.0000$, respectively) increased in both groups at T2 (Group A: T0= 85; T1=108; T2=140. Group B: T0=73; T1=67; T2=105), as reported in Fig. 3. There was a significant intergroup difference at T2 ($p=0.0009$).

Mast cell: the number significantly ($p=0.0007$ and $p=0.002$, respectively) diminished in both groups at T2 (Group A: T0= 32; T1=26; T2= 10. Group B: T0= 41; T1=32; T2= 23). There was a significant intergroup difference at T2 ($p=0.02$).

Bacteria: the number significantly diminished in both groups ($p=0.0000$) at T2 (Group A: T0= 49; T1=24; T2= 10. Group B: T0= 29; T1=32; T2= 6). There was no significant intergroup difference at T2 ($p=n.s.$).

DISCUSSION

This randomized study provided the first evidence that intranasally administered T-LysYal[®] was able to significantly affect nasal symptoms, endoscopic features, and cytological data in patients after FESS for upper airway disorders, including nasal polyps.

These findings may be explained by the complex anti-inflammatory activity exerted by T-LysYal[®]. In fact, hyaluronan is a non-sulfated glycosaminoglycan found in the extra-cellular matrix of all tissues which plays multifunctional role in the reparative process and is essential in the homeostasis of respiratory airways (16-19). The other components of the supramolecular system, such as lysine, and its particular stereochemistry may exert synergic effects, enhancing anti-inflammatory and reparative activities.

In particular, the number of patients with symptoms and endoscopic features, typical for upper airway disorders, significantly decreased after intranasal T-LysYal[®] treatment. These positive effects are consistent with the parallel effect of nasal inflammation pattern. Indeed, intranasal T-LysYal[®] therapy is able to significantly reduce the grade of eosinophilic and neutrophilic infiltrate as well as increasing the quote of well-being epithelial cells

(SIS+ cells). This local anti-inflammatory activity may explain the relevant effect on symptoms (mainly obstruction) and signs (mainly oedema) of nasal inflammation. These outcomes are consistent with previous studies that demonstrated the positive effects of hyaluronate in the treatment of patients after nasal surgery (20-22).

Noteworthy, the effectiveness of T-LysYal[®] lasts also during the follow-up period. This fact is particularly relevant as it demonstrates the profound impact exerted on mucosal repair.

However, the present study has some limitations: firstly, it was an open study, had a restricted number of patients, and there is a lack of the demonstration of effects on pathophysiologic mechanisms. In this regard, there is an ongoing study exploring the effects of nasal T-LysYal[®] on mucociliary clearance that may provide new information on the mechanism of action. On the other hand, this study is randomized and has a follow-up period of observation. However, further studies should be carried out to confirm this initial evidence. In addition, it would be interesting to see whether these long-lasting effects are exclusive to this new intranasal supramolecular system. A direct head-to-head comparison between this new formulation and the most commonly used sodium hyaluronate on patients who have undergone FESS will help to determine whether this system is superior to traditional treatments. Additionally, a head-to-head comparison with lysine hyaluronate in patients who have undergone FESS will help determine whether thymine and sodium chloride are synergistically contributing to the observed results.

In conclusion, the present study demonstrates that intranasal T-LysYal[®] is capable of significantly improving nasal symptoms, endoscopic features, and cytological data in patients undergoing day surgery (FESS) for upper airway disorders, and its effect is long lasting.

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LETTER TO THE EDITOR

NASAL NITRIC OXIDE IN CHILDREN WITH RECURRENT ACUTE OTITIS MEDIA

S. TORRETTA¹, P. MARCHISIO², P. CAPACCIO³ and L. PIGNATARO¹

¹Department of Clinical Sciences and Community Health, Fondazione IRCCS, Ca' Granda Ospedale Maggiore Policlinico, Università degli Studi di Milano, Milan, Italy; ²Department of Physiopathology and Transplantations, Fondazione IRCCS, Ca' Granda Ospedale Maggiore Policlinico, Università degli Studi di Milano, Milan, Italy; ³Department of Biomedical Surgical and Dental Sciences, Fondazione IRCCS, Ca' Granda Ospedale Maggiore Policlinico, Università degli Studi di Milano, Milan, Italy

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Recently, reduced Nasal nitric oxide (nNO) nNO levels have been reported in children with adenoidal hypertrophy predisposing to chronic nasosinus inflammation. Given the strict anatomic and physiopathologic link between the nasopharyngeal and middle ear compartments, and considering the high prevalence of otitis prone children among those affected with chronic adenoiditis, we designed a study aimed to test any possible difference in nNO levels between non-allergic children with and without recurrent acute otitis media (RAOM) associated with chronic adenoiditis. The study involved 54 children with RAOM (44.4% males; mean age= 7.5±3.5 years) and 51 children without RAOM (47.4% males; mean age= 7.0±3.8 years). nNO levels were significantly reduced in children with RAOM compared to children without RAOM (676.9±250.7 ppb vs 831.8±320.4 ppb, respectively; $p=0.02$). Our results could be related to reduced NO production by the ciliated paranasal, nasopharyngeal and middle ear epithelium and the impaired sinusal ostial and Eustachian tube patency due to chronic inflammation, and seem to confirm the involvement of NO pathway in recurrent upper airway infections related to impaired ciliated respiratory mucosa.

Nitric oxide (NO) is a highly reactive mediator involved in regulating blood flow and platelet activity in immunity and in neurotransmission (1). NO is synthesised from L-arginine by the constitutional calcium-dependent endothelial and neuronal NO synthase (eNOS, nNOS), and its inducible calcium-independent isoform (iNOS) (2, 3). As the iNOS expressed by upper respiratory epithelial cells is enhanced by any inflammatory stimulus, NO production is increased during airway inflammation (4). NO therefore acts in the airways as a vasoactive mediator that regulates inflammation, ciliary beat frequency and the conservation of sterility, and plays

a role as an aerocrine mediator in order to maintain adequate ventilation/perfusion matching in the lungs (2, 3).

The levels of nasal NO (nNO), which is the amount of NO produced and spontaneously released by the mucosa of the paranasal sinuses, can be measured by aspirating the air in the nasal cavities (5, 6). nNO is involved in nasal, nasopharyngeal and nasosinus inflammation, as it is impaired in allergic rhinitis and rhinosinus disease (7, 8). In fact, reduced nNO levels have been reported in patients with acute chronic rhinosinusitis (7, 8), probably due to recurrent sinusal infections and to the NO-

Key words: nitric oxide, children, otitis media, adenoid

Mailing address:

Sara Torretta, MD
Department of Clinical Sciences and Community Health,
University of Milan, Fondazione I.R.C.C.S.
Ca' Granda Ospedale Maggiore Policlinico,
Via F. Sforza 35, 20122 Milano, Italy
Tel.: +39 0250320245 Fax: +39 0250320248
e-mail: sara.torretta@gmail.com

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dependent inhibition of inflammatory cell apoptosis that increase the life span of metachromatic elements in the nasal mucosa (9). Recently reduced nNO levels have been reported also in children with adenoidal hypertrophy predisposing to chronic nasosinus inflammation (10). In addition to this, the role of NO in middle ear inflammation has been postulated (11-15), but it is not fully understood. In particular, some evidence suggests the involvement of NO pathway in the development of otitis media with effusion (11-15). Given the strict anatomic and physio-pathologic link between the nasopharyngeal and middle ear compartments, and considering the high prevalence of otitis prone children among those affected with chronic adenoiditis, we designed a prospective cross-sectional study with planned data collection aimed to test any possible difference in nNO levels between non-allergic children with and without recurrent acute otitis media (RAOM) associated with chronic adenoiditis.

MATERIALS AND METHODS

The protocol was approved by our local Ethics Committee and was conducted in accordance with the standards of Good Clinical Practice. Informed consent was obtained from the children's parents or legal guardians.

The study involved non-allergic 3-12-year-old children with chronic adenoiditis defined as adenoidal hypertrophy (i.e. adenoidal hypertrophy $\geq$ grade 3 according to Cassano's classification) (16) assessed by means of flexible nasopharyngeal fiberendoscopy with ongoing nasopharyngeal inflammation/infections (17) (i.e.: at least three episodes of acute adenoiditis requiring antibiotic therapy in the previous six months, or at least four episodes in the previous 12 months) and persistent mucous secretion on the adenoidal surface. The exclusion criteria were patient or their parents' refusal to participate or inability to cooperate; children with fiberendoscopic diagnosis of rhinosinusitis or history of recurrent acute tonsillitis; allergies or asthma documented by positive skin prick test or radioallergosorbent test and serum IgE levels within the previous 6 months; acute respiratory infections during the previous 15 days; the administration of systemic antibiotics and/

or steroids or other medications affecting the airways (Montelukast, inhaled steroids or bronchodilators, nasal steroids or decongestants) during the previous 15 days; chronic systemic or airway diseases (cystic fibrosis, primary ciliary dyskinesia, primary or secondary immunodeficiency).

The demographic and clinical features of each patient were recorded, including history of recurrent acute otitis media (RAOM, defined as at least three documented episode of acute otitis media in the previous six months, or at least four episodes in the previous year) (18), duration of the disease and BMI-weight status (based on percentile range and defined as: underweight if BMI-weight status $\leq$ 4th percentile; normal weight if $5 <$ BMI-weight status $<$ 85th percentile; and overweight status if BMI-weight status $\geq$ 85th percentile). All of the patients underwent a complete ENT examination, including flexible nasal fiberendoscopy using a Pentax endoscope (diameter 2.7 mm) aimed at evaluating the presence of nasopharyngeal purulent discharge and the grade of adenoidal hypertrophy according to Cassano's classification (16). All the patients underwent overnight polysomnography in order to identify those with obstructive sleep apnoea syndrome. In accordance with the international guidelines (19, 20), nNO were measured in parts per billion (ppb) by means of a dedicated chemiluminescence analyser (CDL 88 sp, Ecomedics, Switzerland) using a single-breath on-line method at constant flow. At the parent's discretion, the patient was comfortably seated on a chair or his/her parent's knee, and a nasal olive with a central sampling tube connected to CDL 88 was gently placed against the child's nostril. The child was encouraged to breathe quietly for a few minutes in order to acclimatise before orally inhaling NO-free air (<5 ppb) to total lung capacity over 2-3 seconds from a disposable mouthpiece connected to a dedicated chemiluminescence analyser (CLD 88 sp, Ecomedics, Dürnten, Switzerland) by means of a two-way valve. Then, without any breath holding, the breath was orally exhaled for more than four seconds at a constant flow rate of 50 mL/sec through an expiratory resistance, while targeting a mouth pressure of 10 cm H₂O in order to close the velum and avoid nasal contamination. The test ended with another oral inhalation. The patients were helped to maintain a constant expiratory flow rate by means of a real-time visual aid. nNO were measured

when an nNO plateau of more than two seconds could be identified during exhalation. The mean nNO level in each patient was determined after three exhalations repeated at intervals of >30 seconds. Children with inter-individual variations > 15% among the three tests were excluded. The analyser was calibrated every two weeks using certified 2000 ppb calibration gas. The tests were performed in the morning, and the children avoided food and drink consumption for at least one hour before the test. In the case of acute respiratory infection, or the administration of systemic antibiotics and/or steroids or other medications affecting the airways during the previous 15 days, the tests were postponed until at least 15 days after the discontinuation of the medication and complete clinical recovery.

Descriptive statistics were used to describe the basic characteristics of the patients. The ordinal variables were expressed as mean values and their standard deviation. The possible difference in nNO levels between children with and without history of RAOM was tested using the Wilcoxon-Mann-Whitney test. Statistical significance was set at a p-value of <0.05. The data were analysed

using STATA 10.0 software (StataCorp, College Station, Texas, USA).

RESULTS

The final analysis was performed on the ground of 105 out of 157 children enrolled. Fifty-two children were excluded due to their inability to cooperate or large inter-individual variation during examination. The study therefore included 54 children with RAOM (44.4% males; mean age = 7.5 ± 3.5 years) and 51 children without RAOM (47.4% males; mean age = 7.0 ± 3.8 years). Children with RAOM were comparable to non-RAOM children for all the variables assessed. Demographic and clinical characteristics are reported in Table I. Overnight polysomnography documented simple snoring in all the patients. Mean nNO levels were significantly reduced in children with RAOM compared to children without RAOM (676.9 ± 250.7 ppb vs 831.8 ± 320.4 ppb respectively; $p = 0.02$) (Fig. I).

Table I. Demographic and clinical data.

Variable	RAOM	Non-RAOM	p-value	Total
No. of males (%)	24 (44.4)	24 (47.4)	ns	48 (45.7)
Mean age $\pm$ sd (years)	7.5 ± 3.5	7.0 ± 3.8	n.s.	7.3 ± 3.7
No. of children aged < 5 years (%)	11 (20.4)	10 (19.6)	n.s.	21 (20.0)
No. of underweight weight patients (%)	9 (16.7)	10 (19.6)	n.s.	18.1
BMI-weight status				
No. of normal weight patients (%)	40 (74.1)	38 (74.5)		74.3
No. of overweight patients (%)	5 (9.2)	3 (5.9)		7.6
Mean duration of the disease $\pm$ sd (months)	18 ± 9	21 ± 7	n.s.	20 ± 7
No. of children with Cassano's score = 3 (%)	34	30	n.s.	64
Cassano's score (16)				
No. of children with Cassano's score = 4 (%)	20	21		41
Mean nNO $\pm$ sd (ppb)	676.9 ± 250.7	831.8 ± 320.4	0.02	750.7 ± 284.3

RAOM: recurrent acute otitis media; sd: standard deviation; nNO: nasal nitric oxide; ppb: parts per billion.

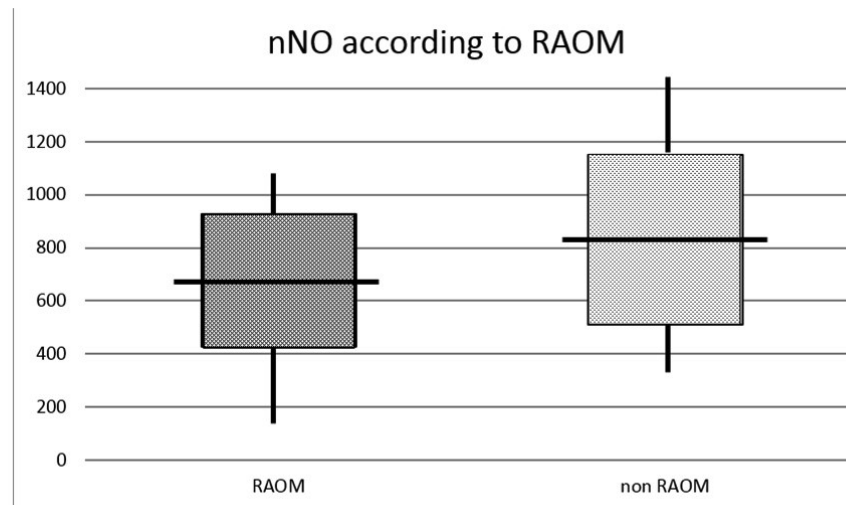


Fig. 1. nNO levels (ppb) in children with and without RAOM. The first and third quartiles are the limits of the box, the inner line is the mean value; the "whiskers" represent the highest and lowest values.

DISCUSSION

The involvement of NO pathway in pathogenesis of middle ear inflammation has been previously postulated and investigated by means of a few experimental studies, mainly performed on *in vitro* or in animal models (11-15). However, previous evidence suggests that NO might have a dual role in this context: both promoting and suppressing middle ear inflammation (15). In particular, NO seems to act as an inflammatory mediator in otitis media with effusion, as middle ear bacterial lipopolysaccharide and cytokines would enhance expression of constitutive iNOS with the following production of free radical NO mediating mucin secretion (21, 22). As a matter of fact, some studies carried out on animal models have documented a promoting effect of NO on middle ear inflammation (12, 13, 23), as it has been found that addition of NO in experimental lipopolysaccharide-induce otitis media in chinchillas increases the mucin concentration in middle ear fluid, and increases mucosal thickness and subepithelial infiltration of polymorphonuclear leukocytes (12). Furthermore, Yilmaz et al. (13) found significantly increased erythrocytes NOS activity and NO levels in a small sample of guinea pigs with experimental otitis media with effusion resulting from histamine

intra-tympanic injection. In addition to these, increased concentrations of NO metabolites were found in mucoid middle ear effusion of a small case-series of patients who had undergone myringotomy and tympanostomy tube insertion (23).

Moreover, Garca and coworkers (24) recently compared serum NO levels in patients with chronic suppurative otitis media with or without cholesteatoma, finding significantly increased NO levels in those with cholesteatoma compared to those without, thus suggesting that increased oxidative stress could play a role in the pathogenesis of chronic middle ear infection. Therefore, the signalling molecule NO, acting as an inflammatory mediator, could elicit the effectors of the innate immune system with activation of defensive mechanisms including phagocytosis, barrier system, and activation of signalling and secreting molecules responsible for quickly identifying and clearing pathogens from the middle ear (25). On the contrary, reduced middle ear NOS mRNA expression was found in 24 otitis-prone children with otitis media with effusion (26), and a study by Granath et al. (15) documented reduced iNOS expression on adenoids obtained from twelve children with otitis media with effusion compared to fourteen children without middle ear disease who had undergone adenoidectomy.

This is the first study evaluating nNO levels in children with chronic adenoiditis associated with RAOM. We found that nasosinus NO levels were significantly reduced in children with RAOM compared to those without ($p=0.002$); likewise, decreased nNO levels have been previously reported in patients with acute nasosinus inflammation compared to healthy controls, and in children with large obstructive adenoids compared to children with non-obstructive adenoids. Decreased nNO levels in children with chronic adenoiditis and history of RAOM here documented could be related to reduced NO production by the ciliated nasopharyngeal and middle ear epithelium and the impaired Eustachian tube patency due to chronic inflammation, resulting in NO entrapment; on the other hand, one may speculate that impaired NO release by the nasopharyngeal, tubaric and middle ear mucosa would result in ciliostasis and ineffective mucociliary clearance predisposing to retention of secretions and pathogens within the middle ear, thus leading to recurrent acute otitis media. With regards to this, it is known that NO plays a role as an intermediate messenger in the ciliated epithelium in response to ciliostimulators such as terbutaline, methacholine, substance P, and endothelin-1, as a significant reduction of *in vitro* ciliary beat frequency has been reported after pre-treatment of adenoidal samples with NOS inhibitors (27). Finally, it is worthwhile to remember that NOS cooperatively acts with Toll-like receptors and cytokines such as interleukin-6, -8, -10, -12, interferon- γ , tumor necrosis factor- α as an inflammatory mediator in the innate immune response (26), and that decreased NO production could predispose to increased susceptibility to recurrent middle ear infections.

In conclusion, our data corroborate previous experience suggesting the involvement of NO pathway in recurrent upper airway infections related to impaired ciliated respiratory mucosa, and firstly document the impairment of nNO in children with history of RAOM.

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LETTER TO THE EDITOR

ACCELERATED BONE TURNOVER IDENTIFIES HEMIPLEGIC PATIENTS
AT HIGHER RISK OF DEMINERALIZATION

A. DEL PUENTE¹, N. PAPPONE^{2,3}, C. SERVODIO IAMMARRONE¹, A. ESPOSITO⁴,
R. SCARPA⁴, L. COSTA^{4,5}, F. CASO^{4,5}, A. BARDOSCIA³ and A. DEL PUENTE⁴

¹Rehabilitation Unit, University Federico II, Naples, Italy; ²Rheumatologic Rehabilitation Unit, S. Maugeri Foundation, IRCCS, Telesse Terme, Italy; ³Rehabilitation Unit, S. Maugeri Foundation, IRCCS, Cassano delle Murge, Italy; ⁴Rheumatology Unit, Department of Clinical Medicine and Surgery, University Federico II, Naples, Italy; ⁵Rheumatology Unit, Department of Medicine DIMED, University of Padova, Padova, Italy

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Immobilization osteoporosis represents a severe complication in hemiplegic patients (HPs), causing fragility fractures, which may occur during rehabilitation reducing functional recovery and survival. The aim of the study was to investigate determinants of bone loss, independent from length of immobilization, which may be useful in early identification of HPs at higher risk of demineralization. Forty-eight HPs of both sexes underwent anthropometric measurements, evaluation of scores of spasticity and of lower limb motory capacity. Laboratory tests were performed. On serum: calcium; phosphorus; creatinine; ALP; iPTH; 25(OH) vitamin-D; sex hormones; $\Delta 4$ -androstenedione; DHEA-S; insulin; IGF-1; FT3; FT4; TSH; c-AMP. On urine: c-AMP and calcium/creatinine ratio. Two bone turnover markers were measured: serum osteocalcin (BGP) and urinary deoxypyridinoline (DPD). Bone mineral density was determined at both femoral necks, defining a percentage difference in bone loss between paretic and non-paretic limb, thus controlling for the complex cofactors involved. Only bone turnover markers significantly and directly correlated with the entity of demineralization, controlling for age, sex and length of immobilization in the multivariate analysis (BGP coefficient estimate=0.008; SE=0.003; p=0.020; DPD coefficient estimate=0.005; SE=0.002; p=0.036). BGP and DPD are not dependent on anthropometric and endocrine-metabolic parameters, disability patterns and duration of immobilization, thus represent independent determinants of the degree of demineralization. A cut-off was defined for BGP and DPD above which subjects show significantly greater risk of demineralization. The immobilization event generates more severe bone loss when it occurs in subjects with higher bone turnover. BGP and DPD measurements may be of primary importance for early identification of HPs at risk, with relevant preventive implications.

The increased survival rate following cerebrovascular accidents that reduce mobility makes osteoporosis due to immobilization a field of the greatest relevance. The severity of this complication lies in the risk of fragility fractures (1). These

events may occur in the rehabilitative phase for the usual passive mobilization, or while attempting to verticalize the patient, or in ambulation and standing exercises (2).

Hip fractures are the predominant type of fracture

Key words: stroke, hemiplegia, osteoporosis, osteocalcin, immobilization, rehabilitation

Mailing address:

Prof. Antonio del Puente,
Rheumatology Unit, University Federico II
Via Pansini, 5, 80131, Naples, Italy
Tel.: +39 081 7463773 - Fax: +39 081 5463445
e-mail: delpuente@unina.it

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(compared to fractures in other sites, such as wrist and vertebral fractures) and they prove to be the most devastating in terms of morbidity and mortality (1). As several studies have shown, survival and recovery of independent mobility after fracture are significantly reduced in patients who had had a previous stroke compared to those who had not (3), further justifying the growing interest of the research on this subject.

However, from a clinical point of view, immobilization osteoporosis is often underestimated and the awareness about this condition should be increased. In fact, fractures are usually the triggering event that focuses attention on bone health of stroke survivors (3).

An interesting aspect that emerges from literature is the existence of a significant inter-individual variability of the demineralizing effect due to immobilization (4, 5). At present, the main determinant of demineralization is generally considered to be duration of immobilization (6), but duration was not found to be consistently correlated with the entity of bone loss (7-9). Therefore, other factors play a crucial role in the pathogenesis of immobilization osteoporosis. Their identification would be highly relevant to define the profile of the subjects at higher risk of demineralization. This would allow to focus therapeutic or rehabilitative intervention to prevent the severe consequences of osteoporosis in stroke patients (3).

The aim of the present study was to investigate the determinants of the degree of bone loss in hemiplegic patients, evaluating the correlation between laboratory data and entity of demineralization. Therefore, it was attempted to identify the other factors besides the length of immobilization affecting the degree of bone loss.

MATERIALS AND METHODS

The present work uses data collected in a previous study, but never analyzed (10). The original project was approved and financially supported by the Italian Space Agency for its relevance on the topic of bone metabolism in conditions of reduced mechanical stimuli.

Methods were extensively described elsewhere. Briefly, 48 individuals suffering from hemiplegia due to stroke were enrolled consecutively among those who were admitted to a specialized hospital over a 9-month period. Exclusion criteria included an elapsed time of longer than 48 months since the stroke; females of child-bearing age; pathologies or intake of drugs known to affect bone metabolism; and inability to maintain the correct position of bone densitometry according to the criteria for quality control our group complies with (10, 11).

The participants were 31 males (mean age 59 years; range 27-84 years) and 17 postmenopausal females (mean age 64.6 years; range 52-74 years). Mean length of immobilization was 9.81 months (± 12.48). In females the average time since menopause was 14.9 years (range 1.7-23.9 years). Mean body mass index (kg/m^2) was 26.90 (± 4.49).

After obtaining informed consent to participate, a medical examination was carried out on each individual, body mass index was measured, and a general questionnaire was submitted. We measured triceps and subscapular skinfold thicknesses on the left side of the body. To take account of the different degrees of disability among patients, we calculated for each subject their score according to the Ashworth scale for spasticity (12) and their scores of lower limb motor capacity according to Lindmark and Harmin (13); the latter comprised a subject's capacity to perform active movements and mobility evaluation.

Bone mineral density (BMD) was measured using dual-energy X-ray absorptiometry (DXA; Hologic QDR 1000) at both femoral necks (14). Bone density in the paretic lower limb was compared with the non-paretic limb. This model allows to overcome a major difficulty in the interpretation of the studies in this field: the presence of various cofactors (genetic, metabolic, endocrine, environmental) (3) affecting bone mineralization, most of which are difficult to evaluate, thus complicating the selection of control subjects (10). In this way, the effect of confounding factors was minimized notwithstanding the limitations related to the loss of nervous stimulation. For each patient we defined a percentage difference in bone loss between the femoral neck of the

paretic limb and that of the non-paretic limb, which corresponded to the following ratio: (normal BMD – paretic BMD)/normal BMD.

For each patient we collected a fasting blood sample and the first morning void. Samples were immediately analysed using standard methods and data was saved on a computer. The following variables were studied. On serum: calcium; phosphorus; creatinine; total alkaline phosphatase; iPTH; 25(OH) vitamin D; total estrogens; 17 β estradiol; estrone; progesterone; testosterone; Δ 4-androstenedione; DHEA-S; insulin; IGF-1; FT3; FT4; TSH; c-AMP. On urine: c-AMP and calcium/creatinine ratio.

In addition, in order to evaluate bone turnover, serum osteocalcin (BGP) was also measured as a marker of bone formation and urinary deoxypyridinoline crosslinks corrected for urinary creatinine (DPD), which is one of the markers of bone resorption more frequently used in patients with stroke (15-17).

In the previous study, (10) when comparing BMD of the paretic limb with BMD of the non-paretic one, a significant bone loss was observed in paretic limb with a mean percentage difference of 6.3% among females (95% CI= 0.35-12.2) and 4% among males (95% CI= 0.72-7.28). As shown, the degree of bone loss in the paretic femur compared with the non-paretic one depends, significantly and directly, on the length of immobilization, even when controlling for age and sex in the regression model (regression coefficient= 0.07; p value= 0.03)(10).

Statistical analysis

Data were analysed using *t*-test and multiple linear regression analysis when needed. The sample size was calculated assuming the conventional value of $\alpha=0.05$ and a value of $\beta=0.20$. The expected difference for the variables of interest between the groups studied was 10%. Given a standard deviation of up to 10% for such variable, the number of individuals needed for each group was calculated to be at least 16.

We analysed the independent determinants of demineralization using models of multiple linear regression. As dependent variable, we used BMD mean percentage difference between non-paretic and paretic limb. Age, sex and duration of immobilization were included as covariates. Among females, age and duration of menopause were strongly correlated. Therefore, when analysing data in the female subgroup, we included age and duration of menopause as covariates in different models, obtaining no differences in the results. For this reason, when reporting data from the female subgroup, we decided to report only results of models using age among covariates.

RESULTS

All study variables listed in the Methods were tested, and Table I shows the data of the only variables reaching statistical significance: the bone turnover markers BGP and DPD. No other laboratory and clinical data collected correlated with the degree of demineralization.

Table I. Independent determinants of demineralization in hemiplegic patients. Multiple linear regression analysis^a.

Independent variable	Coefficient estimate	Standard error	<i>t</i> -value	P
Duration of immobilization	0.002	0.001	2.118	0.040
BGP	0.008	0.003	2.404	0.020
DPD	0.005	0.002	2.163	0.036

^aDependent variable: BMD mean % difference between non-paretic and paretic limb.

Covariates: age and sex (not significant).

Results show that bone turnover markers directly correlate with the entity of demineralization. This indicates that hemiplegic subjects with more accelerated turnover are those who present a greater amount of demineralization, even when controlling for age, gender and duration of immobilization in the multivariate analysis. Similarly significant results were obtained when inserting DPD and BGP in separated regression models (for BGP coefficient estimate= 0.010; SE= 0.003; $p= 0.003$; for DPD coefficient estimate= 0.007; SE= 0.002; $p= 0.006$) and when carrying out the evaluation in the two sexes separately. In particular, when the analysis was performed on the male subgroup, the correlation between bone turnover markers and the degree of bone loss was the only one reaching statistical significance, thus showing an effect that is even greater than that of duration of immobilization.

It was then decided to verify the possible correlation between BPG and DPD and all the other laboratory data and anthropometric parameters. Results show that these two markers of bone turnover do not correlate with any of the study variables, even controlling for age, sex and duration of immobilization. We also evaluated the correlation between the bone turnover markers and the disability pattern measured using Ashworth scale for spasticity and scores of lower limb motor capacity according to Lindmark and Harmin. There were no significant correlations even controlling for age, sex and duration of immobilization.

We then verified whether bone turnover marker values depend on duration of immobilization. The hypothesis was that different durations of immobilization might determine different levels of activity of bone turnover. On the contrary, multiple regression analysis results showed that serum levels of BGP do not correlate with duration of immobilization (coefficient estimate = 0.18; SE = 0.04; $p= 0.7$). Not even DPD urinary levels correlate with length of immobilization (coefficient estimate = 0.02; SE = 0.06; $p= 0.7$). In both models we used age and gender as covariates (not significant). Therefore, notwithstanding the limitation due to the cross-sectional nature of the study, BGP and DPD should be considered independent determinants of

the degree of bone loss in this condition, regardless of duration of immobilization.

In order to obtain a parameter that could be clinically more meaningful, we decided to define for BGP and DPD the cut off above which hemiplegic subjects show a significantly higher risk of developing immobilization osteoporosis. We discretized the variables and used techniques of multiple regression analysis with duration of immobilization, age and sex as covariates. Results show that for BGP this cut off corresponds to the mean value found in the studied population (8.26 ng/ml). This value is within the normality range (5-15 ng/ml) and it approximately corresponds to the limit of the lower tertile. Therefore, immobilized subjects belonging to middle-upper tertiles and over, are at higher risk of demineralization as compared to those with lower values (coefficient estimate=0.073; SE=0.028; $p=0.011$). For DPD, the significant cut off corresponds to the value which is the limit of the upper quintile in our population (10.86 nM DPD/ mM creatinine). This value is approximately 50% greater than the upper limit of normality range (2.5 - 6.5 nM DPD/ mM creatinine). Therefore, immobilized subjects with DPD values 50% greater than the upper limit of normality range are at higher risk of demineralization as compared to those with lower values (coefficient estimate=0.095; SE=0.037; $p=0.013$).

DISCUSSION

Immobilization osteoporosis is a common and severe complication in hemiplegic patients. It generally occurs in the paretic side (10, 18-20). The rehabilitative program in these patients requires a thorough evaluation of the mineralization status in order to avoid potential fractures during mobilization and load bearing exercises (1). International literature indicates the duration of immobilization as one of the main factors in the determinism of bone loss. On the contrary, at present, the literature does not indicate any determinant that may precociously identify hemiplegic subjects who are more likely to develop this complication. This is the aim of our study. Two bone turnover markers (BGP and DPD)

were identified as independent variables directly and significantly correlated with the degree of bone loss after stroke, even when controlling for duration of immobilization, age and sex. In addition, our study defined a cut off for BGP and DPD above which subjects show significantly greater risk of demineralization in the paretic side.

BGP and DPD values did not correlate with immobilization duration. Therefore, notwithstanding the limitation due to the cross-sectional nature of the study, these results indicate that the immobilization event generates more severe bone loss when it occurs in subjects with higher turnover, even when considering duration of immobilization. This is in agreement with the literature that indicates how the effect of immobilization on bone mineral density is worse when it occurs in conditions of increased bone turnover, such as adolescence or immediate post-menopause (3, 10). Therefore, data suggest the existence of an individual profile of increased risk defined by the intensity of bone turnover. Bone turnover activity seems to identify a characteristic that is peculiar to the bone metabolism of each subject. In fact, the risk profile defined by BGP and DPD values is not affected by duration of immobilization; endocrine-metabolic parameters; disability pattern measured using Ashworth scale for spasticity and scores of lower limb motor capacity according to Lindmark and Harmin.

Therefore, the measurement of turnover activity may represent an element of primary importance for the identification of those subjects who are more likely to suffer greater demineralization after stroke.

The present work shows aspects of particular interest: the utilization of a standard method to evaluate bone mineral density; the wide panel of laboratory, anthropometric and functional variables considered; the statistically significant sample size; and, above all, the utilization of each patient as a control for him-herself to evaluate the degree of demineralization, comparing the normal to the hemiplegic limb. This model allows overcoming a major difficulty in the interpretation of the studies in this field: the presence of various cofactors (genetic, metabolic, endocrine, environmental) (3) affecting bone mineralization, most of which are difficult to

evaluate, thus complicating the selection of control subjects (10).

The principal limitation of the study is its cross-sectional nature. Therefore, the data obtained and the considerations about the potential predictive value of bone turnover markers must be interpreted with caution. The data obtained indicate the need for longitudinal studies that may include analysis of further variables involved in the pathogenesis of immobilization osteoporosis, such as serum sclerostine (21), and evaluation of therapeutic approaches.

In conclusion, the present study confirms the importance of immobilization osteoporosis, showing the severe demineralization of the paretic limb. In addition, the data obtained may contribute to a better management of this condition. At present, in fact, the general indication is to carry out a densitometry on every subject with stroke in order to obtain basal values and to re-evaluate it in the follow-up in order to identify those subjects who developed the complication (3, 22-23). Our results indicate, instead, the possibility of early identification of subjects at risk in a simple way, by opportunely measuring bone turnover activity levels in patients with stroke. In these subjects, it will be possible to evaluate different therapeutic options with a preventive aim, such as rehabilitation programs and use of drugs that reduce bone turnover activity.

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LETTER TO THE EDITOR

EPIDEMIOLOGY OF DIABETES MELLITUS IN THE FRAGILITY FRACTURE POPULATION OF A REGION OF SOUTHERN ITALY

A. NOTARNICOLA¹, G. MACCAGNANO¹, S. TAFURI², L. MORETTI¹, L. LAVIOLA³
and B. MORETTI¹

¹Orthopaedics Unit, Department of Basic Medical Science, Neuroscience and Sensory Organs, Faculty of Medicine and Surgery, University of Bari, General Hospital, Bari, Italy; ²Department of Biomedical Sciences and Human Oncology, Faculty of Medicine and Surgery, University of Bari, General Hospital, Italy; ³Department of Emergency and Organ Transplantation, Section on Internal Medicine, Endocrinology and Metabolic Diseases, University of Bari, General Hospital, Bari, Italy

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Increased risk of osteoporosis and its clinical significance in patients with diabetes is controversial. This study aims to increase the data which are available regarding the prevalence of diabetes mellitus in patients affected by fragility fracture in Italy. We retrospectively studied Hospital Discharge Data (HDD) in the Apulian database for the period 2006–2010 to identify a fragility fracture diagnosis in males over 65 years of age and in females over 50. The database was then checked for drug prescriptions to identify those persons who had taken at least one osteoporosis drug. Within this latter group, thanks to hospital admission and prescription records, the subjects affected with diabetes mellitus were identified. Between 2006 and 2010 in Apulia 177,639 patients were hospitalized and diagnosed as having fragility fracture. The greatest number of those fragility fractures were found to be in the 70 to 79 age range (64,917 total; females 56,994, males 7,923). The prevalence of diabetes subjects in Apulia in this period was estimated at 6.5%. In the same region and period 21.1% of subjects affected by diabetes experienced a fragility fracture; in particular, this number was 27% for males and for 20.5% females. This is the first study providing data on the prevalence of fragility fractures and diabetes in the Apulian population. The data confirm that diabetes is a risk factor which influences bone density and risk of fractures and therefore the need of osteoporosis screening and treatment in diabetic patients.

The prevalence of osteoporosis in Italy was reported by the ESOPO study as follows: 23% among all women, ranging from 9% in the 40–49 years of age group to 45% in the 70 and over years of age group; 15% of men aged over 60 also suffered from osteoporosis. Another report estimated that approximately 4 million Italian women and 800,000 men were affected by osteoporosis (1). As the population ages, the incidence of fragility fractures

and chronic and degenerative diseases increases (2). Osteoporosis can significantly impact on the risk of developing fracture. The risk of a fragility fracture increases in the presence of co-morbidity, such as diabetes, cardiovascular disease and rheumatic diseases, which are common age-related conditions that result in significant morbidity in osteoporotic patients and further contribute toward tremendous health problems and economic costs (3, 4).

Key words: osteoporosis, diabetes, fragility, fracture

Mailing address:

Dr Angela Notarnicola,
Orthopaedics Unit, Department of Basic Medical Science,
Neuroscience and Sensory Organs, Faculty of Medicine and Surgery,
University of Study of Bari, General Hospital, Piazza Giulio Cesare 11,
70124 Bari, Italy, Tel./fax: +39 0805592938
e-mail: angelanotarnicola@yahoo.it

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

Many studies have demonstrated osteopenia and increased fracture risk in patients with type 1 and 2 diabetes (5-10). Although skeletal disorders in patients with diabetes mellitus have been reported, there is still controversy over the risk of osteoporosis and its clinical significance in patients with diabetes mellitus (11-13). Diabetes mellitus (DM) is considered a clinical risk factor within the Q-Fracture algorithm which is a validated instrument to assess fracture probability (2, 14).

Although osteoporosis and diabetes mellitus are commonly observed in the elderly population, there is a need for further investigation into the frequency of diabetes in all age groups of patients with fragility fracture. Further investigations are also needed since the majority of older clinical studies focusing on diabetes mellitus and risk of osteoporosis are hampered by methodological problems, such as relatively small sample sizes and heterogeneous study populations (2, 11-13, 15).

The aim of this study is to investigate the prevalence of diabetes mellitus in greater populations of patients with known osteoporosis and fragility fractures and who are from the same geographic area.

MATERIALS AND METHODS

Patients

The present clinical retrospective observational study was approved by the Local Ethics Committee. A survey was carried out of patients referred to the Orthopedics departments of hospitals in a southern region of Italy: Apulia. Apulia has a population of 4,090,266 inhabitants [ISTAT Data of 31 December 2013]. The Hospital Discharge Data database (HDD) was screened for the period 2006–2010 to identify patients with a fragility fracture diagnosis requiring hospitalization. For this retrospective study we selected male patients over the age 65 and female patients over 50 whose HDD reported domestic accidents in the “trauma field” and were not recorded in the “deceased item”.

The absence of specific codes for fragility (mainly osteoporotic) fractures results in a lack of perception of fracture burden, leading to problems in the exact evaluation of osteoporotic fractures in the elderly. The database was checked also for the drug prescription to

reach the persons who assumed at least one drug for osteoporosis and calcium and vitamin D supplements. The database of patients with fragility fracture or osteoporosis drug prescription were cross-checked by using the individual health care code to eliminate the same patients being reported twice. The resulting database provides the prevalent population for the study. The proportion of subjects with osteoporosis and diabetes mellitus was the main study outcome, and was ascertained by performing a data-linkage between the population and the prevailing of the HDD database and drug prescriptions. In particular, the following subjects were classified as diabetic:

- those belonging to the prevailing population for whom hospitalization had been registered with the main cause correlated with diabetes mellitus;
- those with at least one prescription for anti-diabetic agents (ATC A010) ascertained through data-linkage with the regional archive of pharmaceutical prescriptions.

Data were stratified by decade of chronological age (50-59 years; 60-69 years; 70-79 years; $\geq$ 80 years) and gender (male and female).

RESULTS

In the period 2006 to 2010 in Apulia 177,639 patients had been diagnosed with fragility fractures, with the highest number in 2006 (34.66%); in the following year the percentage decreased, as can be seen in Table I.

The analysis of fragility fracture data according to decade of age showed the greatest incidence to be in the 70-79 age group (64,917) and the lowest in the 50-59 age group (17,260) (Table II).

In the study period 2006-2010, there were 36,125 diabetic patients in Apulia, of whom 32,201 were taking oral anti-diabetic drugs and 3,924 insulin. In the prevalent population we found that 21.1% were affected with diabetes mellitus (Table III). This condition tended to increase with age, passing from 9.9% for the 50-59 age group to 24.7% for the over 80 group. The data demonstrated a greater prevalence of diabetes mellitus in males with fragility fracture (27% males vs 20.5% females).

The patients with fragility fracture and diabetes mellitus had the fractures in the following sites: femur (n. 4,139), humerus (n. 1,290), spine (n.

Table I. *Frequency of fragility fracture in the study.*

year	frequency	Percent	Cumulative
2006	61,566	34.66	34.66
2007	30,945	17.42	52.08
2008	29,262	16.47	68.55
2009	28,369	15.97	84.52
2010	27,497	15.48	100
Total	177,639	100	

Table II. *Frequency of fragility fracture according to age and sex.*

Age group	M	F	Total
50-59	n.d.	17,260	17,260
60-69	1,867	41,943	43,810
70-79	7,923	56,994	64,917
=/≥80	6,903	44,749	51,652
Total	16,693	160,946	177,639

Table III. *Percentage of patients affected by fragility fracture and diagnosis of diabetes mellitus, gender differences.*

Affected by			
diabetes	M	F	Total
50-59	0%	9.9%	9.9%
60-69	26.4%	16.8%	17.2%
70-79	28.1%	23.2%	23.8%
=/≥80	25.9%	24.5%	24.7%
tot	27 %	20.5%	21.1%

Table IV. *Fragility fracture in diabetic patients divided by location and diagnosis of diabetes mellitus not complicated and complicated.*

Fracture site	DM non Complicated	DM Complicated	Total
spine	928	124	1,052
humerus	1,140	150	1,290
forearm	681	77	758
femur	3,563	576	4,139
Tibial pilon	682	91	773
Tarsus and Metatarsus	76	21	97
<i>Multifracture</i>	257	42	299
Total	7,327	1,081	8,408

Table V. *Prevalence of diabetes in the different years in the Apulia region (Source: www.epicentro.iss.it/passi/dati/diabete.asp).*

Year	Percentage
2006	Not available
2007	Not available
2008	5.8%
2009	5%
2010	5.5%

1,052), tibial pilon (n. 773), forearm (n. 758), multifractures (n. 299), tarsus and metatarsus (n. 97) (Table IV).

DISCUSSION

Approximately, 5% of the world population is affected with DM, of whom 90% is affected with DM type 2 (16). The data reveal a greater incidence

in females (M:F = 1:1.25). According to ISTAT data, diabetes in Italy in 2012 affected 5.5% of the entire population (2,960,000 patients). The region of Apulia has an incidence of diabetes higher than the national average (Table V). It is generally believed that osteoporosis is underestimated in the diabetic population (13, 17, 18). In literature it is reported that 4.5% of diabetic patients are diagnosed with having osteoporosis (11). The association between

type 2 DM (T2DM) and fracture risk was studied in a Rotterdam cohort (15), which showed an increased risk of more than 30% of non-vertebral fracture in type 2 DM, particularly in patients treated with anti-diabetic agents. Recently-diagnosed DM patients have a 20% higher risk of hip fractures in the first few years following disease onset, compared to non-diabetic patients.

In the Apulian population with a fragility fracture the DM incidence has shown to increase with age, ranging from 9.9% in the 50-59 year age group to 24.7% for people in their 80s. This is coherent with literature which reports a progressive impoverishment of bone stock with age and a consequent increase of risk fracture (19, 20). A difference between the sexes has emerged: the diagnosis of DM patients with fragility fracture is more frequent in males (27%) than in females (20.5%). Furthermore, in our study a greater incidence of fragility fracture emerged in patients with a history of non-complicated diabetes. Our data demonstrate that the incidence of fragility fracture in the Apulian diabetic population is more frequent in the femoral bone with a greater cortical component than in tarsus and metatarsus characterized by a greater trabecular component. The literature suggests that osteoporosis in diabetic patients preferentially develops within the appendicular skeleton with predominantly cortical bone (20).

The main limitation of our data is that HDD does not distinguish specifically between traumatic fractures and fragility fractures. In a subsequent study we intend to study a larger population with fragility fracture, analyzing the orthopedic emergency records of Apulia in order to quantify also those fractures which, since the patients were not hospitalized, were not calculated in the present study. As thiazolidinediones are associated to an increased risk of fractures, it should be interesting to evaluate the effects of different diabetes treatments in this population. The present study does not track the effects of the drug prescription (diabetes and/or osteoporosis treatments) on the fragility fracture. We were unable to know whether the patients effectively assumed the drug or not, therefore we were not able to formulate a hypothesis on the effect of these drugs.

Another general limitation of our study is the lack of densitometry values. As reported above, the osteoporosis diagnosis according to densitometry value can underestimate the incidence of osteoporosis in the diabetic population. For this reason, we chose the fragility fractures as a diagnosis of osteoporosis.

The high incidence of DM in the osteoporotic population confirms how important it is to correct the modifiable risk factors of fragility fracture (physical activity, proper diet, reduction of smoking, etc) and to evaluate the use of anti-osteoporotic treatment. Longitudinal studies may allow us to monitor the efficacy of these strategies to reduce the incidence of fragility fracture.

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LETTER TO THE EDITOR

SODIUM HYALURONATE IMPROVES QUALITY OF LIFE AND NASAL ENDOSCOPY FEATURES IN PRESCHOOL CHILDREN WITH UPPER RESPIRATORY TRACT INFECTIONS

S. MONTELLA¹, E. CANTONE², M. MAGLIONE¹, M. IENGO^{2*} and F. SANTAMARIA^{1*}

¹*Department of Translational Medical Sciences, Federico II University, Naples, Italy;*

²*Department of Neuroscience, Reproductive and Odontostomatologic Science, Section of ENT, Federico II University, Naples, Italy*

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*These two Authors contributed equally to the study supervision

The aim of this prospective, randomised study was to evaluate the effects of nasal douches with sodium hyaluronate on clinical and endoscopic variables, on parental perception of their child's health-related quality of life (HR-QoL), and on parental workdays lost in preschool recurrent upper respiratory tract infections (URTIs). Children aged 2-6 years with recurrent or persistent URTIs underwent at baseline the evaluation of upper respiratory tract symptoms in the previous two weeks, and nasal endoscopy. Parents of enrolled children were assessed for self-perception of their children's HR-QoL using a standardised questionnaire. The same variables were reassessed after a 2-week treatment with either 9 mg sodium hyaluronate plus saline solution or saline alone by nasal douches. Forty of the 48 children enrolled completed the study (22 assigned to the combined treatment). Compared to baseline, the combined treatment resulted in a significant reduction of the prevalence of children with missed daycare days (45% vs 14%, $p=0.04$) and of parents with workdays lost (36% vs 5%, $p=0.02$), and in a significant improvement of HR-QoL score (3.7 vs 2.8, $p=0.004$). At endoscopy, the secretion and mucosal oedema score significantly improved after the combined treatment (6 vs 2, $p<0.001$), and there was a trend towards a reduction of the adenoid hypertrophy score ($p=0.06$). No clinical, HR-QoL or endoscopy changes were found in the saline group. In preschool children with recurrent or persistent URTIs, sodium hyaluronate by nasal douche significantly improves endoscopic features. Additional benefits include the children's HR-QoL and daycare attendance, and parental work.

Upper respiratory tract infections (URTIs) in children account for a high proportion of doctors' surgery visits (1), and result in negative effects on children's education, on the society for parental work absenteeism, and on the public health system for the resources employed in medication consumption (2). URTIs reduce the nasal mucociliary defence by directly damaging the cilia and by changing the rheological properties of the nasal secretions, thus leading to

maintenance or worsening of the inflammatory state (3). Previous studies have suggested that hyaluronic acid may protect airway epithelium and stimulate mucociliary clearance during URTIs (4, 5).

Hyaluronic acid is a non-sulphated glycosaminoglycan made of repeating glucuronic acid and N-acetylglucosamine disaccharide units, linked through alternating beta-1,4 and beta-1,3 glycosidic bonds (6). It is one of the main components of many

Key words: sodium hyaluronate, upper respiratory tract infections, nasal endoscopy, quality of life

Mailing address:

Francesca Santamaria, MD
Department of Translational Medical Sciences,
Federico II University,
Via Sergio Pansini, 5 - 80131 Naples, Italy
Tel.: +39 081 7463495 - Fax: +39 081 7463116
e-mail santamar@unina.it

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extracellular matrices, including respiratory epithelial cells and nasal and tracheobronchial mucosa (7). Because of its biological properties, hyaluronic acid has several clinical applications, such as aesthetic surgery, dermatology, orthopaedics and ophthalmology (8). In spite of such wide employment, only few studies have assessed the effects of hyaluronic acid on chronic or recurrent URTIs (5, 9-15), most of which have provided evidence that hyaluronic acid is beneficial in improving clinical and laboratory outcomes in affected children and adults (5, 10-15). However, none of these studies has assessed its efficacy on the economic burden and health-related quality of life (HR-QoL) in preschool children. Such studies may be very useful as this is the age range with the highest prevalence of URTIs (1).

We hypothesized that in children with URTIs hyaluronic acid not only improves clinical and laboratory outcomes, but also affects health-economic measures and HR-QoL. Therefore, the aim of the current study was to evaluate the effects of nasal douches with sodium hyaluronate on clinical and endoscopic variables, on HR-QoL and on parental workdays lost in preschool children with recurrent or persistent URTIs.

MATERIALS AND METHODS

Study design

This was a single-centre, prospective, randomized, parallel-group study performed in accordance with the principles of Good Clinical Practice and of the Declaration of Helsinki and its amendments. The study protocol was approved by the local review board, and informed written consent was obtained by all patients' parents or legal representatives prior to entry into the study.

Study population

Children aged 2-6 years with recurrent URTIs or persistent rhinorrhoea and/or nasal obstruction were enrolled in the study during winter (16). An URTI was defined by the presence of at least two of the following for at least 24 hours: sore throat; ear pain or discharge; runny nose; cough (dry or barking); hoarse voice; or fever $>37^{\circ}\text{C}$ (17). The infection types included in the study were rhinitis, rhinosinusitis, adenoiditis, tubotympanitis,

otitis media, pharyngitis, and tonsillitis.

Patients were excluded from the study if they had any of the following: cystic fibrosis, primary ciliary dyskinesia, immune defects, autoimmune diseases, genetic syndromes, malformations, allergic rhinitis and/or asthma, neurodevelopmental delay compromising the adherence to treatment, use of nasal hyaluronic acid or saline in the 4 weeks preceding recruitment, ongoing acute infection requiring hospitalization, and any indication for surgery.

Study protocol

During the 2 weeks preceding randomization, no treatment was prescribed to patients consecutively enrolled in the study, but all therapies assumed by them were recorded. We asked the patients' parents or legal representatives to complete a daily diary card on which they had to record every day whether their child had rhinorrhoea, assumed antibiotics, and/or missed that daycare day. They also recorded whether they missed that day from work. Rhinorrhoea severity was then assessed by the clinician according to the following score: 1, no rhinorrhoea; 2, rhinorrhoea occurring rarely; 3, rhinorrhoea occurring for some of the time; 4, rhinorrhoea for most of the time; and 5, rhinorrhoea for all of the time.

After completing the run-in period, parents or legal representatives of enrolled children were assessed for self-perception of their children's HR-QoL using a standardised questionnaire (18). Moreover, all patients underwent fiberoptic nasal endoscopy in order to evaluate: a) adenoid tonsil size graded into four degrees (19); b) presence or absence of nasal turbinate hypertrophy or septum deviation; and c) nasal secretion, mucosal oedema and polyp severity according to the Lund-Kennedy scoring system (20). The otolaryngologist who performed the procedure (E.C.) was blinded to the patients' data.

Recruited patients were alternately assigned to 2 intervention groups and treated for 15 days. Subjects in the first group were treated with high molecular weight sodium hyaluronate (Yabro[®], IBSA, Lodi, Italy) 9 mg/3 mL vial twice a day aerosolized in 2 mL sodium chloride 0.9% using the Rinowash[®] nasal douche device [Air Liquide Medical Systems, Bovezzo (Brescia), Italy], that generates a mass median aerodynamic diameter of approximately 18 μm . Children in the other arm were treated with sodium chloride 0.9% alone (5 mL twice a

day) aerosolized by the Rinowash® device. Compliance to the assigned treatment was evaluated by counting the amount of dispensed and returned vials.

During the treatment period, patients' parents or legal representatives again filled in the daily diary card on which they recorded whether they missed that day from work and whether their child had rhinorrhoea, assumed antibiotics, and/or missed that daycare day. They also took note of the type, number and severity of adverse events. Finally, at the end of the study they again completed the HR-QoL questionnaire, while patients underwent a second fiberoptic nasal endoscopy.

Statistical analysis

Results are expressed as medians and ranges for continuous variables, and as percentages for categorical data. To test differences between the two groups at baseline and after treatment, the Mann-Whitney U-test and Fisher's exact test were used for continuous and quantitative variables and for proportions, respectively. For longitudinal assessments, comparisons were made using the Wilcoxon test for continuous and quantitative variables and Fisher's exact test for dichotomic data. Multivariate logistic analysis was used to measure the effect of combined treatment and monotherapy, adjusted for age and gender; the results of this analysis are presented as odds ratio and 95% confidence interval, with odds ratios indicating the relative increase of the probability of improvement in the outcomes associated with the combined treatment. A two-sided $p < 0.05$ was considered significant. Data were analysed with SPSS-PC 13.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Baseline characteristics

Forty out of the 48 children enrolled (83%) completed the study. Three cases were lost to follow-up, whereas 5 patients allocated to saline alone discontinued the treatment. Therefore, 22 subjects (males, 55%) from the sodium hyaluronate plus saline group and 18 children (males, 61%) from the saline alone group were included in the final analyses. Median age was 4 years (range, 2.3-5.8) for the sodium hyaluronate plus saline group and 3.6 years (range, 1.6-5.1) for the saline alone group ($p =$

0.7). During the 2 weeks preceding randomization, there was no significant difference between the two groups in rhinorrhoea severity score (42 vs 42, $p = 0.4$), number of rhinorrhoea-free days (0.5 vs 0, $p = 0.1$), prevalence of patients with at least one antibiotic course (27% vs 33%, $p = 0.7$) or who missed at least one daycare day (45% vs 50%, $p = 0.9$), prevalence of parents who missed at least one day from work (36% vs 33%, $p = 0.9$), and HR-QoL total score (3.7 vs 3.4, $p = 0.7$). At baseline, we did not find any difference between the two groups of patients in adenoid hypertrophy score (3 vs 3, $p = 0.8$), frequency of nasal turbinate hypertrophy (100% vs 100%) or of septal deviation (59% vs 67%, $p = 0.7$), and Lund-Kennedy score (6 vs 6, $p = 0.9$). None of the enrolled subjects in either group had nasal polyps.

Between-group assessment after treatment

After 2 weeks of treatment, patients assigned to the sodium hyaluronate plus saline group showed significantly lower Lund-Kennedy scores than children from the other group (2 vs 6, $p < 0.001$), while adenoid hypertrophy scores and frequency of nasal turbinate hypertrophy were comparable (2 vs 3, $p = 0.3$; and 86% vs 100%, $p = 0.2$, respectively). No statistically significant difference was found between the two groups in any clinical outcome, even though in the sodium hyaluronate plus saline group there was a trend towards a lower prevalence of parents who missed at least one day from work in the previous 2 weeks (5% vs 28%, $p = 0.07$). Nasal douche was well tolerated by all the enrolled subjects, and none of them experienced adverse events during treatment with either sodium hyaluronate plus saline or saline alone. All subjects had a good compliance to the treatment.

The multivariate logistic model showed a higher probability of improvement (i.e., an OR > 1) with sodium hyaluronate plus saline than with the saline alone for all variables (Fig.1). In particular, with regard to the adenoid hypertrophy and Lund-Kennedy scores, the combined treatment was associated with a probability of success more than 10 times greater than that observed in the other group ($p < 0.05$).

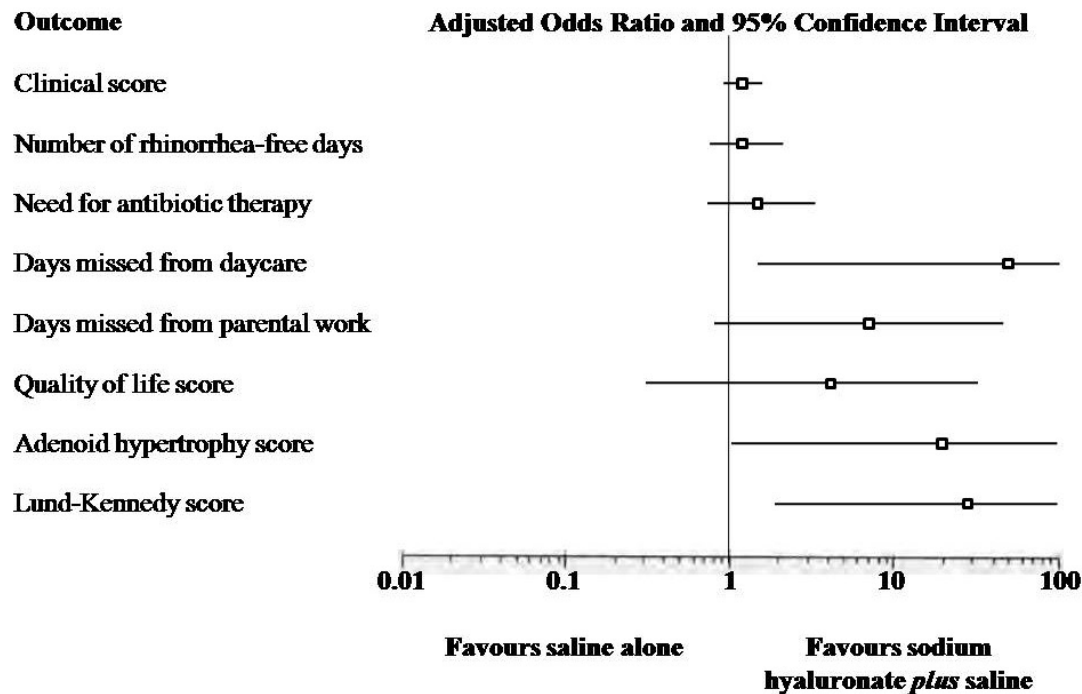


Fig. 1. Findings of the adjusted multivariate logistic analysis showing the odds ratio and 95% confidence interval for the examined clinical and endoscopic variables.

Intra-group longitudinal evaluation

Compared to baseline, at the end of the study there was a significant reduction of the prevalence of children with missed daycare days (45% vs 14%, $p = 0.04$) and of parents with workdays lost (36% vs 5%, $p = 0.02$) in the sodium hyaluronate plus saline group. Moreover, there was also a significant improvement of parental perception of HR-QoL total score (3.7 vs 2.8, $p = 0.004$). In particular, there was a significant difference in the scores of nasal infection (5 vs 4, $p = 0.04$), nasal obstruction (5 vs 4, $p = 0.001$) and emotional distress domains (4 vs 2, $p = 0.03$), while comparable scores for allergy symptoms (4 vs 4, $p = 0.3$) and activity limitations domains (2.5 vs 2, $p = 0.4$) were found. Furthermore, the Lund-Kennedy score significantly improved after treatment (6 vs 2, $p < 0.001$), and we observed a trend towards a reduction of the adenoid hypertrophy score after treatment that, however, did not reach statistical significance (3 vs 2, $p = 0.06$).

We did not find any significant effect on either clinical or endoscopy outcomes after treatment compared to baseline in the saline alone group.

DISCUSSION

This is one of the few studies ever performed to assess the efficacy of nasal washing with sodium hyaluronate on clinical and endoscopic outcomes, and the first report on its effects on HR-QoL in preschool recurrent URTIs. Compared to saline alone, sodium hyaluronate plus saline resulted in a significant reduction of the prevalence of subjects with missed daycare days and of parents with workdays lost, and in an improvement of nasal endoscopy scores, with a likelihood of success more than 10 times greater than controls, and of parental perception of HR-QoL.

The main finding from the current study was that nasal douches with sodium hyaluronate significantly improved the Lund-Kennedy score of rhinosinusitis. Bacteria have a number of mechanisms of adhesion to the human cells, including receptors that bind specific surface ligands (21, 22). Hyaluronic acid can interfere with this ligand-receptor interaction, thus blocking bacterial adhesion (7, 22). It has also a moderate

activity against bacterial biofilm, and consequently a preventive role against respiratory infections (22). Moreover, hyaluronic acid is crucial for airway epithelial integrity and protection against the effect of bacterial factors secreted during infection, and for the mucosal host defence as it stimulates ciliary beating and hence mucociliary clearance and retains and regulates enzymes important for homeostasis at the apical mucosal surface (4, 23).

Preschool children have many respiratory viral infections (1), and this may result in considerable medical expense and loss of daycare time which, from an economic perspective, may interfere with parental professional duties. Our study showed that, unlike the control group, parental perception of HR-QoL significantly improved after treatment with sodium hyaluronate, leading to a significant reduction of the number of workdays lost.

The current study has some limitations. The number of subjects recruited was limited and the period of treatment was short. Moreover, proxy respondents for the HR-QoL survey had to be used, because preschool children often do not volunteer or state their symptoms. However, parents are good surrogates for domains with directly observable behavioural consequences, such as physical suffering, symptoms and activity limitations (18).

In conclusion, this study provides further evidence in support of the effectiveness and safety of sodium hyaluronate administered by nasal douches in preschool children with recurrent or persistent URTIs. Further research on larger populations and on longer treatment periods is needed to confirm the role of sodium hyaluronate in the management of respiratory diseases in childhood.

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LETTER TO THE EDITOR

STUDY OF ORAL CAVITY LESIONS BY INFRARED SPECTROSCOPY

E. GIORGINI¹, C. CONTI², R. ROCCHETTI², C. RUBINI³, S. SABBATINI²,
V. LIBRANDO⁴ and G. TOSI²

¹Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona, Italy; ²Dipartimento di Scienze e Ingegneria della Materia, dell'Ambiente ed Urbanistica, Università Politecnica delle Marche, Ancona, Italy; ³Dipartimento di Scienze Biomediche e Sanità Pubblica, Università Politecnica delle Marche, Torrette (Ancona), Italy; ⁴Dipartimento di Scienze Chimiche, Università degli Studi di Catania, Catania, Italy

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Fourier transform infrared (FTIR) microspectroscopy is considered a useful tool in the biomedical field, for analysing in situ and at cellular level, very small areas of tissues and cells, with minimal sample preparation and without the use of stains or probes. This spectroscopic technique has been successfully applied to analyse biological samples from patients affected by tumoral pathologies, with particular attention to oral cavity lesions. In this study, we describe the application of FTIR microspectroscopy to characterize and discriminate the most recurrent benign and malignant diseases of oral cavity compartment. Infrared maps were acquired on tissues affected by the following pathologies: squamous cell carcinoma, adenoid cystic carcinoma, polymorphous low-grade adenocarcinoma, squamous dysplasia, keratocystic odontogenic tumor, radicular cyst, residual cyst, unicystic ameloblastoma, and ameloblastic fibroma, together with healthy tissue samples (used as control group). The epithelial and connective components of all samples were distinguished and submitted to multivariate analysis. The results were in agreement with histological suggestions.

A variety of experimental techniques are routinely applied in the biomedical field as analytical tools, to get quick information on several pathologies and to understand key changes in the progression of diseases (1). In this context, FTIR microspectroscopy may be considered a non-destructive, label free, highly sensitive and specific tool, which allows to improve knowledge in biomedical research, and, in particular, in cancer diagnosis (2-5). In fact, the whole data-set of information obtained by this technique, opportunely elaborated with advanced computational methods, such as multivariate and statistical analysis (6, 7), allows a

complete analysis of the biomolecular changes and mechanisms involved in specific pathological processes (8-10).

Vibrational spectroscopy studies the interactions of matter with the electromagnetic radiation. Fundamental vibrations, mainly stretching and bending of chemical bonds, can be detected within the middle infrared region MIR (4000-400 cm⁻¹), which includes all the absorption bands of the most relevant biomolecules and cellular metabolites (11-13).

The coupling between FTIR spectrometry and visible microscopy permit the mapping of very restricted

Key words: FTIR microspectroscopy, oral cavity lesions, pattern recognition analysis, multivariate analysis

Mailing address:

Dr Elisabetta Giorgini,
Dipartimento di Scienze della Vita e dell'Ambiente,
Università Politecnica delle Marche,
Via Breccie Bianche - 60100 Ancona, Italy
Tel.: +39 071 2204407 - Fax: +39 071 2204316
e-mail: e.giorgini@univpm.it

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regions of non-homogeneous biological samples, such as cells and tissues, detecting the vibrational motions of the most relevant biomolecules, such as proteins, lipids, carbohydrates and nucleic acids. Besides conventional light sources, synchrotron radiation is sometimes coupled to mono- or multidetector devices, to investigate many cellular processes at single cell levels (14, 15). At the same time, the innovative use of optical fiber systems could have the potential for an intra-operative application of vibrational spectroscopy, to know, in real time and without any contrast agent, the biochemical features of a tissue (16).

Oral mucous membranes and adjacent structures are composed of stratified squamous epithelium, joined by connective tissue to the *lamina propria* and to the submucosa. Squamous cell carcinoma (SCC) is the most recurrent neoplasia, which arises inside this epithelium (squamous metaplasia) with epithelial cells invading vicinal stromal regions (17). The lack of specific histopathological evidence and of a clear aetiology makes its pathogenesis quite complex. At present, despite the considerable improvements in the clinical treatment, it presents a survival rate of approximately 5 years. Other malignant neoplasia, such as adenocarcinoma, unicystic ameloblastoma and ameloblastic fibroma, are often found in the oral cavity compartment, together with some benign lesions. Due to the variety of these pathologies and the difficulty, in some cases, to make a clear and undoubtable diagnosis, it is mandatory to explore new analytical techniques. In this light, based on our recent application of FTIR microspectroscopy for the study of oral cavity diseases, we present an attempt to spectrally characterize and distinguish by using multivariate analysis, the most recurrent benign and malignant lesions of the oral cavity compartment (2, 3, 11).

MATERIALS AND METHODS

Thirty-one tissue samples were collected by surgical resection from the oral cavity of consenting patients and divided as follow: n. 4 G3 grade squamous cell carcinoma (SCC), n. 3 adenoid cystic carcinoma (AdCC), n. 3 polymorphous low-grade adenocarcinoma (PLGA), n. 4 squamous dysplasia (SD), n. 3 keratocystic odontogenic tumor (KOT), n. 3 radicular cyst (RC), n. 3 residual cyst (rc), n. 3 unicystic ameloblastoma (UA), and n.

3 ameloblastic fibroma (AF). N. 2 samples of healthy tissue were also collected and used as control group. This research was approved by the local Ethics Committee, and all the patients signed an informed consent to participate in the study. They received surgical treatment for therapeutic purposes, without any previous pharmaceutical therapy. A preliminary classification of all the samples was carried out according to World Health Organization guidelines, by comparing clinical, radiological and histological outcomes.

For each sample, two adjacent sections (5-7 μm thick), including both the epithelial and connective components, were cryosectioned: the first sections were deposited onto glass slides for histological examination [Haematoxylin and Eosin staining (H&E)], and the second ones were deposited without any fixation process onto CaF_2 optical windows (1 mm thick) and air-dried for 30 min, for infrared analysis (2).

FTIR measurements were carried out by using a Perkin Elmer FTIR Spectrometer Spectrum GX1 interfaced with a Perkin Elmer Autoimage microscope (6X condenser/objective) and equipped with a photoconductive 0.25 mm HgCdTe (MCT) array detector operating at liquid nitrogen temperature, which covers the entire IR spectral range from 4000 to 700 cm^{-1} .

For all the sections, by means of a microscope-television camera, the microphotographs of specific areas were obtained, on which the IR maps, representing the total intensity of the infrared absorption, were acquired in transmission mode and at room temperature (spectral resolution 4 cm^{-1} and spatial resolution 30x30 μm^2) (Spectrum Autoimage 5.1.0 software package, Perkin Elmer). Each spectrum, which corresponded to a single pixel on the maps, was the result of 256 scans. All the spectra were pre-processed to correct the atmospheric contributions of carbon dioxide and water vapour. Background scans were acquired on clean portions of the CaF_2 windows and ratioed against the sample spectrum.

To discriminate the spectra corresponding to different epithelia and connective components, unsupervised hierarchical cluster analysis (UHCA) was applied to all the spectra of each IR map, appositely pre-processed as follow: interpolated in the spectral range 1800-900 cm^{-1} , and second derivative transformed (DII, 9-points smoothing). The Euclidean distance and the Ward's algorithm were used in order to group spectra according

to their similarity (Pyrouette 4.5 software package, Infometrix Corp.). The obtained clusters were localized on the sections by using a topographical method, and their position was cross-validated by the histological examination of the corresponding H&E sections. The spectra of all epithelia-belonging pixels were resubmitted to HCA and PCA analysis (second derivative transformed, 9-points smoothing, spectral ranges 1800-900 and 1350-900 cm^{-1}). The same procedure was adopted for the spectra of connective clusters.

For each kind of epithelium and connective, the average spectra were obtained (two point baseline linear fitted, spectral range 4000-700 cm^{-1}) (Spectrum 10.4 software package, Perkin Elmer). When necessary, Second Derivative (9-points smoothing) and Peak Fitting (Gaussian character) procedures were adopted to isolate meaningful component bands (Grams AI 9.1 software package, Galactic Corp.). Attribution of the bands was carried out according to literature (18, 19).

RESULTS

In Fig. 1, IR absorbance spectra corresponding to healthy epithelium (HE) and connective (HC), with the spectral regions related to lipids, proteins, phosphates and sugars, were reported as an example.

Due to the epithelial nature of some of the studied

lesions, all spectra of the various epithelia were analyzed together by using multivariate analysis, to differentiate between normal and diseased specimens and, moreover, to find possible similarities among the various pathologies. The same procedure was carried out on the corresponding connective tissues (20).

Epithelia spectra

Firstly, the HCA and PCA analysis of all the epithelia-belonging spectra were carried out in the spectral range 1800-900 cm^{-1} (Fig. 2A, B). At a first observation, a satisfactory segregation was obtained for the highly malignant lesions SCC, KOT and AdCC. On the other hand, the following cluster superimpositions were observed: the SD with the AF, and the PLGA with the RC (Fig. 2A).

By limiting the analysis to benign and low malignancy grade lesions in the region of phosphates and carbohydrates (1350-900 cm^{-1}), a better segregation was obtained, even if the UA was split into three different clusters (Fig. 2C, D).

Connective spectra

The IR spectra of connective tissues involved in the above-cited lesions were analyzed by HCA (and PCA, not shown), resulting in a better segregation with respect to epithelia (Fig. 3).

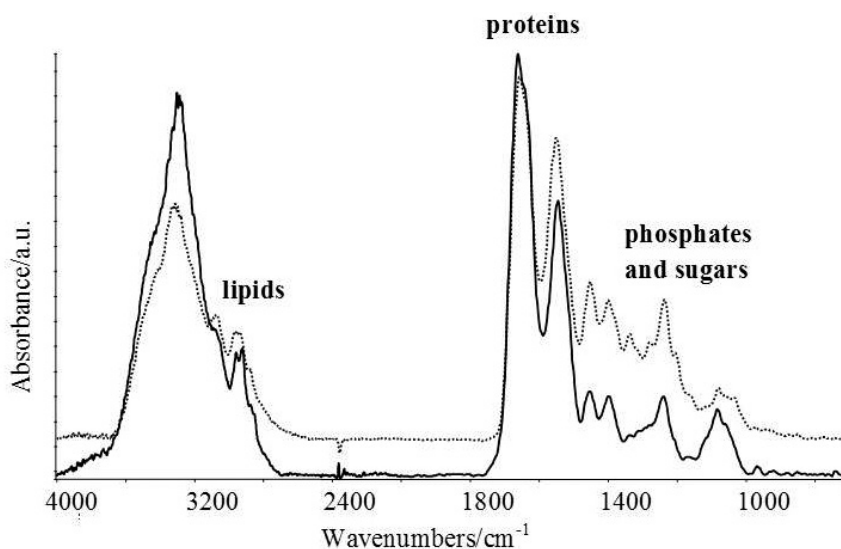


Fig. 1. IR absorbance spectra of healthy epithelium (continuous line) and connective tissue (dotted line) in the spectral range 4000-700 cm^{-1} . The spectral regions corresponding to lipids, proteins, phosphates and sugars are indicated.

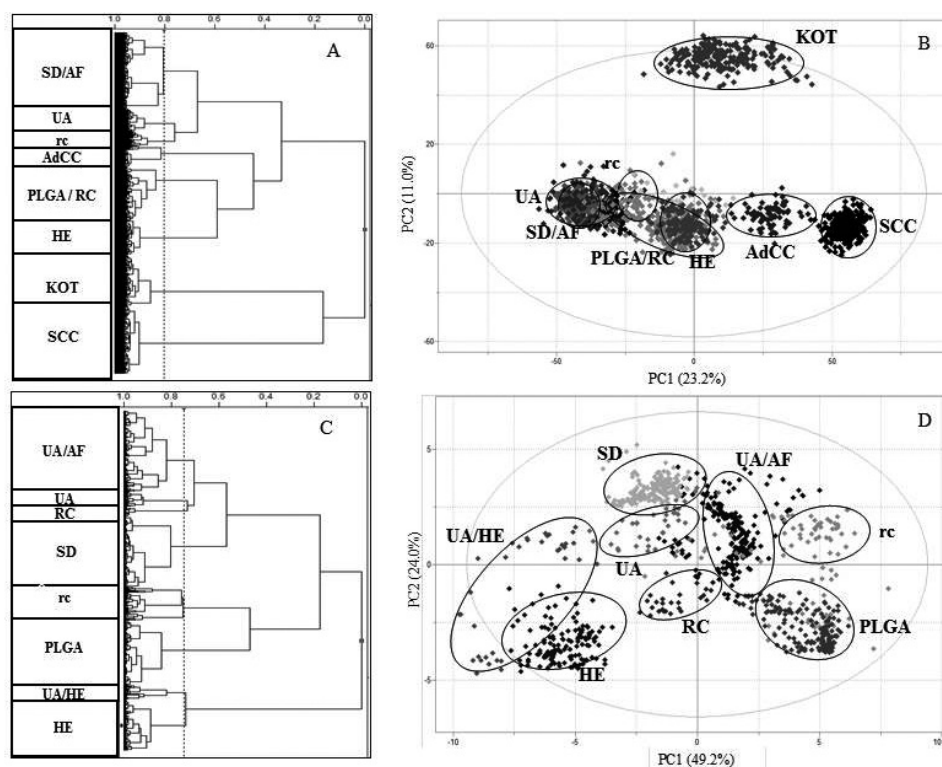


Fig. 2. Multivariate analysis of IR epithelia-belonging spectra: (A) HCA, and (B) PCA in the spectral range 1800-900 cm^{-1} ; (C) HCA, and (D) PCA in the spectral range 1350-900 cm^{-1} . The clusters corresponded to: healthy epithelium (HE); squamous cell carcinoma (SCC); adenoid cystic carcinoma (AdCC); polymorphous low-grade adenocarcinoma (PLGA); squamous dysplasia (SD); keratocystic odontogenic tumor (KOT); radicular cyst (RC); residual cyst (rc); unicystic ameloblastoma (UA); and ameloblastic fibroma (AF).

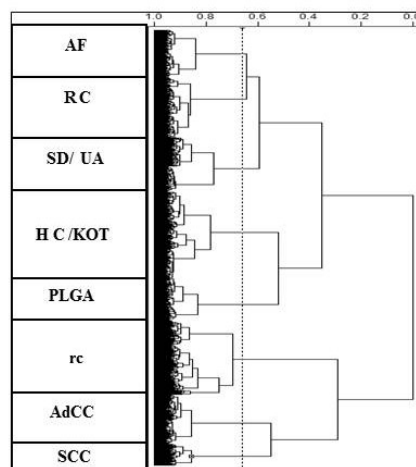


Fig. 3. HCA analysis of IR spectra (spectral range 1800-900 cm^{-1}) corresponding to connective tissues close to the following lesions: squamous cell carcinoma (SCC); adenoid cystic carcinoma (AdCC); polymorphous low-grade adenocarcinoma (PLGA); squamous dysplasia (SD); keratocystic odontogenic tumor (KOT); radicular cyst (RC); residual cyst (rc); unicystic ameloblastoma (UA); ameloblastic fibroma (AF); and healthy connective (HC).

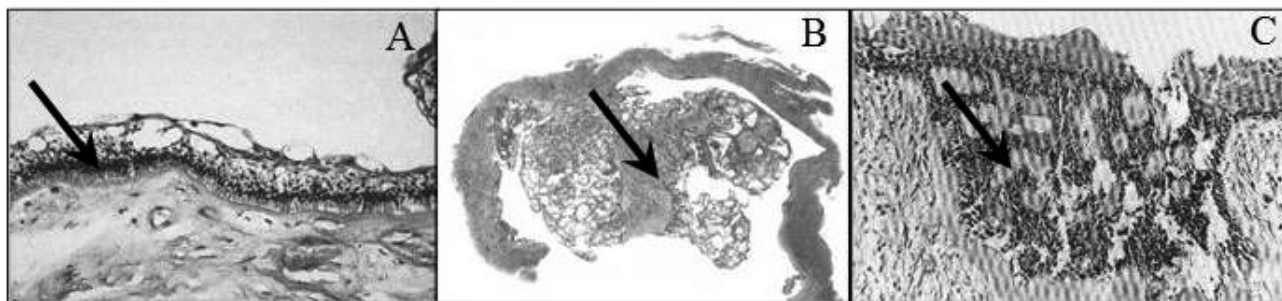


Fig. 4. Microscopic features of the unicystic ameloblastoma (UA): (A) ameloblast-like cells in the basal layer of the cyst; (B) a nodular tumour inside the cyst cavity (unicystic plexiform ameloblastoma); and (C) a thickened coating inside the capsular tissue (mural unicystic ameloblastoma). The black arrows indicate the above cited areas.

DISCUSSION

IR spectra of epithelia and connective tissues of the analysed specimens resulted almost different, the latter showing some typical bands usually referred to collagen, such as the band at 1317 cm^{-1} and the typical tricuspid shape band with maxima absorptions at 1204 , 1240 and 1280 cm^{-1} (20).

The HCA and PCA analysis of epithelia-belonging spectra showed a satisfactory segregation of the clusters corresponding to highly malignant lesions SCC, KOT and AdCC. The superimposition of the SD with the AF, and of the PLGA with the RC resulted in agreement with pathologist's remarks. In fact, from a morphological point of view, the SD and the AF should derive from analogous neoplastic epithelia, the latter being composed by an odontogenic epithelium, similar to the squamous epithelium of the dysplastic one. At the same time, mucous cells are usually present both in the PLGA and in the RC. The splitting of the UA into three different clusters, shown in Fig. 2 C, D, could be ascribed to the different ways of growth of this epithelium: surrounding the lumen (as in healthy samples), expanding itself as an intraluminal plexiform proliferation in the connective tissue (like in AF) and infiltrating the stroma of the cyst wall (Fig. 4).

According to HCA and PCA of connective-belonging spectra, the superimposition of the SD with the UA, and of the KOT with HC spectra, can be conceivable with the fact that connective tissue is not tumoral in nature, but it can be altered by the tumor itself.

FTIR microspectroscopy can result a valid complementary tool in clinical diagnosis and prognosis to study inflammatory and cancer lesions. In fact,

it is able to detect even early biochemical changes before morphological modifications can be seen under light microscope. The treatment of IR data-set with multivariate analysis is mandatory to afford a quick understanding of the biochemical features of the different compartments of oral cavity from mouth to salivary glands.

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ERRATA CORRIGE

Vol. 29 No. 4, page 969: the author D. Cairolì should read D. Carioli.
The Authors apologise for any inconvenience caused.