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## EDITORIAL

**WIDESPREAD MEMBRANE POTENTIAL CHANGES AND CARDIORESPIRATORY SYNCHRONIZATION INVOLVED IN ANXIETY AND SLEEP-WAKE TRANSITIONS**R. JERATH<sup>1</sup>, S.M. CEARLEY<sup>1</sup> and M. JENSEN<sup>2</sup><sup>1</sup>*Charitable Medical Healthcare Foundation, Augusta, GA, USA;*<sup>2</sup>*Georgia Regents University, Augusta, GA, USA**Received December 12, 2015 - Accepted August 4, 2016*

Located within the ascending reticular activating system are nuclei which release neurotransmitters such as acetylcholine, serotonin, dopamine, and norepinephrine. These nuclei have widespread projections that extend into the limbic system and throughout cortex. Activation of these neurotransmitters during awake states leads to arousal, while inhibition leads to the loss of consciousness experienced during slow-wave sleep. Previously, we proposed a mechanism in which cardiorespiratory synchronization may underlie the widespread hyperpolarization that occurs throughout the brain during slow-wave sleep. We further propose that a similar homeostatic mechanism may be involved in sleep-wake transitions and maintaining various arousal states including rapid eye movement sleep, waking, and anxiety. Widespread depolarization associated with more rapid, shallow breathing and desynchronized cardiorespiratory oscillatory activity may underlie waking, anxiety, and rapid eye movement sleep states. The exact voltage values of these widespread membrane potential changes remain unknown and possibly highly variable between different neural areas and cell types. Here, we place these consciousness states on a spectrum of approximated widespread membrane potential values with anxiety states being the most depolarized, followed by waking states, and rapid eye movement sleep. We propose that although these widespread membrane potential changes are minor, they may underlie transitions between and maintenance of varying levels of arousal. Further research on these mechanisms could provide insights into how the brain functions. This homeodynamic arousal mechanism involves the established feed-forward and feedback signaling between the ascending reticular activating system and the hypothalamus, as well as the modulation by cardiorespiratory oscillatory feedback from the body. Understanding the basic mechanisms responsible for the states of sleep, waking, and anxiety could lead to better treatment options in health and disease.

Although extant knowledge about the ascending reticular activating system (ARAS) provides evidence of its involvement in regulating sleep and wake cycles, exactly how these processes work is ambiguous. In this paper, we further expand on our previously proposed model of cardiorespiratory synchronization, and propose that high levels of

cardiorespiratory synchronization can modulate various brainstem nuclei and lead to the widespread hyperpolarization that occurs throughout the brain during slow-wave sleep. We apply this theory to other states of consciousness including rapid eye movement (REM) sleep, waking, and anxiety. We suggest that widespread depolarization may underlie

*Key words: anxiety, ARAS, cardiorespiratory synchronization, consciousness, membrane potential changes, sleep*

*Mailing address:*

Ravinder Jerath, M.D.  
Charitable Medical Healthcare Foundation,  
2100 Central Ave, Suite #7  
Augusta, GA, USA 30904  
e-mail: rj605r@aol.com

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various degrees of rapid and shallow breathing and decreased levels of cardiorespiratory synchronization that occur during states of REM sleep, waking, and anxiety. These consciousness states may be better understood on a spectrum ranging from hyperpolarization and depolarization of neuronal membrane potential. This spectrum places slow-wave sleep on the more hyperpolarized end of the spectrum, and places REM sleep, waking, and anxiety on the more depolarized end of the spectrum. We propose that widespread homeodynamic membrane potential changes may correspond with, and could likely underlie the various states and associated neural activity of the aforementioned states of consciousness.

#### *Ascending reticular activating system*

The ARAS consists of a group of nuclei in the brainstem (such as the locus coeruleus, raphe nuclei, pedunculopontine nucleus (PPN), and parabrachial nucleus; as well as various thalamic nuclei, the hypothalamus, and basal forebrain) (1) responsible for transitions in waking and sleeping. Consciousness is a result from activation of the ARAS and the subsequent changes in the brainstem and cortex (2). This system connects the brainstem and the cortex and extends from the pontine reticular formation through the mesencephalic tegmentum and ends in the intralaminar nuclei of the thalamus (3).

The ARAS and brainstem are made up of various nuclei that are involved in neurotransmitter systems that spread throughout the brain (Fig. 1). The release of neurotransmitters by these nuclei can launch rapid and widespread changes throughout the brain. The cardiovascular and respiratory centers are also located in the medulla adjacent to these other nuclei. The respiratory center consists of the nucleus of the solitary tract, nucleus ambiguus, and other pons nuclei. Neurons from the nucleus of the solitary tract innervate the reticular formation, hypothalamus, thalamus, limbic system, and other areas throughout the brain (4).

#### *Neurotransmitters and the sleep-wake cycle*

Changes in the sleep-wake cycle are initiated, maintained, and regulated by neurotransmitters

(produced by neurons) and located within the brainstem and hypothalamus (5) (Fig. 2). Acetylcholine (ACh) is released by the central nervous system and has both excitatory and inhibitory effects (6), and levels are higher during REM sleep (7).

Gamma-aminobutyric acid (GABA) in the “pontine reticular formation can promote wakefulness and inhibit REM sleep” (7), as can serotonin and dopamine.

Norepinephrine is more active during wakefulness and less active during slow-wave sleep, and helps regulate REM sleep (8), but initiates deactivation of REM sleep when it is activated in the locus coeruleus (9). In response to stimuli, norepinephrine neurons in the locus coeruleus react either by: i) exhibiting moderate tonic firing and phasic firing during non-stressed waking states, or ii) exhibiting high tonic firing and phasic firing dysregulation during stress (10).

Analogously related, epinephrine is active during wakefulness, and elevated levels are associated with stress and the sympathetic response (11). Injection of epinephrine into the locus coeruleus results in fewer REM periods but an overall extension of the sleep cycle (12).

Lastly, orexin neurons are also important in sleep because they help to: i) modulate sleep-wake transitions; ii) transitions between the sleep states (13); and iii) innervate areas throughout arousal pathways (14).

Nuclei in the VLPO contain neurons that use GABA, and fire only during NREM and REM sleep, and vagal outflow to the heart and body is dominated by the parasympathetic system during NREM sleep, while sympathetic dominance occurs during REM (15). Furthermore, EEG evidence has shown that during slow-wave sleep, parasympathetic vagal activity of the heart and slow waves strongly interact (15).

#### *Cardiorespiratory synchronization and slow-wave sleep*

Most studies on the control of arousal and sleep-wake cycles focus on the role of efferent signaling from various areas of the brain, such as the



hypothalamus (Fig. 3). We recognize the important role of these brain structures in these sleep processes and transitions; however, we focus on the proposed modulation of these sleep processes via afferent feedback such as cardiorespiratory signaling to the brainstem, which is largely ignored. Brain structures such as the hypothalamus are also involved in modulating breathing (16) therefore influencing this afferent signaling, but detailed discussion of modulation through higher brain areas is beyond the scope of this paper.

Bartsch et al., found that the degree of cardiorespiratory phase synchronization (defined as consistency of number of heartbeats with breathing cycles during the same phases) increased by 400% during the transition from REM to deep sleep (17). There are also strong hyperpolarizing forces that lead to widespread inhibition throughout the brain within the cortex, thalamus, ARAS, hypothalamus, and amygdala that prevent external stimuli from reaching the cerebral cortex in order to provide the body with protection while sleeping (18).

This widespread hyperpolarization could result from modulation of brainstem nuclei by slow, deep breathing and high levels of cardiorespiratory synchronization (15), and could partly stem from hyperpolarizing currents originating from slowly adapting stretch receptors in the lungs (19), and widespread inhibition during prolonged inspirations of slow, deep breathing (20).

#### *Cardiorespiratory synchronization and widespread hyperpolarization*

We propose that widespread hyperpolarization may underlie the restorative properties of slow-wave sleep, as well as the propagation of delta waves, and functional disconnection of the thalamus and cortex that occur in slow-wave sleep. During slow-wave sleep, when the ARAS is inhibited (18), there is a decrease in the release of acetylcholine, norepinephrine, and serotonin (9). As a comparison, during REM sleep, the cholinergic nuclei from the pons-midbrain junction are active while serotonin is inactive. Furthermore, synchronous oscillations within spindle and delta frequencies takes place

when thalamic neurons hyperpolarize during the onset of sleep, and shift to burst firing mode (21).

During slow-wave sleep the thalamus transitions from tonic firing to burst firing mode synchronizing with the cortex. During awake states, tonic firing from the thalamus transmits information to the cortex (22). This rhythmic bursting is thought to play an important role in promoting the slow-waves observed during slow-wave sleep (23).

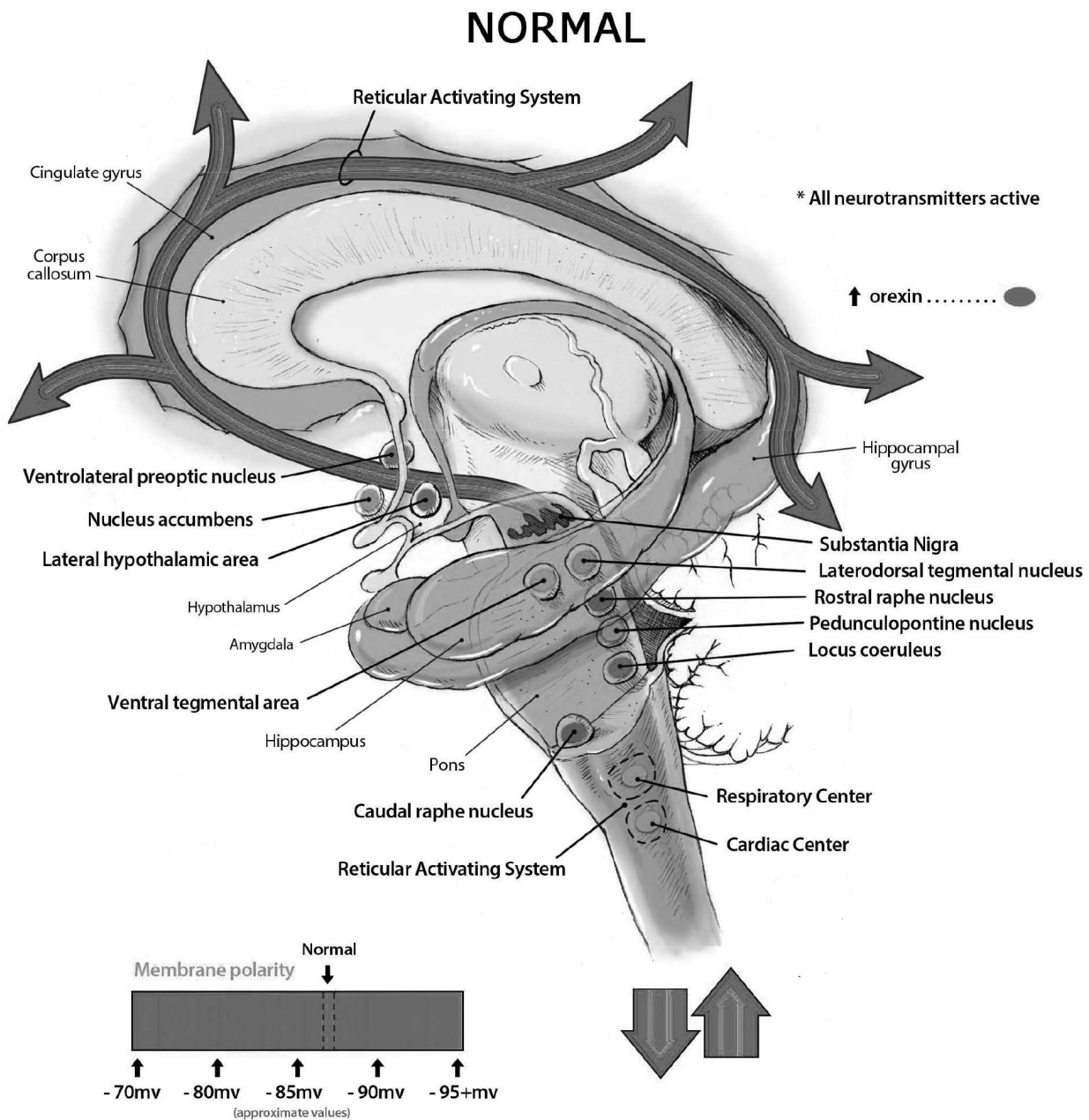
#### *Widespread depolarization and REM*

REM sleep is characterized by increased parasympathetic activity and variable sympathetic activity associated with an increased activation of certain brain functions. During REM sleep there is widespread depolarization throughout the brain. The ARAS depolarizes (24) and activates other brain regions such as the thalamus (25). The beta-gamma band activity may be generated by the membrane potential responsiveness of PPN neurons that fire at gamma band frequency when depolarized during waking and REM states (26), and hyperpolarization of these neurons via GABAergic inhibition may promote slow-wave sleep. There is also increased activity in the limbic system and cardiac and respiratory centers of the brainstem (27).

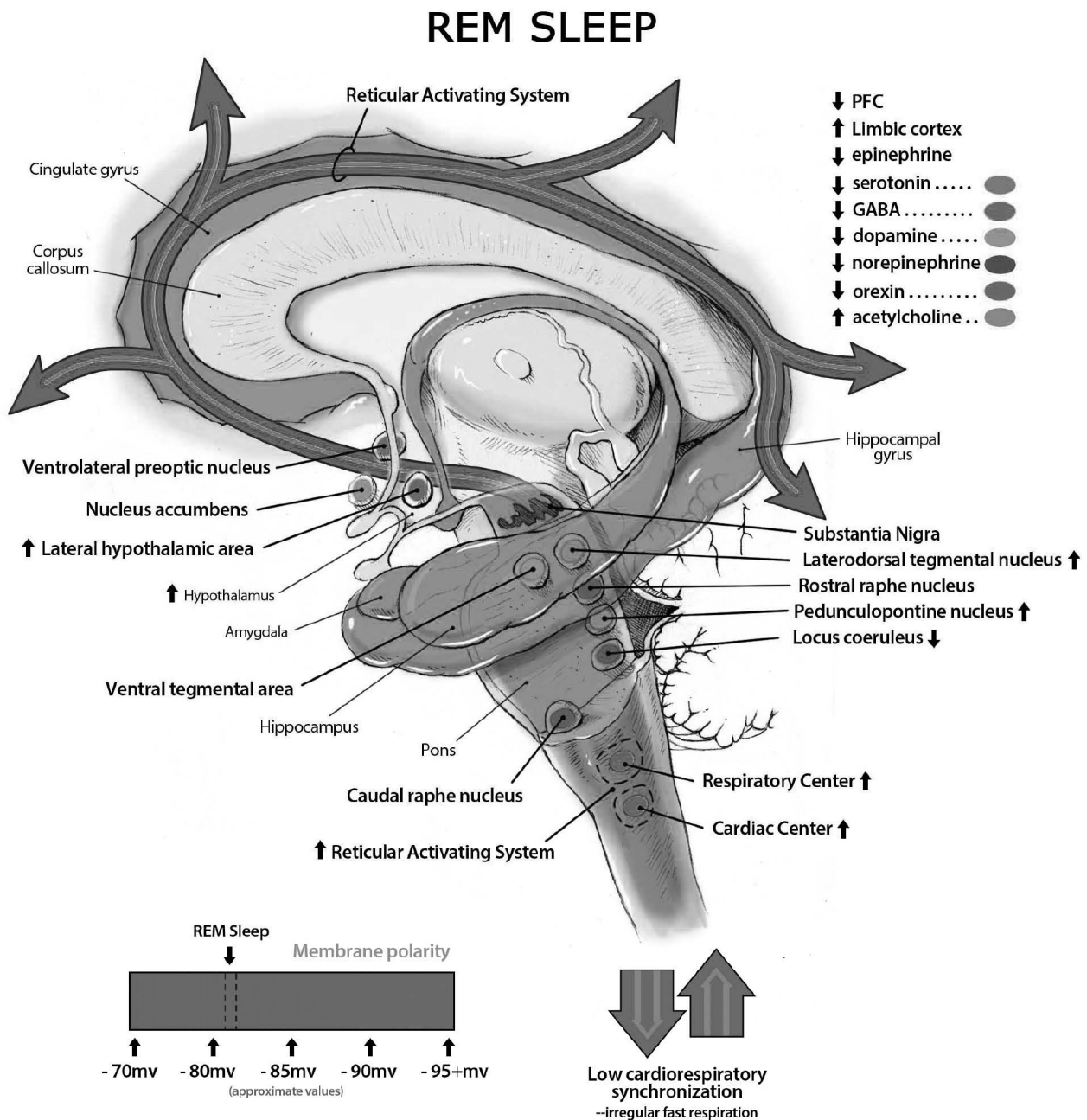
During REM sleep cardiorespiratory synchronization decreases and breathing becomes more shallow and rapid than in slow-wave sleep (28). We propose that REM sleep is initiated when limbic and amygdala activity synchronize with the cardiorespiratory system, leading to widespread depolarization within the ARAS via a cholinergic system. This leads to activation of visual and parietal responses without the activation of certain waking cognitive areas and processes. We suggest that the hypothalamus is highly involved in maintaining normal REM and slow-wave sleep by increasing the frequency and depth of breathing—which in turn leads to widespread modulation of resting membrane potentials throughout the brain—and modulates shifts in sleep cycle phases.

#### *Waking and paradoxical sleep*

Waking and REM sleep states are characterized

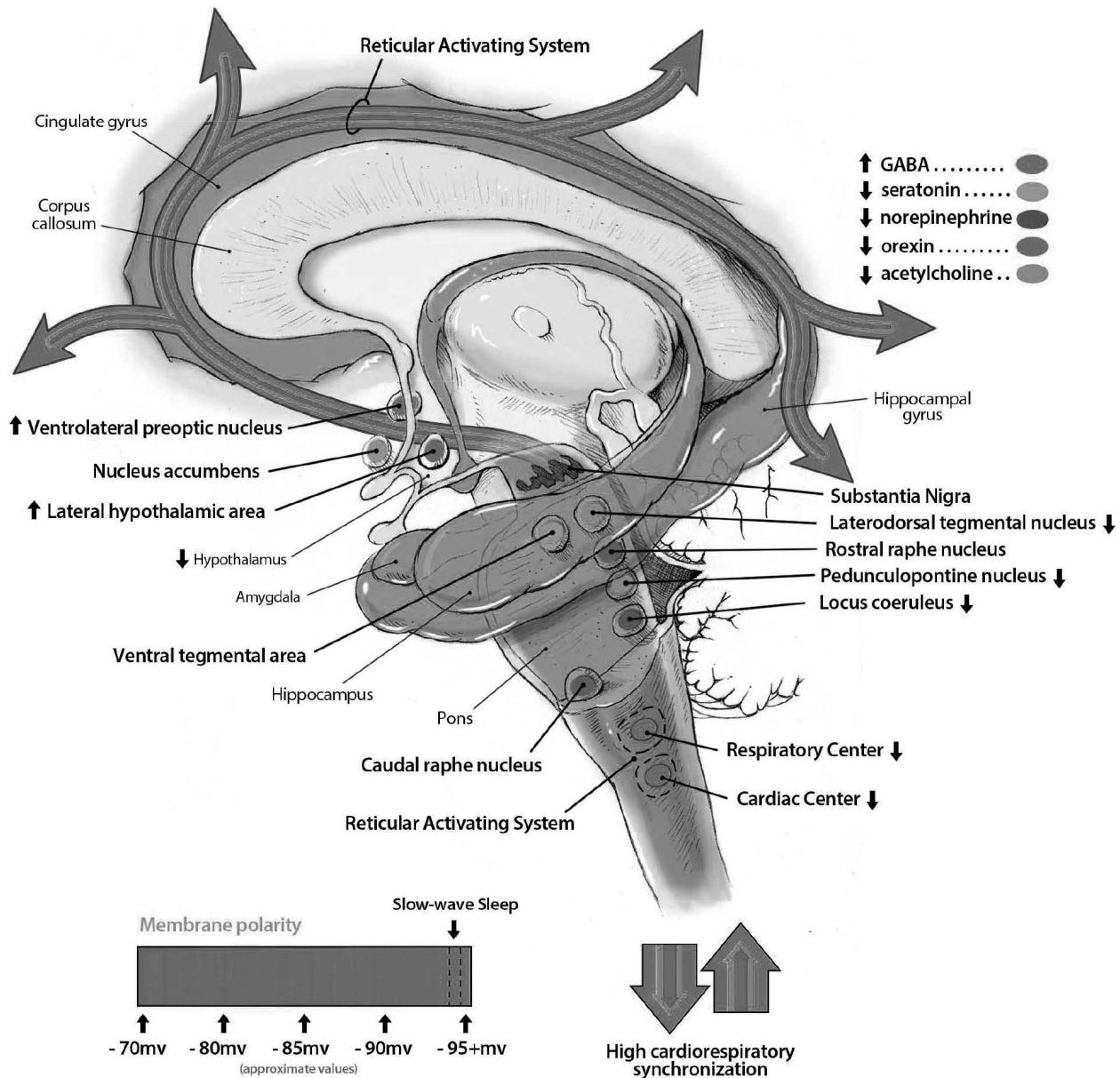


**Fig. 1.** Ascending Reticular Activating System Activity in Normal States. During normal states, there are average levels of cardiorespiratory synchronization and respiratory and heart rates. There is increased orexin activity and all other neurotransmitters are active. The cardiorespiratory and brainstem activity exhibited during awake states likely results in a normal baseline membrane polarity throughout the ARAS, limbic system, and the rest of the brain which can be compared to other consciousness states. (Approximate membrane potential values shown are used to demonstrate the degree of the shift in membrane potentials between states. Actual membrane potential values likely vary amongst different cell types.)



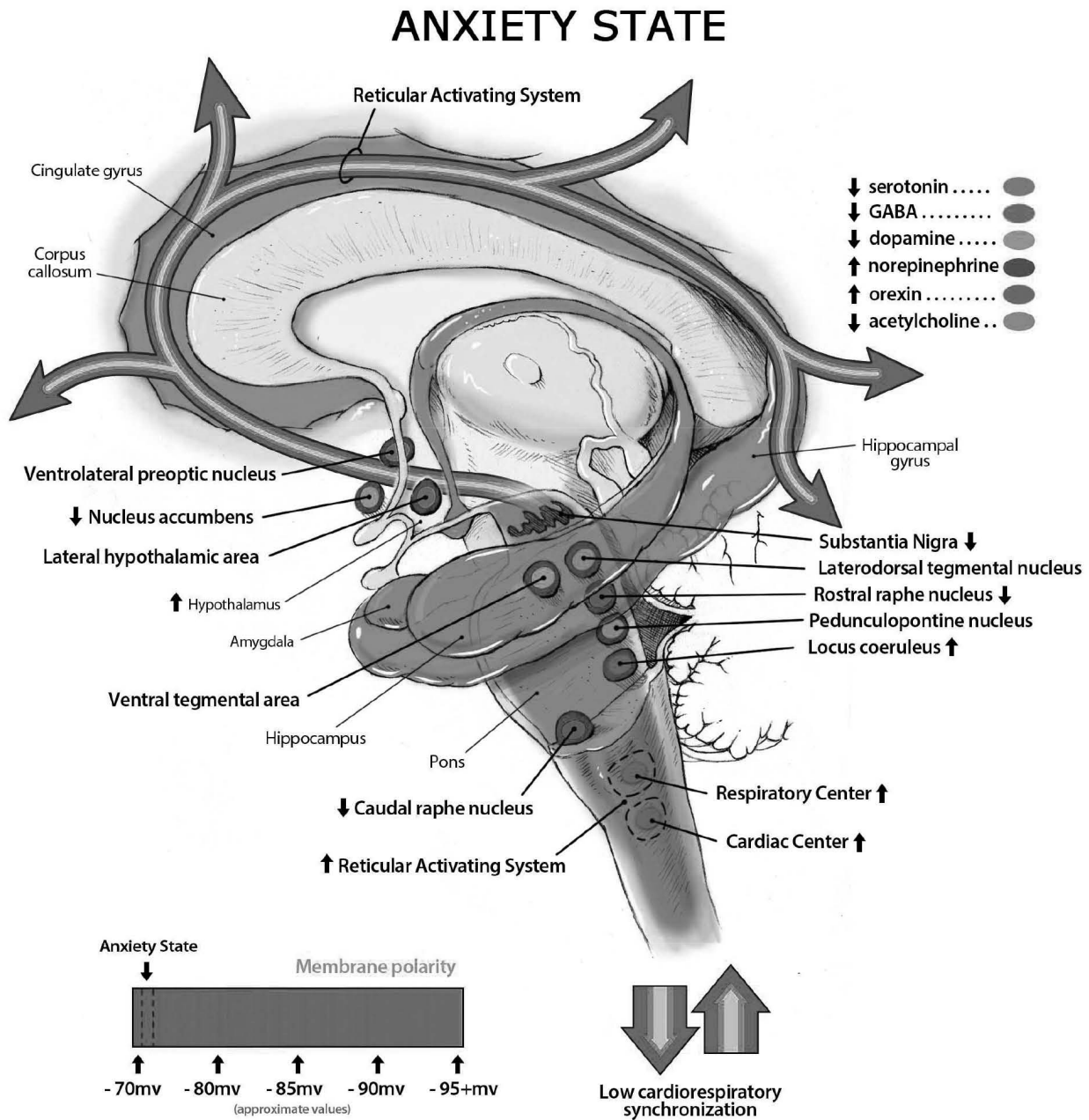
**Fig. 2.** Ascending Reticular Activating System Activity in REM Sleep States. The light coloring of the brainstem and ARAS illustrate the relatively depolarized state exhibited during REM sleep. REM sleep states exhibit widespread depolarization but are slightly less depolarized than anxiety states (approximately -80 mV). During REM sleep, levels of cardiorespiratory synchronization are low and respiratory and heart rates are high and more chaotic. During this state there is increased activity in the limbic system but the prefrontal cortex remains deactivated. There are decreased levels of most neurotransmitters; however, there is acetylcholine activation. Low levels of cardiorespiratory synchronization and fast, irregular breathing may underlie the widespread depolarization throughout the ARAS, limbic system, and some other areas of the brain during REM sleep. (Approximate membrane potential values shown are used to demonstrate the degree of the shift in membrane potentials between states. Actual membrane potential values likely vary amongst different cell types.)

## SLOW-WAVE SLEEP



**Fig. 3.** Ascending Reticular Activating System Activity in Slow-Wave Sleep. The light coloring of the brainstem and reticular activating system illustrate the widespread hyperpolarization that occurs throughout the brain during slow-wave sleep (at approximately -95 mV). The highest levels of cardiorespiratory synchronization are experienced during slow-wave sleep and respiratory and heart rates are very low during this state. This leads to increased levels of GABA and decreased levels of norepinephrine, orexin, serotonin, and acetylcholine. These changes may underlie the widespread hyperpolarization that occurs throughout the ARAS, limbic system, and cortex during slow-wave sleep. (Approximate membrane potential values shown are used to demonstrate the degree of the shift in membrane potentials between states. Actual membrane potential values likely vary amongst different cell types.)





**Fig. 4.** Ascending Reticular Activating System Activity in Anxiety States. The dark coloring of the brainstem and ascending reticular activating system illustrate the relatively depolarized state exhibited during anxiety states. Anxiety states lie on the farthest end of the membrane polarity spectrum and exhibit the greatest depolarization of all states described (at approximately -70 mV). During anxiety states, levels of cardiorespiratory synchronization are low and respiratory and heart rates are higher and more chaotic. This leads to increased levels of norepinephrine and orexin while levels of serotonin, GABA, dopamine, and acetylcholine decrease. This may result in widespread depolarization throughout the ascending reticular activating system, limbic system, and rest of the brain. (Approximate membrane potential values shown are used to demonstrate the degree of the shift in membrane potentials between states. Actual membrane potential values likely vary amongst different cell types.)

by high frequency beta-gamma band activity, and are modulated by part of the ARAS called the pedunculopontine nucleus (PPN) (26). Recent studies have shown that cholinergic neurons: i) synchronize with theta oscillations; ii) fire during active waking and paradoxical sleep, modulating cortical activation and plasticity; and iii) cease firing during slow-wave sleep (29). Furthermore, wakefulness and paradoxical sleep both show higher levels of high-frequency activity in areas of the thalamus and cortex than in slow-wave sleep. Wakefulness results from the modulation between monoaminergic and cholinergic cells, with thalamic and cortical neurons becoming hyperpolarized when these cells decrease their firing rates, eventually leading to slow-wave sleep.

However, paradoxical sleep results from an increase in firing rates of cholinergic neurons that release neurotransmitters in the thalamus and cortex, depolarizing them and interrupting slow-wave sleep to an almost awake state. It is possible that this state creates an altered perceptual experience due to a lack of full spatio-temporal binding, and when combined with levels of high-frequency activity in the medial pulvinar nucleus, creates dreams (30). Therefore, we suggest that vagal and cholinergic activity can lead to dreaming during paradoxical sleep because of synchronization between basal forebrain neurons bursting rates and theta oscillations during waking and paradoxical sleep.

#### *GABA and vagal innervation*

Several vital organs are innervated by the vagus nerve, which relays sensory, visceral, and physiological information to the central nervous system (31). Vagal nerves are the primary nerves for the parasympathetic system and project into the SA and AV nodes, and the cardiac muscle and the brain receives more vagal afferent information cardiovascular system than from any other organ (32). The cardiac vagal neurons modulate heart rate and production of respiratory sinus arrhythmia, and vagal activity can slow down or stop the heart (due to a decrease in spontaneous depolarization of SA and VA nodes upon release of Ach) (32).

GABA is the primary “inhibitory transmitter in central nervous system,” and nearly 20% of the brain’s neurons are GABAergic (33). During inspiration, GABAergic neurons in the ventral medulla receive bursts of excitatory neurotransmission (34). When respiration rates become fewer than 8.5 breaths per minute, during sighing, or during deep breaths, low frequency heart rhythm oscillations are generated by vagal activity, suggesting sympathetic activity and perhaps providing an assessment of sympathetic and parasympathetic system balance (32).

#### *Cardiorespiratory synchronization and anxiety*

We propose that cardiorespiratory synchronization is on the opposite end of the spectrum, and is the mechanism that underlies the symptoms of anxiety. Our theory suggests that widespread depolarization throughout the brain during REM sleep can also occur during times of stress and states of anxiety, caused by rapid and shallow breathing, along with low levels of cardiorespiratory synchronization. We propose that the rapid, shallow breathing that occurs in both REM (35) and anxiety states may underlie widespread neural depolarization that simultaneously occurs with hypothalamic modulation and neurotransmitter release in the ARAS. This process is homeodynamic, and overall changes occur through: i) feed-forward controls from the hypothalamus and ARAS; and ii) feedback controls from the cardiorespiratory system.

The autonomic nervous system and respiratory activity are interrelated, and are based on membrane potential changes (36). Anxiety states are associated with rapid breathing, while slow, deep breathing (*Pranayama* and meditation) has been shown to attenuate symptoms (36). It has been shown that meditation increases the parasympathetic component of heart rate variability (37), reflecting increased vagal activity. We suggest this indicates that stimulation of the vagus nerve via *Pranayama* and meditation techniques may lead to widespread neural inhibition via modulation of the ARAS, and increasing levels of inhibitory GABA.

We hypothesize that widespread depolarization of the ARAS activates the cholinergic system and the associated limbic and striate cortical activity and



subsequent dream sleep (Fig. 4). Stimulation of the cholinergic neurons of the pons-midbrain junction causes desynchronization of EEG oscillatory activity, and wakefulness while stimulation of the thalamus causes sleep (9). Therefore anxiety states and REM sleep states likely exhibit more widespread neural depolarization, while slow-wave sleep states exhibit more widespread hyperpolarization and inhibition via ARAS modulation and cardiorespiratory oscillatory activity.

### CONCLUSION

Previously we proposed that high levels of cardiorespiratory synchronization influence brainstem nuclei and lead to the widespread hyperpolarization that occurs throughout the brain during slow-wave sleep. In this paper, we have further expanded on this model to explain neural activity during other conscious states including REM sleep, normal awake states, and anxiety. Furthermore, more shallow, rapid breathing and unsynchronized cardiorespiratory oscillations may lead to the slight but widespread depolarization that occurs during REM sleep, waking, and anxiety states, with REM states being the most depolarized, followed by anxiety and waking states. Further study of these possible homeodynamic neural membrane potential changes and their role in maintaining states of consciousness and transitions between states would allow for a better understanding of widespread neural processes, as well as how the brain functions as a whole.

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## EDITORIAL

# MAST CELL AND CANCER WITH SPECIAL EMPHASIS ON IL-37 AN ANTI-INFLAMMATORY AND INHIBITOR OF INNATE IMMUNITY: NEW FRONTIERS

F. CARINCI<sup>1</sup>, G. LESSIANI<sup>2</sup>, E. SPINAS<sup>3</sup>, S.K. KRITAS<sup>4</sup>, G. RONCONI<sup>5</sup>, AI. CARAFFA<sup>6</sup>  
and P. CONTI<sup>7</sup>

<sup>1</sup>*Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy;* <sup>2</sup>*Center of Intensive Rehabilitation, "S. Agnese", Pineto (TE), Italy;* <sup>3</sup>*Department of Surgery and Odontostomatological Sciences, University of Cagliari, Italy;* <sup>4</sup>*Department of Microbiology and Infectious Diseases, School of Veterinary Medicine, Aristotle University of Thessaloniki, Macedonia, Greece;* <sup>5</sup>*UOS Clinica dei Pazienti del Territorio, Policlinico Gemelli, Roma, Italy;* <sup>6</sup>*Department of Pharmacology, University of Perugia, Perugia, Italy;* <sup>7</sup>*Immunology Division, Postgraduate Medical School, University of Chieti-Pescara, Chieti, Italy*

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**Mast cells (MCs) are mediators of allergy and inflammation and participate in the growth of cancer cells. MCs can promote both neoangiogenesis and tumor growth. They increase in the stroma of certain tumors where they can be recruited by tumor-derived chemoattractants, such as monocyte chemoattractant protein-1 (MCP-1), RANTES and stem cell factor (SCF) to selectively secrete inflammatory molecules including chemical mediators and cytokines (TNF, IL-6 and IL-1). However, MC differentiation pathways and heterogeneity in cancer are still poorly understood. Human interleukin 1 (IL-1) plays a pivotal role in the pathogenesis of many diseases and functions, including host response to microbial invasion, injury inflammatory processes, immunologic challenges and cancer. Inflammation around the tumor includes the infiltration of mast cells and facilitates cancer growth. MCs are activated by IL-1 which can be produced by certain cancer cells and stimulate the stromal cells to selectively release IL-6, contributing to the development of Th-17 cells and increasing inflammation. IL-37, mainly generated by macrophage cell line, is an IL-1 family member which binds IL-18 receptor  $\alpha$  (IL-18R $\alpha$ ) chain, and acts as a natural inhibitor of immune responses. IL-37 down-regulates cJun induced by IL-1, pro-inflammatory signals and reduces the expression of the mitogen-activated protein kinase (MAPK) p38 $\alpha$ , STAT transcription factors and p53, affecting cellular differentiation and proliferation. In the present study we report the relationship between inflammatory mast cells, cancer and the beneficial effect of IL-37.**

In over 25 years Charles A. Dinarello discovered several inflammatory and anti-inflammatory cytokines including IL-37 which abrogates pro-inflammatory cytokines and inhibits innate immunity (1-3). Traditional cancer therapies are known to cause

immunological complications and other side effects, therefore, new therapeutic approaches arouse great interest in researchers and the general population.

In 1996 we published a study regarding the role of IL-1 on mast cell (MC) degranulation cultured with

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*Mailing address:*

Prof. P. Conti,  
Immunology Division, Postgraduate Medical School,  
University of Chieti-Pescara, Chieti, Italy  
Tel./Fax: +39 0871 574136  
e-mail: pconti@unich.it

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a natural inhibitor of IL-1: interleukin-1 receptor antagonist (IL-1RA), describing an additional biological activity of IL-1 on histamine and 5HT released from a cancer cell line rat basophilic leukemia cells (RBLc) cultured *in vitro* (4). Over 12 years ago, we also reported that MCs could either promote or inhibit tumor growth and act as a new target for the adjuvant treatment of solid tumors through the selective inhibition of cancer-promoting molecules (5).

Today, in this article, we study the potential of inhibiting inflammatory cytokines with an anti-inflammatory cytokine, such as IL-37, contributing to therapy of neoplastic disorders.

Mast cells derived from a bone marrow precursor are primary effectors in allergic reactions and mediate innate and adaptive immunity (6). They are activated by binding to IgE cross-link FcεRI high affinity receptor ( $K_d = 10^{-10}$  M) and secrete numerous vasoactive, chemoattractant, and inflammatory molecules as well as growth factors, and participate in inflammation and tumor development (7-8) (Fig. 1). In addition, MCs can be activated through Toll Like Receptor (TLR), and TLR2 stimulation has been shown to induce MC degranulation. The TLR-dependent pathway engages mitogen-activated protein kinase (MAPK) s and IKK, resulting in activation of transcription factors such as AP-1 and NF-κB that participate in the induction of pro-inflammatory cytokines (9-10). Furthermore, enhancement of IgE production by antigen is abrogated in *Kit<sup>W-sh/W-sh</sup>* MC-deficient mice and restored by MC reconstitution (9). MC binding IgE lead to receptor aggregation with an intracellular signal initiating several phosphate transfer reactions through the activation of Src, Syk and Tec (tyrosine kinases proteins). The phosphorylation leads to the activation of phospholipase Cγ (PLCγ) which is an important mediator for the intracellular calcium release and protein kinase C (PKC) activity. The production of inositol triphosphate (IP3) activates GTPase which regulates MAPK, extracellular signal regulated kinase (ERK) and c-Jun N-terminal kinase (JNK). These reactions stimulate NF-κB and AP-1 which leads to the production of chemokines and cytokines. Inhibitors of FcεRI binding have been

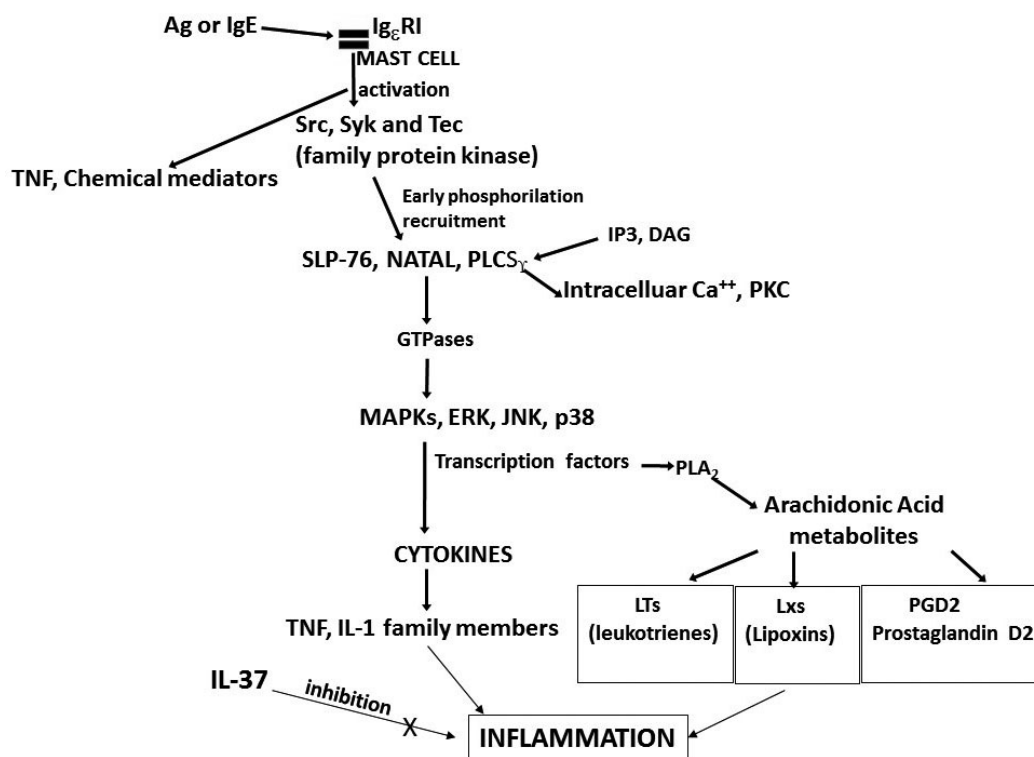
identified and an anti-IgE therapeutic antibody is used to treat severe allergic and inflammatory dysfunctions (9).

The basic secretagogues, such as substance P, LPS, toxins, IL-1, essentially activate the MCs *in vitro* and *in vivo* through a single receptor, MrgprB2, the orthologue of the human G-protein coupled receptor (GPCR) MrgprX2, causing the release of MC products such as histamine and anti-cancer TNF (11).

Cancer development cannot be induced in mast cell-deficient mice due to their positive effect on angiogenesis. Therefore, MCs are involved in cancer angiogenesis, and some MC-stabilizers may cause tumor hypoxia and tumor-cell apoptosis. These results suggest that MCs are strongly involved in cancer development and they are essential for tumor growth. In addition, some other mast-cell mediators, such as VEGF, heparin, and PDGF, may promote tumor growth.

Nowadays, it does not appear that there are effective blockers to prevent degranulation and activation of MCs and eventually their new inhibitor mediators would be important. The discovery that IL-37 blocks innate immunity may also have an inhibitory effect on mast cells mediating the development of cancer (12). We now know that inflammatory immune cells which serve to exacerbate the malignant disease are attracted by chemokines at the site of inflammation. In particular, mast cells are attracted by the chemokine monocyte chemoattractant protein 1 (MCP-1/CCL2) and RANTES (13). This observation was reported by us many years ago and it showed that in mice the injection of MCP-1 and RANTES under the skin resulted in a recruitment of inflammatory MCs that mediate a severe inflammation. The injection of human MCP-1 or RANTES in genetically W/W<sup>v</sup> mast cell-deficient mice did not provoke any appreciable inflammatory reaction or mast cell extravasation, suggesting the specificity of these chemokines for mast cells (13). These studies provide direct *in vivo* evidence that hrRANTES has significant proinflammatory activity on mast cells and suggest that the antagonist(s) of MCP-1 and RANTES may have inhibitory biological effects on inflammatory conditions.





**Fig. 1.** Inhibitory effect of IL-37 on mast cell inducing inflammation.

MCs can be activated in response to inflammation, pathogens and trauma but only in the presence of interleukin-1 (IL-1) and tumor necrosis factor (TNF). Activated MCs lead to selective release of mast cell mediators (14).

The field of MC-oncology represents the intersection of cancer and allergic disease. MCs promote both neoangiogenesis and tumor growth; in addition, they mediate cancer cell development and metastases formation, through heparin-like molecules and cytokine generation. The formation of cancer cells leads to the accumulation of MCs that provoke inflammation and is associated with poor prognosis.

In cancer, the number of mast cells is increased in the stroma of tumors and may accumulate in reaction to the tumor. They can be recruited by tumor-derived chemoattractants, such as MCP-1, RANTES and SCF, to selectively secrete molecules beneficial to the tumor, such as growth factors, histamine (mitogenic H1 and an immunosuppressant H2), heparin (neovascularization agents), VEGF, IL-8,

and proteases, that favor new blood vessel formation and metastases. MC recruitment is implicated in the activation of the Myc oncogene protein in mice, which is a proteolytic transcription factor involved in tumor angiogenesis and cell proliferation, inducing a fast development of certain tumors (15). Myc activation induces MCP-1/CCL2 which chemoattract MCs.

MCs can secrete chemical inflammatory mediators and generate IL-1 and IL-6, which are pro-inflammatory compounds. IL-1 can induce selective secretion of IL-6 from human cultured mast cells without degranulation which induce development of T<sub>H</sub>17 cells, stimulate secretion of VEGF and promote angiogenesis and tumor growth. IL-1 produced by certain tumors stimulates the stromal cells to release IL-6 which promotes the survival, growth and expansion of the of tumor cells (16).

The possibility of early inhibited IL-1 in cancer has a significant impact on tumor inflammation and prognosis. Blocking IL-1 $\beta$  with IL-37 would reduce inflammation as well as IL-6 activity

which, in turn, is implicated in this process. MC-deficient W/W<sup>v</sup> mice show a decreased rate of tumor angiogenesis, demonstrating the importance of these cells and their mediators, such as TNF and VEGF, in cancer development. MCs are the only cell type in which preformed cytokine TNF is stored in secretory granules and can activate IL-1 and T cells participating in the immune response.

IL-1 requires proteolytic cleavage for its activation and release. The cysteine protease caspase 1 is cleaved and activated from its pro-form. Therefore, pro-IL-1 is cleaved by caspase 1 to generate the active form which is normally conserved within the cell, bound to the plasma membrane, or localized in the nucleus.

IL-1 family members share structural properties with the extracellular immunoglobulin-like domains and intracellular Toll-IL-1 receptor domain. The NF- $\kappa$ B, AP-1 transcription factors and MAPK pathways are activated by IL-1 ligand to a chain of the IL-1 receptor (IL-1R), an effect that can be inhibited by IL-37. IL-1 stimulates a variety of different cell types via specific receptors. IL-1R binds both IL-1 $\alpha$  and IL-1 $\beta$  and has structural homology with the immunoglobulins not directly involved in antigen recognition.

Targeting IL-1 has been considered as a therapeutic target for toxic shock, atopic rheumatic and autoimmune diseases, allergy, inflammation and cancer.

It has been reported that the IL-1 family includes 11 members and IL-37 is a new member that regulates innate and adaptive immune systems to promote anti-inflammatory response.

IL-37, formerly termed IL-1 family member 7 (IL-1F7), is an orphan member of the IL-1 ligand family and requires the receptor IL-18R $\alpha$  to inhibit innate immunity which exerts an anti-inflammatory activity. Innate cells, including airway epithelial cells, granulocytes, NK cells, T cells, macrophages, mast cells and dendritic cells, orchestrate allergy, inflammation and cancer. Therefore, IL-37 is a ligand of the IL-1 family which binds to IL-18-binding protein and possesses in general an anti-inflammatory activity and anti-innate immunity properties. IL-18R $\alpha$  is a ligand-binding chain required for the anti-inflammatory activity of IL-

37, and is needed to transmit the anti-inflammatory signal of IL-37. IL-18R $\alpha$ -deficient mice displayed constitutive NF $\kappa$ B and JNK activation. Blocking IL-1 $\beta$  would reduce inflammation and IL-6 activity which, in turn, promotes the survival and expansion of cancer cells (4).

The gene expression of pro-inflammatory molecules, along with co-stimulatory molecules, chemotactic factors, NF- $\kappa$ B signaling, MMP-13, G-CSF and TRAF1 are lowered by IL-37. This cytokine strongly inhibits LPS-induced expression of IL-1 $\alpha$ , IL-1 $\beta$ , TNF, IL-23, IL-17, IL-18, IFN- $\gamma$ , and CC chemokines including MCP-5/CCL12. Intracellular IL-37 inhibition of activated peripheral blood mononuclear cells (PBMC) increases the generation of inflammatory cytokines including IL-1, TNF, and IL-18, *in vitro*. IL-37 increases Treg and inhibits the capacity of antigen presenting cell (APC) to activate naïve T cells. In cancer and chronic inflammation, inflammatory cells and chemical mediators are present, contributing to the pathogenesis of the disease. Moreover, IL-37 results inactivated in cancer, an effect that provokes the loss of its inhibitory activity (17).

TLR and the receptors for IL-1 are important in inflammatory and cancerous processes and they are orchestrated by NF- $\kappa$ B, where IL-37 plays a protective role, in particular in different murine models of cancer (18). In spite of mTOR enhanced activation after IL-1 epithelial cell stimulation, IL-37 may act through the inhibition of TLR signaling. Therefore, IL-37 is implicated in an anti-cancer activity by suppressing IL-1, and TLR-induced mTOR mediate the development of the cell cycle. In addition, it has been reported that cancer tissues express lower levels of IL-37 compared with healthy tissues. Thus, increasing the levels of IL-37 in patients suffering from cancer disease may have a better prognosis.

In this regard, since we ignore the effects of IL-37 on other cell types and receptors, it would be interesting to know, in addition to the effects reported here, the effect of IL-37 on MC activation receptors in allergic and cancer diseases, its molecular mechanisms and side-effect(s), which are highly important.

In this article we review the relationship between

IL-37, mast cells and tumor cells and report that IL-37, in addition to having an impact on allergy and asthma, may also inhibit mast cell inflammation and cancer development by affecting innate and adaptive immunity.

IL-37 blocks mast cell activation and TH2 cell-directed allergic inflammatory response, suggesting that this anti-inflammatory cytokine may be critical for allergic inflammation. Therefore, IL-37 is a novel target for the pathogenesis and therapy of allergic disorders and cancer development, emerging as an inhibitor of innate and adaptive immunity.

Continued discoveries in the immunopathogenesis of inflammation, allergy and cancer provide optimism for the development of efficacious therapeutic immunological compounds (19). Thus IL-37, a novel immunomodulatory and therapeutic cytokine, can lead to a beneficial effect on the pathogenesis of cancer, allergy and inflammation. These studies would certainly provide clinical positive effects.

However, the management and the immunological treatment of cancer remain controversial.

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## DIFFERENTIAL EXPRESSION AND DISTRIBUTION OF CYTOKERATINS AND VIMENTIN IN BUCCAL POUCH MUCOSAL CELLS DURING REAL-TIME CELL PROLIFERATION: RESEARCH BASED ON A PORCINE MODEL

A. BRYJA<sup>1</sup>, M. DYSZKIEWICZ-KONWIŃSKA<sup>2</sup>, A. CHACHUŁA<sup>3</sup>, S. CIESIÓŁKA<sup>3</sup>,  
W. KRANC<sup>1</sup>, D. BUKOWSKA<sup>4</sup>, P. ANTOSIK<sup>4</sup>, M. BRUSKA<sup>1</sup>, M. NOWICKI<sup>3</sup>,  
M. ZABEL<sup>3,5</sup> and B. KEMPISTY<sup>1,3</sup>

<sup>1</sup>Department of Anatomy, Poznan University of Medical Sciences, Poznan, Poland; <sup>2</sup>Department of Biomaterials and Experimental Dentistry, Poznan University of Medical Sciences, Poznan, Poland;

<sup>3</sup>Department of Histology and Embryology, Poznan University of Medical Sciences, Poznan, Poland; <sup>4</sup>Institute of Veterinary Sciences, Poznan University of Life Sciences, Poznan, Poland;

<sup>5</sup>Department of Histology and Embryology, Wroclaw Medical University, Wroclaw, Poland

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In recent years, buccal pouch oral mucosa cells were used as a source of potential biological grafting material in advanced tissue engineering. However, there are several limitations in the process of graft fabrication: donor and recipient patient availability as well as an incomplete knowledge of *in vitro* procedures related to tissue surgical recovery, *in vitro* cell culture (IVC) and/or tissue processing in “human somatic cell therapy.” Therefore, the animal model for oral mucosa grafting is still recognized as a source for xenografts and a useful model for biomedical research. In this study, the porcine buccal pouch oral mucosa cells were used in analysis of the stromalization/epithelialization process during short-term, *in vitro* real-time cell proliferation. We evaluated cytokeratin 18 (CK18), cytokeratin 8 + 18 + 19 (panCK), and vimentin (Vim) expression as epithelial and stromal cell markers, respectively. The porcine buccal pouch oral mucosa cells were cultured *in vitro* for 168 h, and the protein expression/distribution was analyzed every 24 h during real-time cell proliferation. In our analysis of protein expression using fluorescence intensity (FI), followed by confocal microscopic observations, we found the highest expression of CK18 occurred after 24 h of IVC, panCK after 72 h, and Vim after 48 h of IVC, as compared to other cultivation periods. We also found a substantial increase in Vim expression (3-4 fold) as compared to CK18 and panCK, and all of the investigated proteins were distributed in the cellular cytoplasm. The lag phase of cell proliferation occurred during the first 24 h of IVC, whereas the log phase was observed between 24 h-120 h of IVC. Throughout 7 days of IVC, statistically significant differences were found in Cell Index (CI) of the analyzed cells. Increased Vim expression in buccal pouch oral mucosa cells, as compared to CK18 and panCK, suggested that the stromal cells substantially predominated during *in vitro* cell cultivation. This may be a result of significant specificity of porcine oral mucosa cells isolated from the buccal pouch.

*Key words: cytokeratins, vimentin, buccal pouch mucosal cells, porcine*

### Mailing address:

Dr Bartosz Kempisty, associate professor,  
Department of Histology and Embryology,  
Department of Anatomy, Poznan University of Medical Sciences,  
Poznan, Poland, Swieczickiego 6 St., 60-781 Poznan, Poland  
e-mail: etok@op.pl

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In recent years, buccal mucosal grafts (BMG) have been successfully used in urological reconstruction as well as in target tissue substitution in a large field of regenerative medicine (1). The development of tissue biotechnology has led to *in vitro* produced grafts for urethroplasty and for several types of human cosmetic deformities (2). The BMG were recently used as a long-term structurally and functionally stable model in penile skin grafts (3). However, BMG technology is still restricted by cellular viability, the proper structure of grafts that replace deformed tissues, proliferation and/or time-dependent differentiation of cells in culture, production efficiency of primary donor tissue *in vitro*, as well as quality and thickness of the transplanted grafts. Although the application of mucosal grafts in urethroplasty has been popular among biotechnologists and urologists, the possibility of developing a mucosal-tissue specific technology in other fields of medicine requires further investigation. Few reports exist which indicate the use of mucosal grafts in dentistry and oral/maxillofacial surgery (4). In addition, the application of mucosal grafts in oral cavity tissue reconstruction has limitations, mainly related to adnexal structures and various patterns of tissue keratinization (5). Moreover, it has been observed that the split-thickness oral cavity skin graft produced on collagen dermal equivalents is significantly restricted by cellular viability, therefore it is not sufficient for intraoral clinical application. Recently, the search for equal and the most similar animal model for culturing the buccal mucosal tissue *in vitro* opens new possibilities for advanced oral tissue engineering (6).

Although the culture of hamster, rat, and canine buccal mucosa cells were the most frequently used experimental model, research based on the domestic pig model is recently of high interest. It was found that primary culture of porcine ovarian granulosa cells may be a potential biomedical model for human research (7-11). Therefore, we proposed the use of the porcine buccal mucosa cells primary cultivation system as a possible model for further application in human research.

Furthermore, the expression of cytokeratins (CK18, pCK) was used in the comparable assays of keratinization pattern of pig buccal mucosa dur-

ing short-term cell cultivation and real-time proliferation. Cytokeratin 18 (CK18), also described as KRT18 or type I intermediate filament (IF), is expressed in adults in a variety of epithelial tissues. Cytokeratin 18, when co-expressed with CK8 (type II keratin), belongs to the IF proteins that are first expressed during murine development (12). Both of these IFs are produced in similar proportions. One of the most frequent complications of microsurgical buccal pouch mucosa preparation from pigs is the isolation of both epithelial and stromal layers of the mucosa. For this reason, vimentin expression, a marker of stromal cells, was assessed in order to check the proportion of cells in culture.

## MATERIALS AND METHODS

### *Animals*

A total of 32 crossbred Landrace gilts, were used in this study. The animals were housed under the same conditions. The local Ethics Committee approved the experiment.

### *Cell isolation and culture*

After slaughter, samples of buccal pouch mucosa were obtained within 40 min and transported to the laboratory. The excised tissue was washed twice in Dulbecco's phosphate buffered saline (D-PBS) (137 mM NaCl, 27 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4). The surface of the buccal pouch was surgically removed using sterile surgical blades. The cell suspension obtained from this digestion was filtered through mesh to remove non-dissociated tissue fragments and then incubated with 0.05% collagenase I (Sigma Aldrich, Madison, USA) for 25 min at 38°C in a shaking water bath. Isolated cells were washed three times by centrifugation (10 min at 200 g) with Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich, Madison, USA) supplemented with gentamicin (20 µg/mL) and 0.1% BSA. The epidermal side of the mucosa was gently scraped with a scalpel to detach the keratinocytes. The resultant cell suspension was filtered through a 400-micron mesh and then washed three times by centrifugation (10 min at 200 g) with supplemented DMEM. The final cell pellet was resuspended in DMEM supplemented with 10% fetal calf serum (FCS; Sigma Aldrich, Madison, USA) and 10,000 U mL<sup>-1</sup> penicillin G,

10 mg mL<sup>-1</sup> streptomycin, and 25 µg mL<sup>-1</sup> amphotericin B (antibiotic antimycotic solution; Sigma Aldrich, Madison, USA). Cell viability was 90 to 95% as determined by trypan blue staining (Sigma Aldrich, Madison, USA). The cells were cultured at 38.5°C in a humidified atmosphere of 5% CO<sub>2</sub>. Once the keratinocyte cultures attained 70–80% confluency, they were passaged by washing with PBS, digested with 0.25% trypsin/ethylenediaminetetraacetic acid (Cascade Biologics), neutralized by a 0.0125% trypsin inhibitor (Cascade Biologics), centrifuged, and resuspended at a seeding density of 2x10<sup>4</sup> cells/cm<sup>2</sup>. The culture medium was changed every three days.

*In vitro porcine buccal pouch mucosal cell cultivation using a real-time cell analyzer (RTCA)*

Cells were isolated from 32 gilts, which were divided into 8 groups (4 gilts in each group) and each group is represented by 2 replicates in this experiment. The recovered buccal pouch mucosal cells were transferred into an E-Plate 16 of a real-time cell analyzer (RTCA, Roche-Applied Science, GmbH, Penzberg, Germany), which consisted of an RTCA analyzer, an RTCA SP station, and RTCA software. The cells were then cultured in 200 µl of standard porcine culture medium that consisted of Dulbecco's Modified Eagle's Medium (DMEM/F12, Sigma-Aldrich, USA) as mentioned above. The cells were cultured for 0–168 h at 38.5°C under 5% CO<sub>2</sub> in air. The cultivation medium was replaced after 96 h of culture. After each period of cultivation, the cells were treated with trypsin (0.25% trypsin in a balanced salt solution, Sigma-Aldrich, St. Louis, MO, USA), and the collected pool of proliferating cells was used for confocal microscopy and RT-qPCR analysis. After each cultivation period, the cell index (CI) was used to evaluate the relative and quantitative changes in electrical cell impedance. The cell status was determined using RTCA software.

*Confocal microscopic observations of protein expression and distribution*

Both at recovery and during each step of *in vitro* culture (every 24 hours), mucosal cells were collected and fixed using an acetone-methanol (1:1, v/v) solution for 10 min at -20°C and washed three times in PBS/PVP (0.2%). To block non-specific binding, the samples were incubated in 3% BSA in PBS with 0.1% Tween-20 for 30 min at room temperature (RT). Mucosal cells were incubated for 1 h

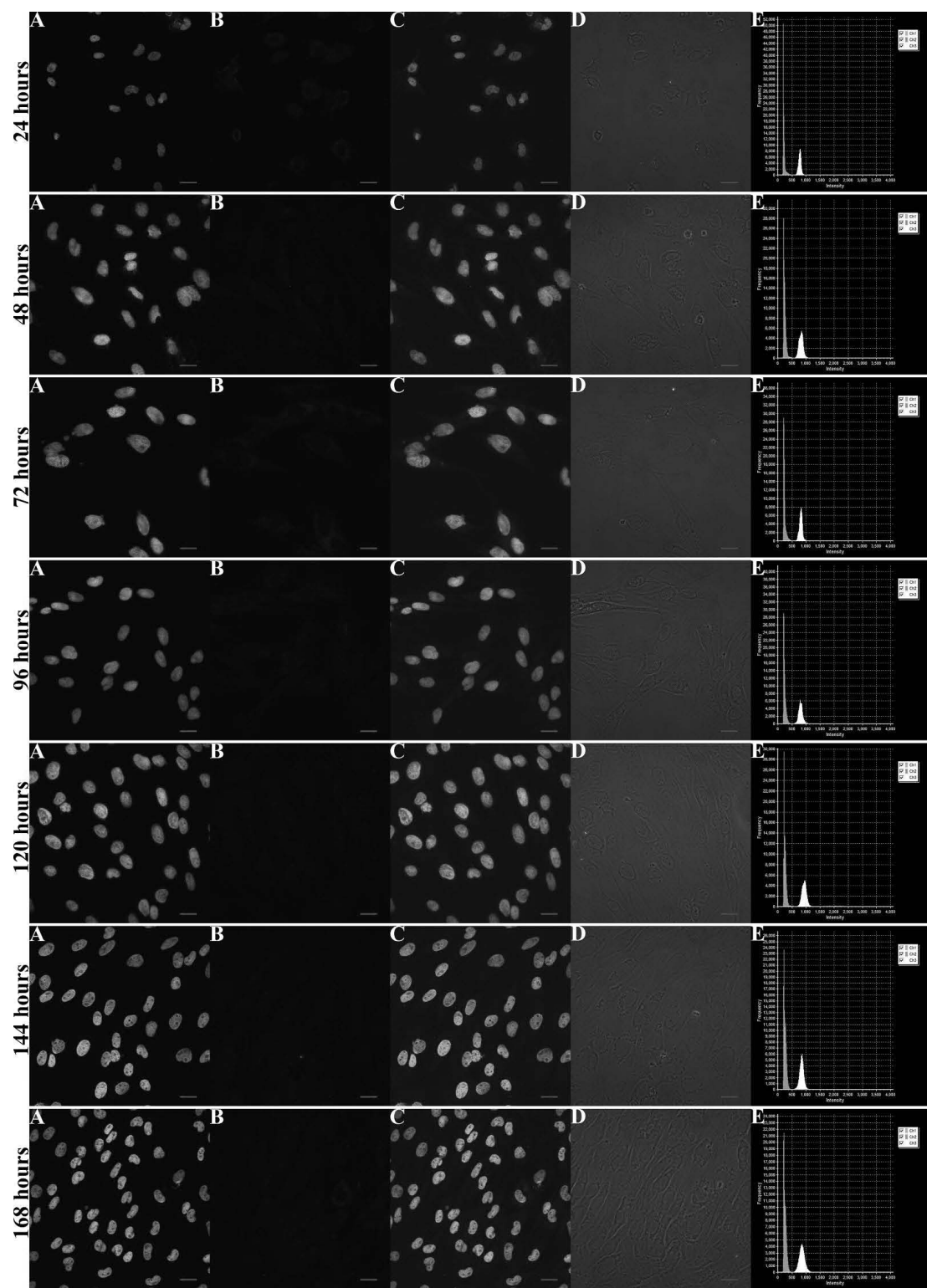
at RT with a mouse monoclonal anti-vimentin antibody [VI-10] (Abcam), mouse monoclonal anti-cytokeratin 8 + 18 + 19 antibody [2A4] (Abcam), and mouse monoclonal anti-cytokeratin 18 antibody (Dako Denmark A/S). The antibodies were diluted 1:500 in PBS with 1.5% BSA and 0.1% Tween-20. After several washes with PBS containing 0.1% Tween-20, the samples were incubated with MFP488-labeled goat anti-mouse IgG (H+L) (MoBiTec GmbH, Germany) diluted 1:500 in PBS with 0.1% Tween-20 for 1 h at RT. After washing in PBS with 0.1% Tween-20, the mucosal cells were suspended in 0.1 ml of mineral oil containing 1.5 µg/ml of DAPI, mounted on an anti-fade medium on glass slide, and examined by an LSN 510 confocal system with an Olympus Fluoview 10i microscope. All confocal microscopic images were analyzed using Imaris 7.2 (BitPlane, Zurich, Switzerland) software. The protein expression pattern was estimated as the fluorescence intensity measured by Imaris 7.2 program.

*Statistical analysis*

A one-way ANOVA followed by Tukey's post-hoc test was used to compare the results of the real-time quantification of the proliferation index. The experiments were carried out in a minimum three replicates. The results quantifying the cell proliferation index were obtained using an RTCA system. The differences were considered to be significant at \**P*<0.05, \*\**P*<0.01 and \*\*\**P*<0.001 for the quantitative analyses of proteins expression. The software program GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA, USA) was used for the statistical calculations.

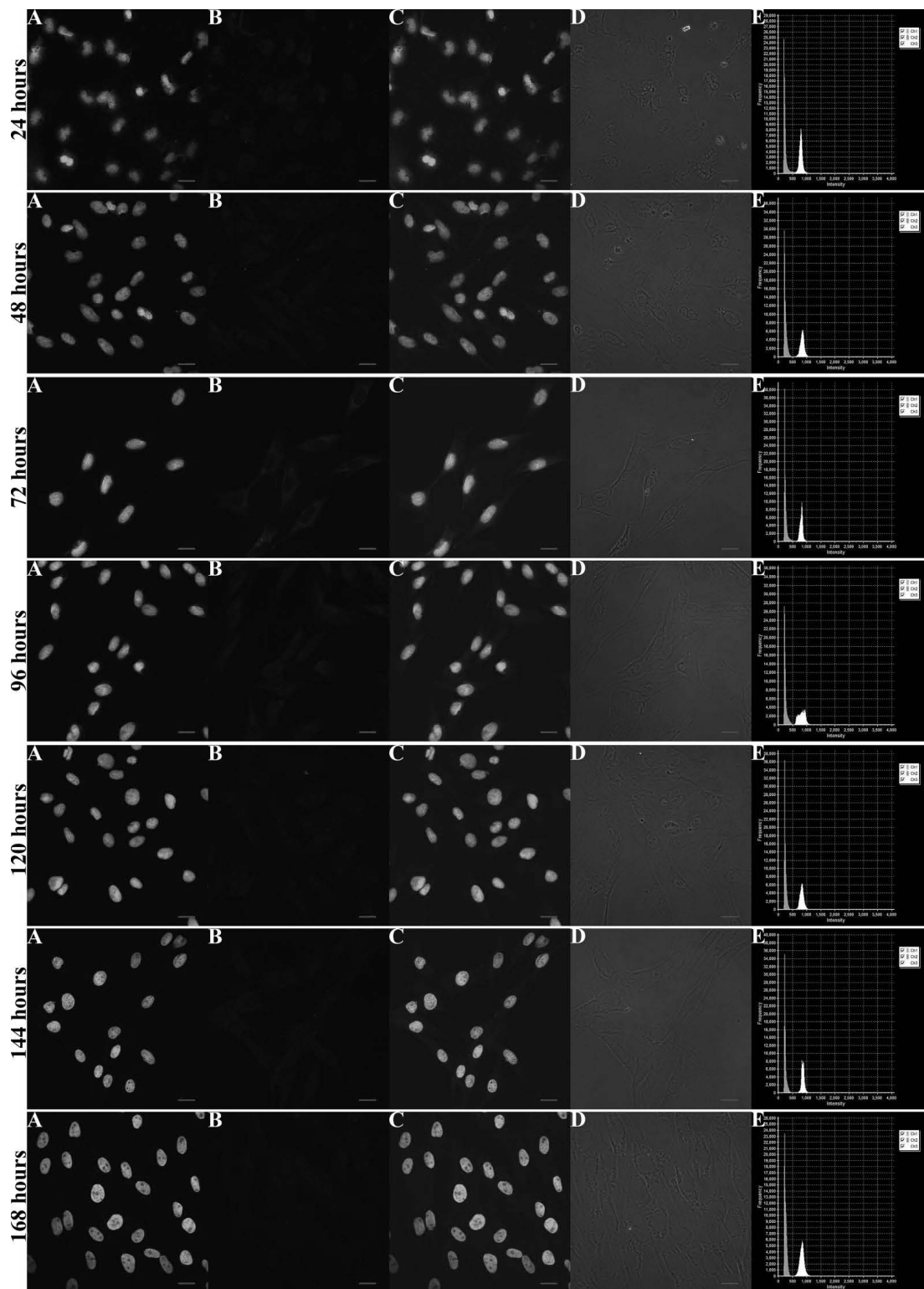
## RESULTS

Protein expression and distribution were analyzed using Imaris 7.2 (BitPlane, Zurich, Switzerland) software, and the results were presented as a fluorescence intensity value (FI). The expression of CK18 was increased after 24 h of IVC as compared to 48 h and 72 h (*P*<0.001, *P*<0.05, respectively), (Figs. 1 and 4A). When comparing 24 h to 96 h, 120 h, 144 h and 168 h, a significant increase was also observed of CK18 expression (*P*<0.01). The expression of panCK was higher at 72 h of IVC as compared to 24 h and 48 h (*P*<0.001), 96 h (*P*<0.05), 120 h (*P*<0.001),



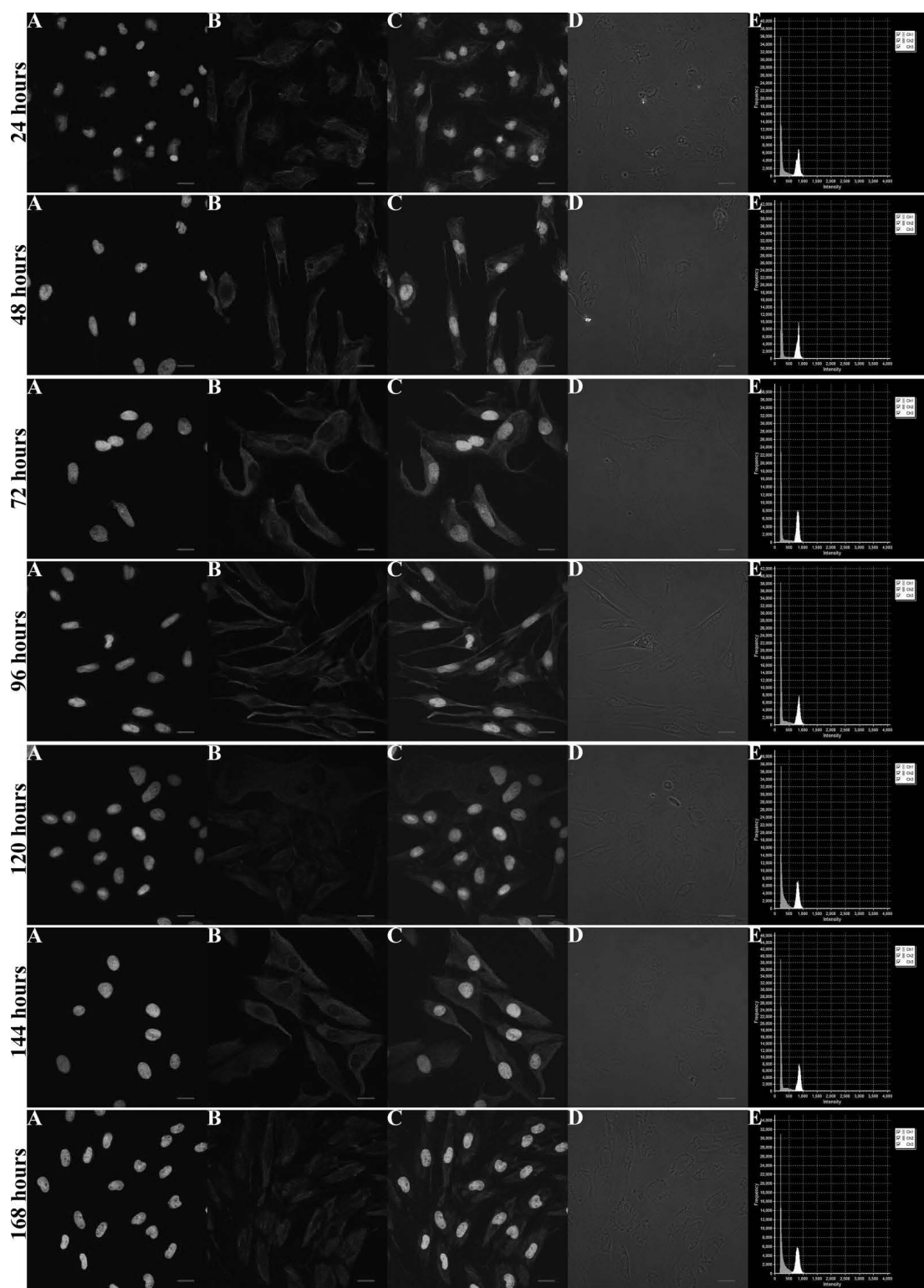
**Fig. 1.** Confocal microscopic observation of CK18 protein levels and cellular distribution in porcine buccal pouch oral mucosal cells during 168 h of culture. The scale bars represent 20  $\mu\text{m}$ . A) DAPI; B) antibody stained cells; C) merge; D) Nomarski; E) histogram.



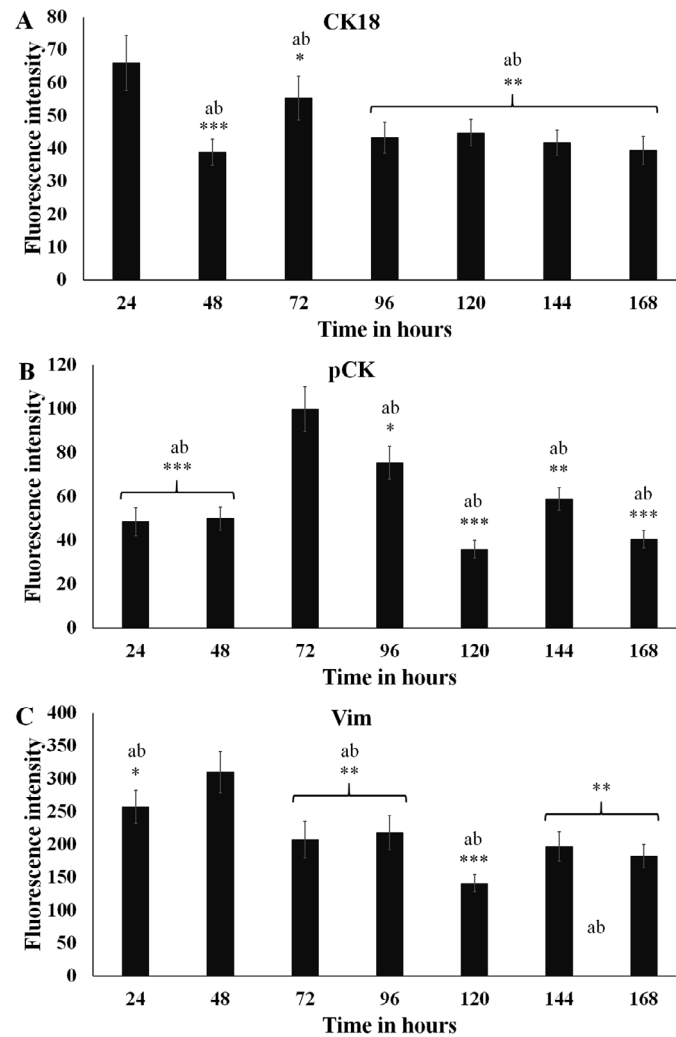


**Fig. 2.** Confocal microscopic observation of pCK protein levels and cellular distribution in porcine buccal pouch oral mucosal cells during 168 h of culture. The scale bars represent 20  $\mu\text{m}$ . A) DAPI; B) antibody stained cells; C) merge; D) Nomarski; E) histogram.

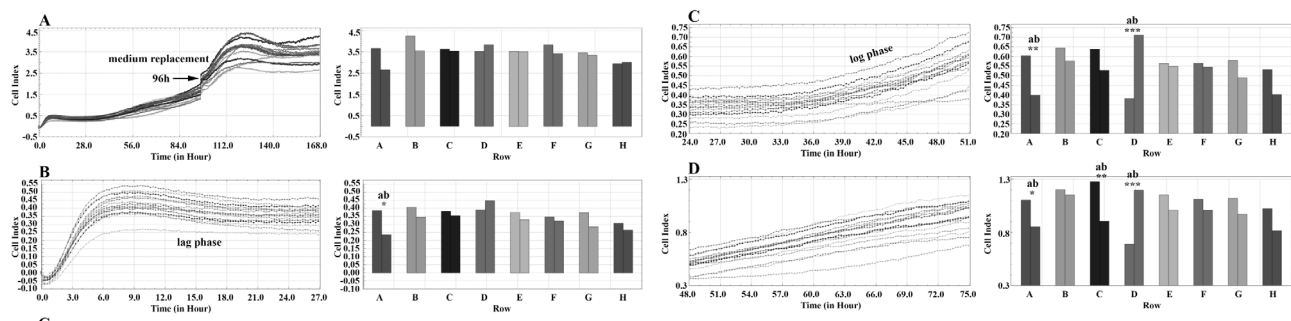




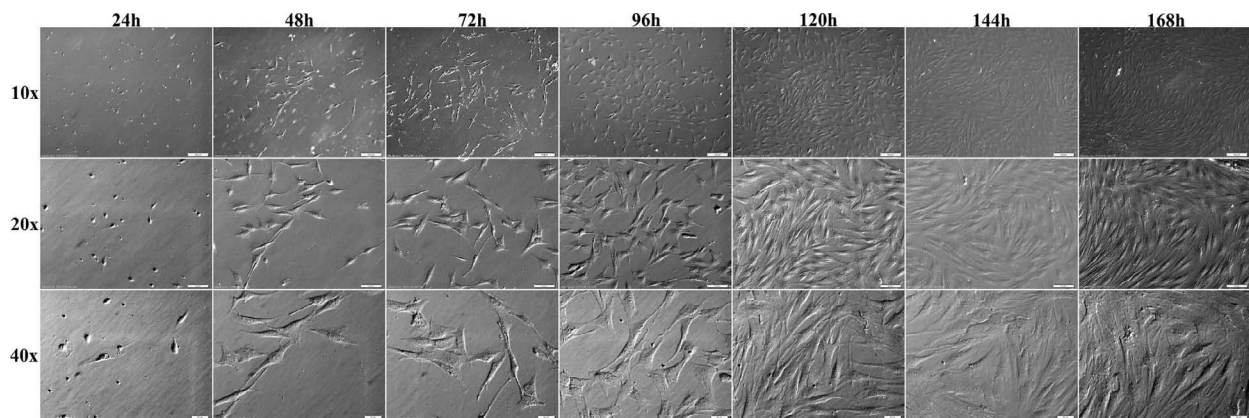
**Fig. 3.** Confocal microscopic observation of Vim protein levels and cellular distribution in porcine buccal pouch oral mucosal cells during 168 h of culture. The scale bars represent 20  $\mu$ m. A) DAPI; B) antibody stained cells; C) merge; D) Nomarski; E) histogram.



**Fig. 4.** Fluorescence intensity (FI) of CK18, pCK, and Vim protein expression. The fluorescence intensity (FI) of CK18 (A), pCK (B), and Vim (C) was analyzed by using Imaris 7.2 (BitPlane, Zurich, Switzerland) software after 24–168 h of in vitro culture. Different superscripts display significant difference at the level of \* ( $P < 0.05$ ), \*\* ( $P < 0.01$ ), and \*\*\* ( $P < 0.001$ ).



**Fig. 5.** Cell proliferation index (CI) of porcine buccal pouch mucosal cells cultivated for 168 h. The experiment consisted of eight replicates involving the cultivation of the same population of collected cells. At each step, the cell proliferation index was assessed for real-time in vitro cultivation for the time periods of 0–168 h (A), 0–24 h (B), 24–48 h (C), 48–72 h (D), 72–96 h (E), 96–120 h (F), 120–144 h (G), and 144–168 h (H). The differences were considered to be significant at the level of \*  $P < 0.05$ , \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$ . The different superscripts (ab) described significant differences.



**Fig. 6.** Cellular proliferation, *in vitro* culture (IVC) monitoring, and Nomarski phase/contrast images. Magnifications shown are 10x, 20x, and 40x.

144 h ( $P<0.01$ ), and 168 h ( $P<0.001$ ) (Figs. 2 and 4B). The FI of Vim was increased after 48 h of IVC compared to 24 h ( $P<0.05$ ), 72-96 h ( $P<0.01$ ), 120 h ( $P<0.001$ ), and 144-168 h ( $P<0.01$ ), (Figs. 3 and 4C).

Real-time proliferation was analyzed using the RTCA system during 168 h *in vitro* culture of buccal pouch mucosal cells (Fig. 5A) and defined as the Cell Index (CI). During the first 24 h of IVC, cells reached the lag phase of proliferation (Fig. 5B). Shortly after 24 h of IVC, they then proliferated and reached log phase, which was stable up to 120 h of IVC (Fig. 5C). The medium was replaced only once, 96 h after the start of IVC. The experiment was performed in eight replicates. In the first proliferation period (0h-24 h), we found a difference in proliferation in one of eight groups ( $P<0.05$ ). Between 24 h and 48 h, we observed differences in two groups ( $P<0.01$ ,  $P<0.001$ , respectively), whereas between 48 h and 72 h, various proliferation CI were detected in three groups ( $P<0.05$ ,  $P<0.01$ , and  $P<0.001$ , respectively), (Figs. 5, C and D). When analyzing periods between 72 h-96 h and 96 h-120 h, differences were observed in two of eight groups ( $P<0.01$ ,  $P<0.05$ ,  $P<0.001$ , respectively), (Fig. 5, E, F, and G). Between 120 h-144 h, various CI in three groups were determined, ( $P<0.001$ ,  $P<0.01$ ), whereas between 144 h-168 h, differences of CI were found in two groups ( $P<0.001$  for both). The cell proliferation and changes of cellular morphology during short-term culture were also observed (Fig. 6).

## DISCUSSION

It was observed by Lindberg et al. (13) that keratinocyte markers, such as several cytokeratin, are well expressed in human oral epithelium and epithelial xenografts during IVC. Therefore, identification of epithelial and/or stromal markers in buccal pouch mucosa is often highly associated with keratinization of these cells *in vitro*. Moreover, the induction of the keratinization process and marker gene expression analysis in keratinized and/or non-keratinized epithelium has pre-clinical and clinical significance. Recently, the application of mucosal grafts in animal and human models was thoroughly investigated (14, 15). In this case, experiments performed on porcine buccal pouch mucosal cells model cellular epithelialization, stromalization and/or keratinization. This allows for a further possibility of graft or xenograft application, an area of increasing scientific interest.

Recently, human reconstructive and/or regenerative medicine involved developed and highly advanced tissue engineering characterized by preparation of autologous grafts (16, 17). Tissue grafting of oral mucosa or skin grafts are used in clinical trials such as dental implant surgery, periodontal surgery, and many types of reconstructive surgeries (18, 19). Moreover, the most critical limitation of human grafting and application of tissues as “human somatic cell therapy” are to maintain the tissues well-defined anatomical structure and physiological

functionality when it leaves the operating room following autologous transplants (20). The final product is fabricated from a well-constructed, biological oral mucosa graft. Although the current knowledge of human oral mucosa grafting in regard to cell and tissue *in vitro* culture (IVC), as well as the procedures of grafting and graft fabrication following transplants, are well recognized, the molecular regulations of important *in vitro* mechanisms still require further investigation. Research has been carried out utilizing an animal model as a replacement source of human tissue grafts. The availability of animal tissue, the potential for generating genetic modifications within cell culture, and the ability to have cells/tissues in culture for a prolonged time or a long storage period for the next transplant allows this to be a convenient model in human research based on oral mucosa grafts (21).

In this study, molecular experiments were carried out that explain the course of two important processes regarding oral grafting: stromalization and epithelialization of porcine buccal pouch mucosal cells. What is more, it is widely known that CK18, panCK and Vim are crucial for the process of epithelialization and stromalization of connective tissue, thus analyzing their expression is crucial for tissue engineering. The cells were isolated following slaughter and were cultured for short periods. The expression of stromalization and epithelialization markers was analyzed in relation to cellular *in vitro* real-time proliferation. Since we observed the highest expression of vimentin during cultivation, it is suggested that the process of cell stromalization was predominant. Indeed, stromal cells, such as fibroblast-like cells, predominate during the culture. Moreover, the cuboidal-epithelial-like cell subpopulation was barely visible during culture, with a significant appearance of spindle-shaped cells. The lack of these cell types in culture was accompanied by decreased expression of epithelialization markers such as CK18 and panCK. Huang et al. (22) focused on the normal hamster buccal pouch cells (HBP) as a potential model for human buccal squamous cell carcinoma (SCC). First, they isolated the subpopulation of stem/stromal cells from normal HBP and differentiated these cells into adipogenic, chondrogenic, and osteogenic lineages. These

experiments showed the maintenance of significant stemness specificity of stem/stromal cells collected from normal HBP. Moreover, the expression of stem cell (SC) markers such as CD29, CD90, and CD105 in HBP display their transdifferentiation/differentiation capability and stem cell origin. Contrary to our results, they observed 90% of cells stained positively for cytoplasmic keratin and only 10% for vimentin. In conclusion, we may suggest that morphological changes, molecular modifications, and the existence of stem/stromal cell marker expression demonstrate the highest specificity and plasticity of normal buccal pouch oral mucosa cells, which may be used as potential targets in clinical trials involving radio-, and/or chemotherapy in both humans and animals.

Moreover, Martín-Cano et al. (23) used panCK, CK1, CK4, CK13, and filaggrin as potential stemness markers of the oral mucosal epithelium. They used oral mucosal epithelium as an autologous graft for epithelium with a structure comparable to native urethra. The SC subpopulation during the IVC of oral mucosal epithelium successfully underwent the transdifferentiation process. Moreover, the generation of urethral epithelium from oral mucosa was observed by expression of SC markers at both the histological and immunofluorescence levels.

In summary, we conclude that the expression of cytokeratins and vimentin in oral mucosal cells and/or stromalization-epithelialization of these tissues in both humans and animals is significantly associated with generation of tissue-engineered oral mucosa equivalent used as a potential graft in microsurgery. Furthermore, the stemness specificity of oral mucosal cells, as well as the significant plasticity of the epithelium, may be applicable as a graft in reconstructive and/or regenerative human medicine.

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## REMISSION EFFECT OF VITAMIN C ON ISOFLURANE-INDUCED APOPTOSIS AND ITS MECHANISM

J. HU<sup>1</sup>, BF. GAO<sup>1</sup>, HQ. LI<sup>1</sup>, ZZ. ZHANG<sup>1</sup>, YL. LEI<sup>1</sup>, GQ. SUN<sup>1</sup> and DM. GUO<sup>2</sup>

<sup>1</sup>Anesthesia Department, Shandong Jiaotong hospital, Jinan, Shandong, China;

<sup>2</sup>Shandong University School of Public Health, Jinan, Shandong, China

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**This study aims to discuss the remission effect of vitamin C on isoflurane-induced apoptosis of rats and its possible mechanism of action, to provide a theoretical basis for postoperative cognitive impairment. Reactive oxygen species (ROS) detection, adenosine triphosphate (ATP) test, MTT method and Morris water maze were applied for detection tests. For data statistics, double factor analysis of variance (ANOVA) and post hoc Bonferroni test were adopted. It was found that vitamin C could slow down the isoflurane-induced accumulation of ROS in H4-APP cells; moreover, it could relieve the activation of caspase-3 and increase cell survival rate to inhibit the occurrence of apoptosis, indicating that ROS was the source of cell toxicity. On the other hand, vitamin C could protect the cells with its antioxidant effect. It was proved that vitamin C could remit isoflurane-induced apoptosis and relieve the decline in learning and memory ability of rats.**

Discovered and recognized as a rare disease in the early 20<sup>th</sup> century, Alzheimer's disease has a history of more than 100 years (1). It has now developed into a common of senile neurodegenerative disease, with its incidence rate second only to heart disease, tumor and cerebrovascular disease (2). In 2011, it was announced by the Alzheimer's Association that about 36.5 million people suffer from dementia in the world, with an average survival of only 5.9 years (3). The main clinical manifestation of Alzheimer's disease is progressive dementia (4). Postoperative cognitive dysfunction is a common central nervous system complication after clinical surgery (5). Within a period after operative anesthesia, patients are usually found with memory impairment, anxiety and character changes and older patients are more likely

to suffer from this complication (6). Postoperative cognitive dysfunction can lead to mental anxiety, complication aggravation and prolong recovery time of patients (7). Besides age, anesthesia is the biggest risk factor for patients' memory (8). Each year approximately 66 million patients aged over 65 years need to undergo surgery with anesthesia (9).

We applied reactive oxygen species (ROS) detection, adenosine triphosphate (ATP) test, 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) method and Morris water maze in this study. With vitamin C as the antioxidant, we performed cellular level tests and animal behavior tests which proved that vitamin C could relieve isoflurane-induced apoptosis as well as memory deterioration of rats (10).

*Key words: vitamin C, cognitive impairment, isoflurane, apoptosis*

*Mailing address:*

Dr Baofeng Gao,  
Anesthesia Department, Shandong Jiaotong Hospital,  
No.12, Wuyingshanzhong Road, Tianqiao District,  
Jinan, Shandong, 250031, China  
e-mail: hujinghj55@163.com

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INTEREST RELEVANT TO THIS ARTICLE.**

## MATERIALS AND METHODS

### *Experimental materials*

The experimental materials included human neural glioblastoma H4, rat neuroblastoma B104, high glucose Dulbecco's modified eagle medium (DMEM), isoflurane, pancreatin, Hanks balanced salt solution (HBSS), Dulbecco's phosphate buffered saline (DPBS), cell culture supplies, Caspase-3 antibody, Actin antibody, Tris- HCl, 4× lithium dodecyl sulfate (LDS), polyvinylidene fluoride (PVDF) membrane, JC-1 dye, TMRE dye and reactive oxygen species/reactive nitrogen species (ROS/RNS) detection kit.

### *Experimental equipment*

The equipment included a fear condition system (FCS), a vortex oscillator, a low temperature high speed centrifuge, an ultra centrifuge, a flow cytometry C6, protein electrophoresis apparatus, a SpectraMax plus microplate reader, TE70 half-dry transfer box, and ChemiDox XRS gel imaging system.

### *Cell recovery*

After being taken out of the liquid nitrogen, the cryogenic vial was shaken in a water bath (37°C) until it melted, and 10× culture medium containing 10% fetal bovine serum (FBS) was added; after full blending, the mixture was centrifuged to remove the supernatant. Then, culture medium with 10% FBS was added, and the mixture was mixed evenly. The mixed liquid was added to a culture flask, and culture medium with 10% FBS was added. Then, the culture flask was placed in an environment at 37°C and containing 5% CO<sub>2</sub> (11) to make neuroblastoma cells of the rats recover.

### *Cell cultivation*

Medium was changed every day according to the observed state of cells. First, the culture flask was disinfected with an alcohol lamp; then, the cap was removed, and the culture solution was poured out carefully. And then, the culture flask was washed with phosphate buffer solution (PBS) twice, and PBS was discarded. Finally, 10 ml of DMEM culture solution (10% FBS) was added (12).

### *Cell propagation*

When cells grew by about 80%, the culture medium was discarded, and the culture flask was washed with PBS twice. Then, trypsin (0.25%) and ethylene diamine tetraacetic

acid (EDTA) digestion juice (0.02%) were added to the culture flask. After approx. 3 min, when the cells became round, DMEM culture solution (10% FBS) was added to stop the digestion reaction. Cells were blown off the flask wall carefully. The cell suspension was then transferred to a centrifuge tube with a pipetting tube for 5-min centrifugation at room temperature. The supernatant was discarded carefully, and DMEM (10% FBS) was added and mixed evenly, then the cell suspension was evenly added to new culture flasks. After the addition of culture solution in a ratio of 1:3, the culture flasks were placed in a cell incubator for further cultivation (13).

### *Cryopreservation of cells*

On the day before cryopreservation, primary cells or cells which had 1~2 times of passage were spread in fresh culture solution; trypsin (0.25%) was used to digest the cells, then, DMEM (containing 10% FBS) was used to adjust cell density to  $1 \times 10^6 \sim 2 \times 10^6$ /ml; dimethyl sulfoxide (10%) was added and mixed evenly; then, it was placed in a frozen depository, followed by sealing and labeling, and immediately placed in a refrigerator at -20°C for 2 h, then, it was kept at -40°C overnight and was preserved in liquid nitrogen (14).

### *Treatment of cells*

On the day before anesthesia, processing on cells, human glioblastoma cell line H4-APP cells or rat neuroblastoma cell lines B104 were inoculated in the corresponding culture plates. The following day, the cell culture plates were placed in a closed anesthesia box connected with anesthetic duct passage; then, the box was placed in a large incubator at 3°C. The experimental treatment group was treated with 2% isoflurane, 21% oxygen and 5% carbon dioxide for 6 h, while the control group was placed in a cell culture box for 6 h (15).

### *Vitamin C treatment on cells*

When the cross linking of vitamin C and anesthesia was carried out, fresh vitamin C solution was prepared and placed in the cell culture medium for 30 min before anesthesia processing on cells; then, the vitamin C solution was incubated with the cells at 37°C (16).

### *Detection of reactive oxygen species*

H4-APP cells were inoculated in a 96-well plate ( $3.0-4.0 \times 10^4$  each well); samples in each group were processed

in 3-5 duplicate wells. They were placed in a 37°C cell culture box for one day. Next, 1 × DCFH-DA working fluid was prepared. DPBS was drawn and used to wash the cells 3 times in the 96-well plate, the remaining culture medium was removed. After DPBS was drained out, 100 µl of 1 × DCFH-DA working fluid was added. Then, they were cultured in an incubator at 37°C for 30 min. The experiment was performed in a lucifugal environment.

The excess DCFH-DA working fluid was drained out, and staining fluid was washed off with DPBS. Then, 100 µl of blank culture medium was added. The control group was cultured under the environment of 21% oxygen and 5% carbon dioxide at 37°C for 6 h, while the experimental group was cultured under the environment of 21% oxygen, 5% carbon dioxide and 2% isoflurane at 37°C for 6 h. The experiment was performed in a lucifugal environment. Afterwards, the cell culture plate was taken out and washed carefully once with DPBS and 100 µl of new DPBS and 100 µl of cell lysis buffer were added; after quick mixing, 150 µl of the liquid was transferred to a black 96-well plate, and a microplate reader was used to obtain the reading at 480nm/30 nm. Each experiment was set up with a group of H<sub>2</sub>O<sub>2</sub> positive control (17).

#### *Adenosine triphosphate detection test*

H4-APP cells were inoculated in a 96-well plate, 0.8 - 1.0 × 10<sup>6</sup> each well; each group of samples was processed in 3 to 5 duplicate wells; then, they were cultured in a cell culture box at 37°C for one day. The staining liquid was gently washed off with DPBS; then, the samples were added to 1 ml of blank culture medium. The control group was cultured in 21% oxygen and 5% carbon dioxide at 37°C for 6 h, while the experimental group was cultured in 21% oxygen, 5% carbon dioxide and 2% isoflurane at 37°C for 3 h.

The cells were narcotized; according to the experimental instructions, firefly luciferase solution was prepared in a lucifugal environment.

Standard ATP was prepared and diluted in gradient; then, it was added to the left side of a 96-well culture plate, 100 µl each well. On one hand, gradient standard ATP could be used to examine the activity of the firefly luciferase and verify the experimental accuracy; on the other hand, according to fluorescence readings of ATP, content of ATP in the samples could be calculated. Three hours later, the cell culture plate was taken out and washed once with DPBS. Then, 800 µl of cell lysis buffer was added. After 5-min incubation, a

micropipette was used to transfer the cells to a 96-well culture plate, 100 µl each well.

In a lucifugal environment, 100 µl of firefly luciferase solution was added to the standard ATP and sample wells, and the edge of the culture plate was patted gently. After being placed at room temperature for 15 min, the microplate reader was used to calculate the content of ATP in the sample (18).

#### *Detection of cell survival rate with MTT method*

H4-APP cells were inoculated in a 96-well plate, 1.0×10<sup>4</sup> each well; each group of samples was set up with 3 to 5 duplicate wells; they were cultured in a cell incubator at 37°C for 24 h.

The cells were gently washed with DPBS 3 times to remove the remaining culture medium. After DPBS was drained out, they were added to 100 µl of blank culture medium. The control group was cultured in 21% oxygen and 5% carbon dioxide at 37°C for 3 h, while the experimental group was cultured in 21% oxygen, 5% carbon dioxide and 2% isoflurane at 37°C for 6 h.

After 6-h anesthesia treatment, the culture medium was drained out, and the cells were washed with 100 µl of DPBS once. Then, 10 µl of MTT solution (5 mg/ml) was added to each well; and the cells were cultured at 37°C. Four hours later, the culture was terminated. The culture supernatant in the wells was carefully drained out and discarded. Then, 150 µl of dimethylsulfoxide (DMSO) was added to each well; after 10-min oscillation, the crystal substance fully dissolved.

At the wavelength of 490 nm, optical density (OD) of each pore was determined and recorded. The mean value of OD values of three duplicate wells was used to calculate cell survival rate according to the following formula: cell survival rate = (OD value of experimental group – OD value of blank control group) / (OD value of normal cell control group – OD value of blank control group) (19).

#### *Behavior test on aged rats with Morris water maze*

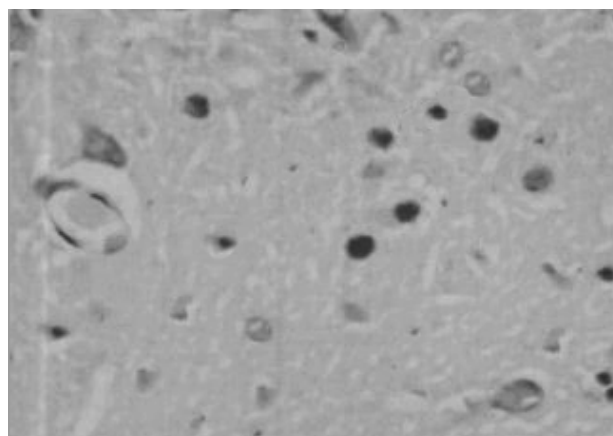
A Morris water maze (20) was made using a round bucket with a diameter of 1 m and a height of 0.5 m and a removable transparent glass platform with a diameter of 8 cm. The platform was sunk into the water for 2 cm. The four directions of the round bucket were pasted with different markers as four water inlets. The water temperature was controlled at 20±2°C. In the experiment, thirty-six SD rats aged 24 months were divided into an oxygen group (n = 12),



a vitamin C + 1.8% isoflurane group ( $n = 12$ ) and a 1.8% isoflurane group ( $n = 12$ ). The Morris water maze experiment was performed on the rats to test their ability of learning and memory. The rats were put into the Morris water maze; the time spent on searching the platform (latent period) was recorded as the index for reflecting the learning and memory ability of the rats. After the rats found the platform, the rats stayed at the platform for 30 s. If the rats did not find it in 120 s, then the latent period was denoted as 120 s and the rats were guided to board the platform and stay for 30 s to do spatial learning and memory. Finally, the time of the rats escaping latent period in every time of training was recorded. In the process of the experiment, a quiet environment should be ensured and the positions of light source and objects around the Morris water maze remained unchanged. At the end of every training day, the maze was cleaned to eliminate odour hint.

#### Data statistics

There were no less than 3 duplicate wells in each of the above experiments, and each experiment was repeated 3 times ( $n \geq 3$ ). Double factor analysis of variance (ANOVA) was applied to evaluate the interaction effects between the blank test groups (control group and isoflurane treatment group) and cross linking groups (control group + vitamin C group and isoflurane + vitamin C treatment group). Post hoc Bonferroni test was applied for the pairwise comparison between groups. In the process of mapping, the



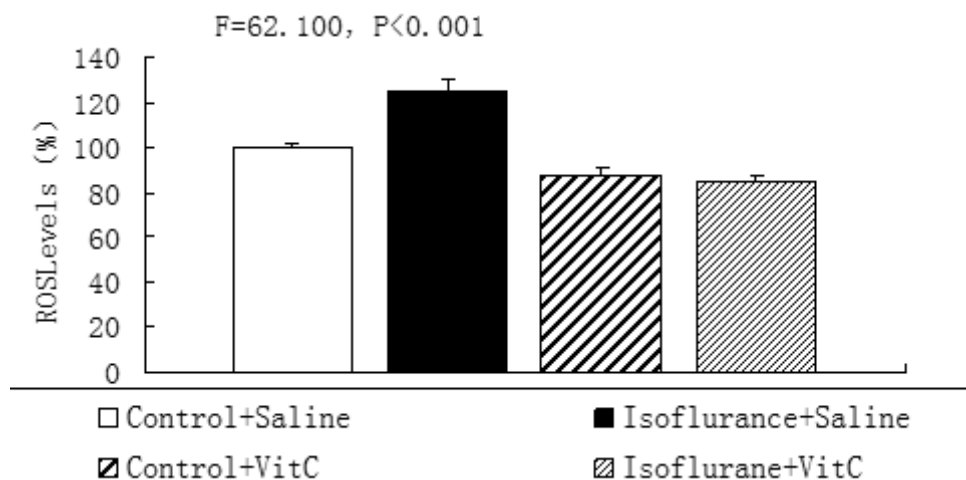
**Fig. 1.** Light microscope image of cortical neurons of a rat.

control group data was the radix (100%), while all the other groups of data were turned into the percentage with regard to the control group.

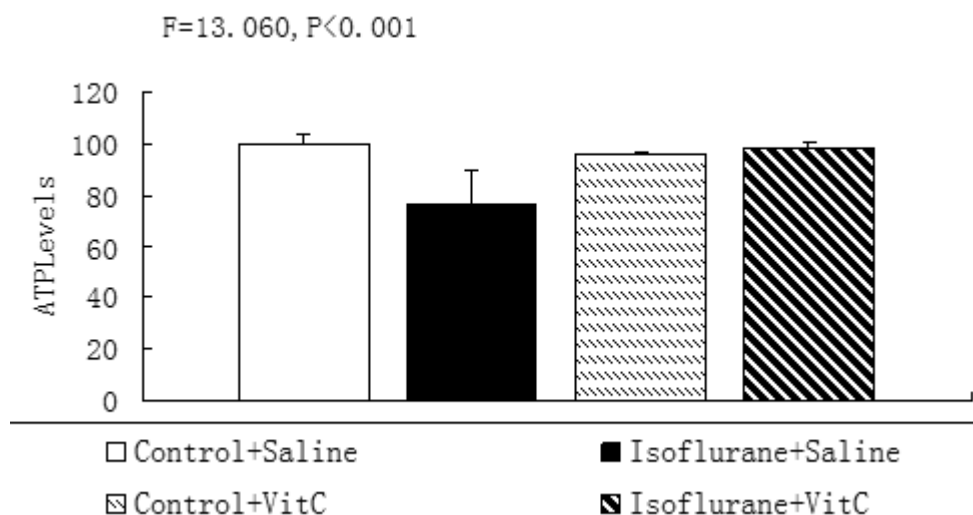
## RESULTS

### Hippocampal and cortical nerve cells of rats

Under the light microscope, it was observed that the nuclei (relatively large) of normal non-apoptotic cells turned blue due to the counterstaining with hematoxylin. The apoptotic cells shrank in the shapes of oval, triangle or fan; the nuclei turned brown or sepia; nuclear pyknosis was irregular in shape and size; there was occasionally crescent-shaped chromatin.



**Fig. 2.** Vitamin C relieved the increase of isoflurane-induced ROS level in H4-APP cells.



**Fig. 3.** Vitamin C relieved the isoflurane-induced decrease of intracellular ATP content.

Some of the cells disintegrated into apoptotic bodies of different sizes. Neural cells of rat cortical area are shown in Fig. 1. The test results indicated that the apoptotic cells of rat cortical areas were rare.

#### *Detection of the effect of vitamin C in the anesthesia process*

In this study, ROS determination test was applied to explore the effect of vitamin C in the anesthesia process with isoflurane. The results are shown in Fig. 2. In comparison with the control + saline group, intracellular ROS content increased markedly in the isoflurane + saline group. According to the double factor analysis of variance, there was difference in the correlative factors. Post hoc Bonferroni test showed that after vitamin C was added, the basic content of ROS in the control + vitamin C group decreased slightly in comparison with the control + saline group.

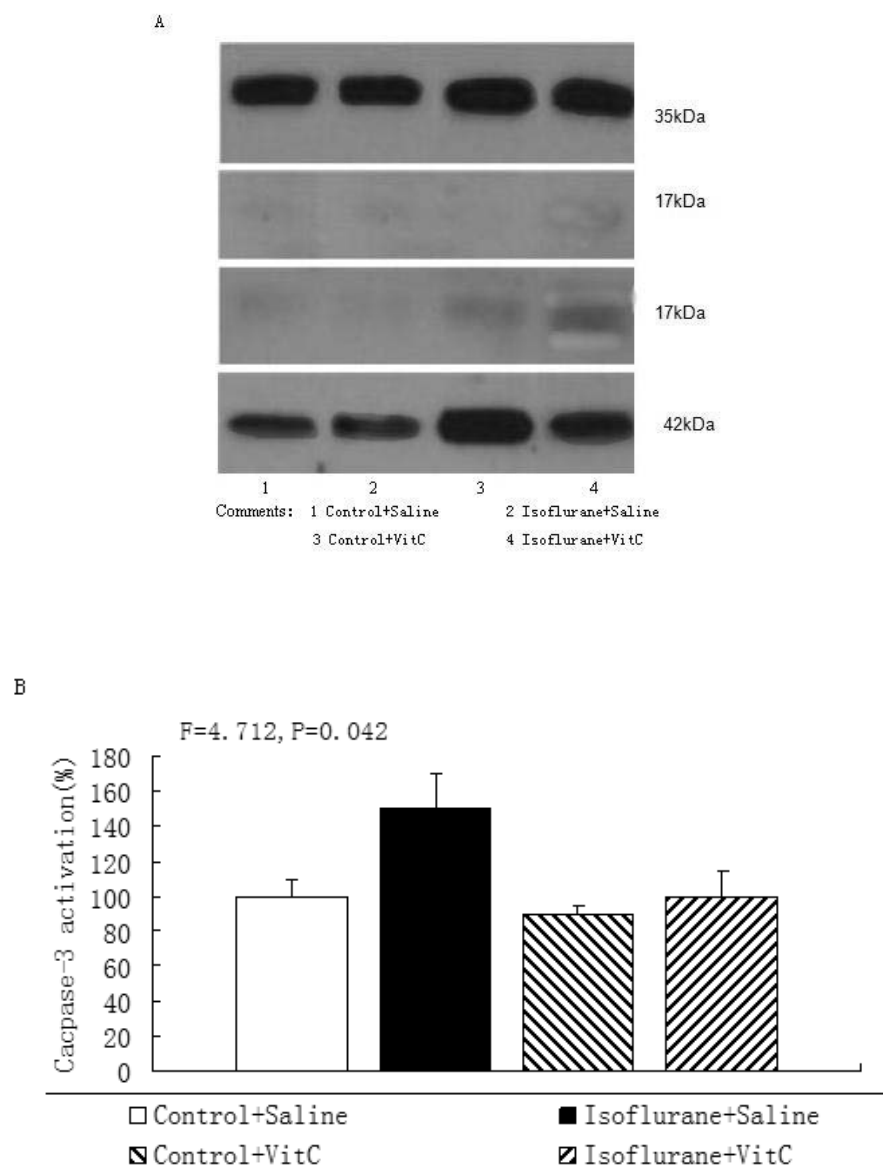
#### *Detection test of intracellular adenosine triphosphate content*

As shown in Fig. 3, in comparison with the control + saline group, intracellular ATP content decreased significantly in the isoflurane + saline group. According to double factor analysis of variance, there was difference in the correlative factors. According to the Bonferroni test, after vitamin C was

added, there was no significant difference in ATP level of the control + vitamin C group and control + saline group; in comparison with isoflurane + saline group, ATP level in the isoflurane + vitamin C group decreased significantly.

#### *Detection of the activation of caspase-3 with Western blot method*

In this study, Western blot method was applied to detect the activated Caspase-3 to determine whether vitamin C had an inhibitory effect on the activation of caspase-3 in the cells treated with 2% isoflurane. The results are shown in Fig. 4A. In comparison with the control group, after being treated with 2% isoflurane, active caspase-3 protein content increased. After pre-treatment with vitamin C, the increase of active caspase-3 protein content was relieved in the 1.8% isoflurane + vitamin C group. Fig. 4B shows the gray statistical analysis on Western blotting results. It can be seen that in comparison with control + saline group, activity ratio of caspase-3 in the cells of the isoflurane + saline group increased significantly after treatment with 2% isoflurane. Double factor analysis of variance showed that there was difference between the correlative factors. Post hoc Bonferroni test showed that after vitamin C was added, in comparison with the control + saline group, there was no significant difference in the



**Fig. 4.** Vitamin C relieved the intercellular activation of caspase-3 induced by isoflurane.

activation ratio of caspase-3 in control + vitamin C group, while the activation ratio of caspase-3 in the isoflurane + vitamin C group decreased significantly in comparison with the isoflurane + saline group.

*Analysis of cell survival rate*

In order to explore the protective capability of vitamin C on cells, this study adopted MTT test to detect the survival rate of H4-APP cells treated with isoflurane. The results are shown in

Fig. 5. In comparison with the control + saline group, cell survival rate decreased significantly in the isoflurane + saline group. The double factor analysis of variance showed that there was difference in the correlative factors. Post hoc Bonferroni test showed that there was no significant difference in cell survival rate between the control + vitamin C group and the control + saline group after vitamin C was added. In comparison with the isoflurane + saline group, cell



**Fig. 5.** Vitamin C improved isoflurane-induced decrease of cell survival rate.

**Table I.** The average escape latent incubation period of the rats in each group ( $n = 12$ , mean  $\pm$  SD).

| Group               | Oxygen group      | Vitamin C+1.8%<br>isoflurane group | 1.8% isoflurane<br>group |
|---------------------|-------------------|------------------------------------|--------------------------|
| 1 <sup>st</sup> day | 59.31 $\pm$ 32.41 | 58.73 $\pm$ 33.72                  | 60.25 $\pm$ 28.65        |
| 2 <sup>nd</sup> day | 23.62 $\pm$ 13.92 | 44.15 $\pm$ 17.90**                | 47.40 $\pm$ 20.93**      |
| 3 <sup>rd</sup> day | 14.85 $\pm$ 8.35  | 26.83 $\pm$ *11.54                 | 27.05 $\pm$ 11.61*       |
| 4 <sup>th</sup> day | 7.51 $\pm$ 4.41   | 9.15 $\pm$ 2.89                    | 10.42 $\pm$ 4.21         |
| 5 <sup>th</sup> day | 7.30 $\pm$ 2.38   | 8.01 $\pm$ 2.54                    | 9.20 $\pm$ 1.25          |
| 6 <sup>th</sup> day | 6.73 $\pm$ 2.25   | 7.39 $\pm$ 2.07                    | 7.26 $\pm$ 2.76          |

survival rate of the isoflurane + vitamin C group increased significantly; in comparison with the control + saline group, it declined slightly with no significant difference.

#### *Behavior test on aged rats with Morris water maze*

With the increase of training time in the Morris water maze experiment, the average escape period was inclined to shorten. In the vitamin C +1.8% isoflurane group and the 1.8% isoflurane group, the average escape periods of the rats on the second and the third days were extended in comparison with the oxygen incubation group, with a significant difference ( $p < 0.05$ ), especially on the second day ( $p < 0.01$ ); there was also a remarkable difference between the vitamin C +1.8% isoflurane group and the 1.8% isoflurane group, suggesting that vitamin C could relieve the isoflurane induced learning and memory

decline on rats. On the first day and during the fourth and sixth days, there was no significant difference among the three groups ( $p > 0.05$ ), as shown in Table I.

## DISCUSSION

Memory impairment is the main clinical symptom of patients with postoperative cognitive disorder, indicating that the central nervous systems of the patients are affected during operative anesthesia (21). Considering the dependence of clinical operations on anesthesia and the neural inhibition effect of anesthetics, it is believed that, except for the age factor, anesthesia is most likely to lead to postoperative cognitive disorder (22).

Based on the experiments, we found that under normal physiological conditions, the generated ROS could be remitted with the antioxidant effect



of vitamin C. Moreover, vitamin C could relieve isoflurane-induced symptoms effectively, such as inhibiting the increase of ROS level, relieving the decrease of intercellular ATP content, inhibiting the activation of intracellular caspase-3 and relieving the decrease of cell survival rate.

In this study, it was found that isoflurane had no significant influence on the long-term cognitive function of aged rats, which differed from the previous research result that the spatial memory impairment in aged rats could last for about two weeks (23, 24). The difference in the results might be associated with specific ages of the rats, concentration of inhaled anesthetics, drug duration and the sample size.

With vitamin C selected as the antioxidant, this study observed the effect of vitamin C on isoflurane-induced cytotoxicity and cognitive function disorder based on cellular level tests and animal behavior tests. The experimental results show that vitamin C can relieve isoflurane-induced ROS accumulation and inhibit apoptosis at the cellular level (25). Moreover, vitamin C can improve isoflurane-induced decline of learning and memory ability in animal behavioral models (26). Hopefully, this study can provide experimental data and a theoretical basis for the prevention and treatment of isoflurane-induced postoperative cognitive disorder

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## ASSOCIATION BETWEEN EXPRESSION OF CUMULUS EXPANSION MARKERS AND REAL-TIME PROLIFERATION OF PORCINE FOLLICULAR GRANULOSA CELLS IN A PRIMARY CELL CULTURE MODEL

S. CIESIÓŁKA<sup>1</sup>, J. BUDNA<sup>1</sup>, A. BRYJA<sup>2</sup>, W. KRANC<sup>2</sup>, A. CHACHUŁA<sup>1</sup>,  
M. DYSZKIEWICZ-KONWIŃSKA<sup>3</sup>, H. PIOTROWSKA<sup>4</sup>, D. BUKOWSKA<sup>5</sup>, P. ANTOSIK<sup>5</sup>,  
M. BRUSKA<sup>2</sup>, K.P. BRÜSSOW<sup>5</sup>, M. NOWICKI<sup>1</sup>, M. ZABEL<sup>1,6</sup> and B. KEMPISTY<sup>1,2</sup>

<sup>1</sup>Department of Histology and Embryology, Poznan University of Medical Sciences, Poznan, Poland; <sup>2</sup>Department of Anatomy, Poznan University of Medical Sciences, Poznan, Poland; <sup>3</sup>Department of Biomaterials and Experimental Dentistry, Poznan University of Medical Sciences, Poznan, Poland; <sup>4</sup>Department of Toxicology, Poznan University of Medical Sciences, Poznan, Poland; <sup>5</sup>Institute of Veterinary Sciences, Poznan University of Life Sciences, Poznan, Poland; <sup>6</sup>Department of Histology and Embryology, Wroclaw Medical University, Poland

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Folliculogenesis is a compound process that involves both ovarian follicle growth and oocyte development, which is tightly attached to the follicular wall. During this process, cells that form the follicle structure undergo substantial morphological and molecular modifications that finally lead to differentiation and specialization of ovarian follicular cells. The differentiation of ovarian cells encompasses formation of follicle, which is composed of theca (TCs), mural granulosa (GCs), and cumulus cells (CCs). It was previously hypothesized that GCs and CCs represent undifferentiated and highly specialized follicular cells, respectively, which may have similar primordial cell origins. In this study, we investigated the expression pattern of cumulus expansion markers such as COX2, HAS2, PTX3, and TSG6 in porcine GCs during short-term, *in vitro* culture. We hypothesized that these genes may display an important function in GCs in relation to cellular real-time proliferation. The expression pattern of COX2, HAS2, PTX3, and TSG6 was evaluated after using RT-qPCR in relation to confocal microscopy observations of protein expression and distribution during real-time proliferation of porcine follicular GCs. The COX2 and HAS2 mRNAs were highly expressed after 120 h of *in vitro* culture (IVC), whereas PTX3 and TSG6 mRNAs were increased during the first 24-48 h of IVC ( $P<0.001$ ,  $P<0.01$ ). Conversely, all of the encoded proteins were highly expressed after 144-168 h of IVC as compared to other culture periods ( $P<0.001$ ,  $P<0.01$ ). When analyzing the real-time proliferation of GCs *in vitro*, we observed a logarithmic increase of cell proliferation between 0 h and 120 h of IVC. However, after 120-168 h of IVC, the cells reached the lag phase of proliferation. Since it is well accepted that porcine GCs undergo luteinization shortly after 24-48 h of IVC, the expression pattern of investigated genes indicated that Cox2 and Has2 are independent from the LH surge, but their increased levels may be upregulated by cell proliferation *in vitro*. Moreover, higher expression of PTX3 and TSG6 during first 24 h and/or 48 h of IVC suggested that their levels are accompanied by porcine GCs luteinization process.

Folliculogenesis and oogenesis include the process of female gamete and ovarian follicular cell growth. Oocyte development is orchestrated

by the induction of several morphological and biochemical changes, finally leading to oocytes reaching the MII stage (1). In addition, the process

*Key words:* pig, granulosa cells, expansion markers, real-time cell proliferation

### Mailing address:

Bartosz Kempisty PhD,  
Department of Histology and Embryology,  
Department of Anatomy, Poznań University of Medical Sciences,  
6 Śwęcickiego St., 60-781 Poznań, Poland  
Tel./Fax: +48 61 8546419/+48 61 8546440,  
e-mail: bkempisty@ump.edu.pl

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of oocyte maturation *in vivo* and/or *in vitro* is the most crucial step toward successful fertilization and further growth and development of embryos in preimplantation stage (2). It is well recognized that the maturation of oocytes and achievement of the MII stage is associated with follicle growth, as determined by the development of the follicular granulosa cells (GCs) (3, 4). During long stages of folliculogenesis, the preantral follicles reach the antral stage, which is associated with differentiation of granulosa cells into two subpopulations: mural granulosa cells lining the follicular wall and cumulus-granulosa cells that closely surround the oocyte. There is available data that indicates the role of GCs in oocyte development and conversely, the influence of oocyte on proliferation and differentiation of GCs (5). It was found in several studies that denuded oocytes are unable to resume meiosis and reach the MII stage since cumulus-granulosa cells do not surround them (6-8). Therefore, it has been suggested that bidirectional communication between oocyte and surrounding cumulus-granulosa cells is one of the most important factors that influence growth and development of both oocytes and GCs (9). The growth of oocytes and somatic GCs is simultaneously regulated by the secretion of specific hormones, such as LH, FSH, and progesterone, which are also involved in the regulation of ovulation as well as differentiation of these cells. It was found that GCs differentiate into luteal cells both *in vivo* and during primary *in vitro* cultivation (10). The LH surge, which is stimulated by the pituitary gland, induces the process of luteinization, which is associated with the differentiation of granulosa cells into luteal cells. It is not yet fully recognized whether the luteinization process is associated with cumulus-granulosa cell expansion in an *in vitro* primary culture model. However, there are data indicating that cumulus expansion is associated with increased expression of gene markers such as pentraxin 3 (PTX3), cyclooxygenase 2 (COX2), HAS2, and TSG6 (11, 12).

In our recent studies, we have shown the expression and distribution of connexins that are responsible for proper oocyte-GCs dialog in porcine GCs during real-time, *in vitro* cell proliferation (13-17). These

experiments indicated that the proliferation, as well as the differentiation, of GCs in a porcine model is not always associated with expression of genes important for differentiation processes, such as the LHR, FSHR and/or progesterone receptors (13-17). Therefore, it is suggested that *in vitro* proliferation of GCs is a complex process regulated by various mechanisms orchestrated by different and specific pathways. In this study, mRNA expression of cumulus expansion markers COX2, HAS2, PTX3, and TSG6, as well as distribution of encoded proteins in relation to GC real-time proliferation in a primary cell culture model, was investigated.

## MATERIALS AND METHODS

### *Animals*

A total of 40 crossbred Landrace gilts with a median age of 170 days and weight of 98 kg were used in this study. All animals were housed under identical conditions. The experiment was approved by the local Ethics Committee.

### *Collection of porcine ovaries and in vitro culture of granulosa cells*

Ovaries (n=80) and reproductive tracts were recovered at slaughter and transported to the laboratory at 38°C in 0.9% NaCl within 30 min. The ovaries of each animal were placed in PBS supplemented with fetal bovine serum (FBS; Sigma-Aldrich Co., St. Louis, MO, USA). Thereafter, single preovulatory large follicles, with a diameter estimated greater than 5 mm (n=300), were opened into a sterile Petri dish by puncturing using a 5 ml syringe and 20-G needle, and the cumulus-oocyte complexes (COCs) and follicular fluid (FF) were recovered. The follicular fluid was the used to isolate GCs, whereas the COCs were discarded.

Culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, USA), 2% fetal calf serum (FCS) (PAA, Linz, Austria), 10 mg/ml ascorbic acid (Sigma-Aldrich, USA), 0.05 µM dexamethasone (Sigma-Aldrich, USA), 200 mM L-glutamine (Invitrogen, USA), 10 mg/ml gentamycin (Invitrogen, USA), 10,000 units/ml penicillin and 10,000 µg/ml streptomycin (Invitrogen, USA). Cells were cultivated at 38.5°C under aerobic conditions (5% CO<sub>2</sub>). Once the adherent cells were more than 80% confluent, they were detached with 0.05%



trypsin-EDTA (Invitrogen, USA) for 3 min and counted using a Z2 counter or cell viability analyzer (Vi-Cell XR 2.03; both Beckman Coulter, USA). For our experiments,  $3 \times 10^6$  cells per dish were used for culture, and  $5 \times 10^3$  cells were utilized per one well of the E-Plate in RTCA system.

#### *In vitro GCs culture using a real-time cell analyzer (RTCA)*

Granulosa cells recovered from follicular fluid (after puncturing of single follicles) were transferred into an E-Plate 16 of a real-time cell analyzer (RTCA, Roche-Applied Science, GmbH, Penzberg, Germany), which consists of an RTCA Analyzer, an RTCA SP Station, and RTCA Software. Granulosa cells were then cultured in 200  $\mu$ l of the standard culture medium as mentioned above. The cells were cultured for 0-168 h at 38.5°C under 5% CO<sub>2</sub> in air. The cultivation medium was replaced every 44 h. The pool of GCs before *in vitro* culture (IVC) and after 168 h of IVC was treated with trypsin (0.25% trypsin in a balanced salt solution, Sigma-Aldrich, St. Louis, MO, USA) and used for further confocal microscopy analysis. Additionally, after each step of cell culture, the Cell Index (CI) and the normalized CI were used to evaluate the relative and quantitative changes in electrical cell impedance. The cell status was determined using RTCA software. Once the cells were attached (lag phase defined as first 24 h of culture), the electrical impedance was measured. Since the cells started with log phase (logarithmic increase of proliferation occurs between 24-48 h of culture), the electrical impedance was increased. The CI or normalized CI was measured from the electrical impedance of proliferative cells.

#### *Confocal microscopic observations of protein distribution*

At recovery, after every 24 h during 168 h of *in vitro* culture, GCs were collected and fixed using an acetone-methanol (1:1, v/v) solution for 10 min at -20°C and washed three times in PBS/PVP (0.2%). To block non-specific binding, the samples were incubated in 3% BSA in PBS with 0.1% Tween-20 for 30 min at room temperature (RT). Granulosa cells were incubated for 1 hour at RT with a goat polyclonal anti-COX2 (N-20) antibody, rabbit polyclonal anti-HAS2 (H-60) antibody, rabbit polyclonal anti-PTX3 (H-300) antibody, and goat polyclonal anti-TSG6 (N-20) antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies were diluted 1:500 in PBS with 1.5% BSA and 0.1% Tween-20. After

several washes with PBS containing 0.1% Tween-20, the samples were incubated with anti-COX-2, anti-HAS2, anti-PTX3, and anti-TSG-6 primary antibodies and were treated with MFP488 goat anti-rabbit IgG (H+L) and MFP488 donkey anti-goat IgG (H+L) diluted 1:500 in PBS with 0.1% Tween-20 for 1 hour at RT. After washing in PBS with 0.1% Tween-20, the GCs were stained with 0.1  $\mu$ g/ml 4,6-diamino-2-phenylindole (DAPI, UltraCruz Mounting Medium, sc-24941; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 10 ml of mineral oil containing 1.5  $\mu$ g/ml of DAPI, mounted in an anti-fade medium on glass slides, and examined by an LSN 510 confocal system with an Olympus Fluoview 10i microscope. All confocal microscopic images were analyzed using Imaris 7.2 (BitPlane, Zurich, Switzerland) software. The protein expression pattern was estimated as the fluorescence intensity measured by the Imaris 7.2 program.

#### *Real-time quantitative polymerase chain reaction (RT-qPCR) analysis*

Total RNA was isolated from GCs before and after IVC using an RNeasy mini column from Qiagen GmbH (Hilden, Germany). The RNA samples were resuspended in 20  $\mu$ l of RNase-free water and stored in liquid nitrogen. RNA samples were treated with DNase I and reverse-transcribed (RT) into cDNA. RT-qPCR was conducted in a LightCycler real-time PCR detection system (Roche Diagnostics GmbH, Mannheim, Germany) using SYBR® Green I as a detection dye, and target cDNA was quantified using the relative quantification method. The relative abundance of PTX3, COX2, HAS2 and TSG6 transcripts in each sample was standardized to the internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For amplification, 2  $\mu$ l of cDNA solution was added to 18  $\mu$ l of QuantiTect® SYBR® Green PCR (Master Mix Qiagen GmbH, Hilden, Germany) and primers (Table 1). One RNA sample of each preparation was processed without the RT-reaction to provide a negative control for subsequent PCR.

To quantify the specific genes expressed in the GCs, the expression levels of specific oocyte mRNAs in each sample were calculated relative to PBGD and ACTB. To ensure the integrity of these results, the additional housekeeping gene, 18S, was used as an internal standard to demonstrate that PBGD and ACTB mRNAs were not differentially regulated in GC groups. The gene for 18S

rRNA expression has been identified as an appropriate housekeeping gene for use in quantitative PCR studies. Expression of PBGD, ACTB, and 18S mRNA was measured in cDNA samples from isolated GCs.

#### Statistical analysis

A one-way ANOVA followed by Tukey's post-hoc test was used to compare the results of the real-time quantification of the proliferation index. The experiments were carried out in a minimum of two replicates. The results quantifying the cell proliferation index were obtained using an RTCA system. The differences were considered to be significant at  $*P<0.05$ ,  $**P<0.01$ , and  $***P<0.001$  for the RT-qPCR and RTCA analyses. The RTCA results were evaluated by comparing the results obtained in six replicates of the same recovered granulosa cell population. Statistical calculations were applied to the highest normalized proliferation index at each time point for each investigated group to compare the results. The software program GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA, USA) was used for the statistical calculations.

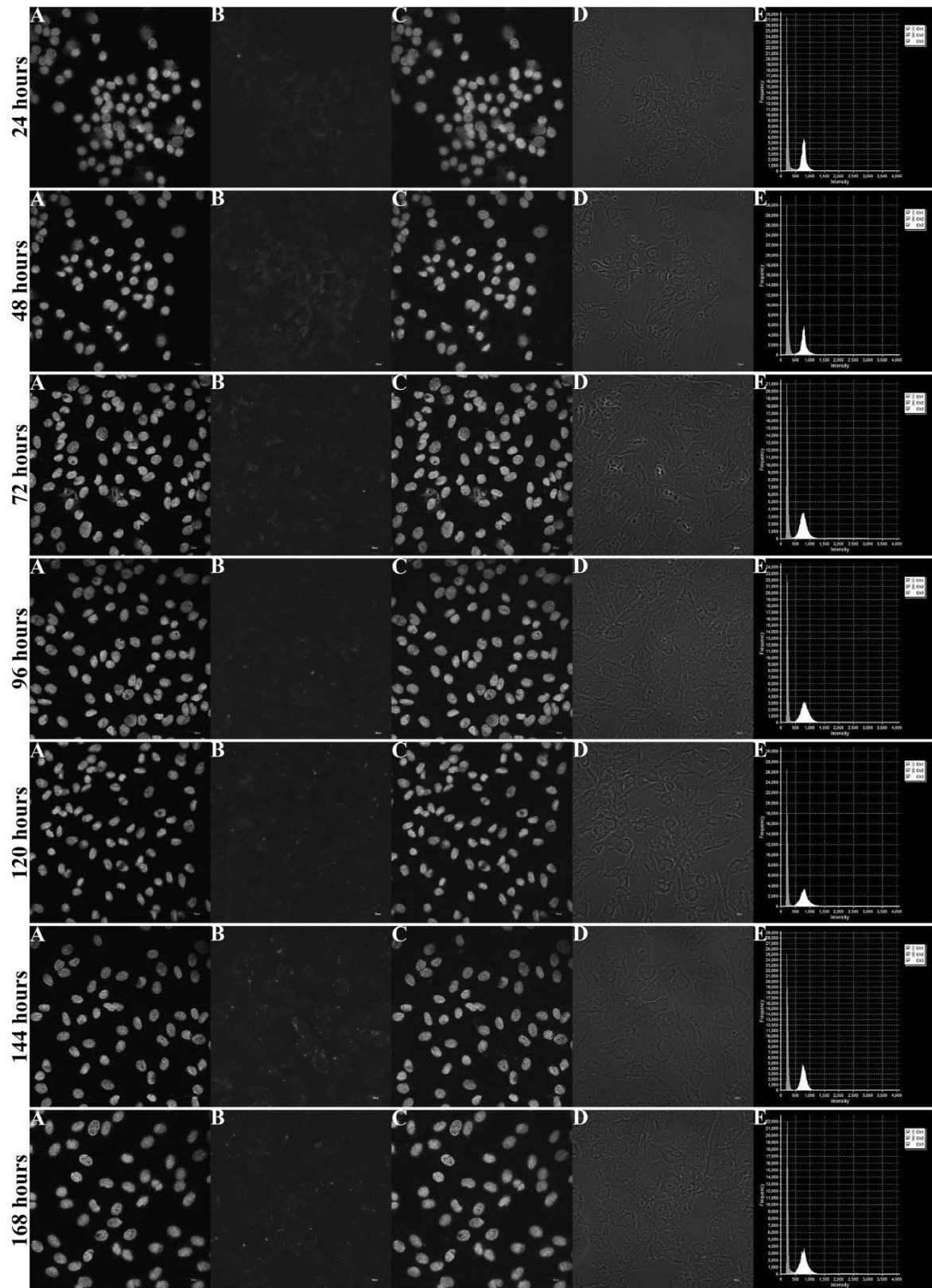
## RESULTS

Expression of COX2, HAS2, PTX3, and TSG6

mRNA and protein was analyzed using RT-PCR reaction and fluorescence intensity. The mRNA and encoded proteins levels were analyzed after each 24 h during 7 days of *in vitro*, real-time porcine follicular GC culture. We have performed immunofluorescence assay to image distribution of analyzed proteins within cells (Figs 1-4). The expression of COX2 protein measured by fluorescence intensity was higher after 144 h of IVC as compared to 24 h, 48 h, and 120 h ( $P<0.01$ ) and at the level  $P<0.001$  compared to 72 h and 96 h (Fig. 5). We found increased expression of HAS2 after 168h of IVC compared to 24 h and 48 h ( $P<0.001$ ) and compared to 72 h, 96 h, and 120 h ( $P<0.01$ ) (Fig. 5). Similarly, the expression of PTX3 was increased after 144 h and 168 h of IVC as opposed to other cultivation periods ( $P<0.001$ ) (Fig. 5). The increased level of TSG6 after 144 h of IVC compared to 24 h, 48 h, 72 h, 96 h, ( $P<0.001$ ), and 168 h ( $P<0.01$ ) was also observed (Fig. 5). Furthermore, a significant change was also observed in GCs morphology. During short-term IVC GCs changed from a cuboidal shape to a spindle shape. The expression of COX2, HAS2, PTX3 and TSG6 mRNA before and after each 24 h of IVC was monitored (Fig. 6). The expression of COX2 and HAS2 was increased after 120 h of IVC as compared

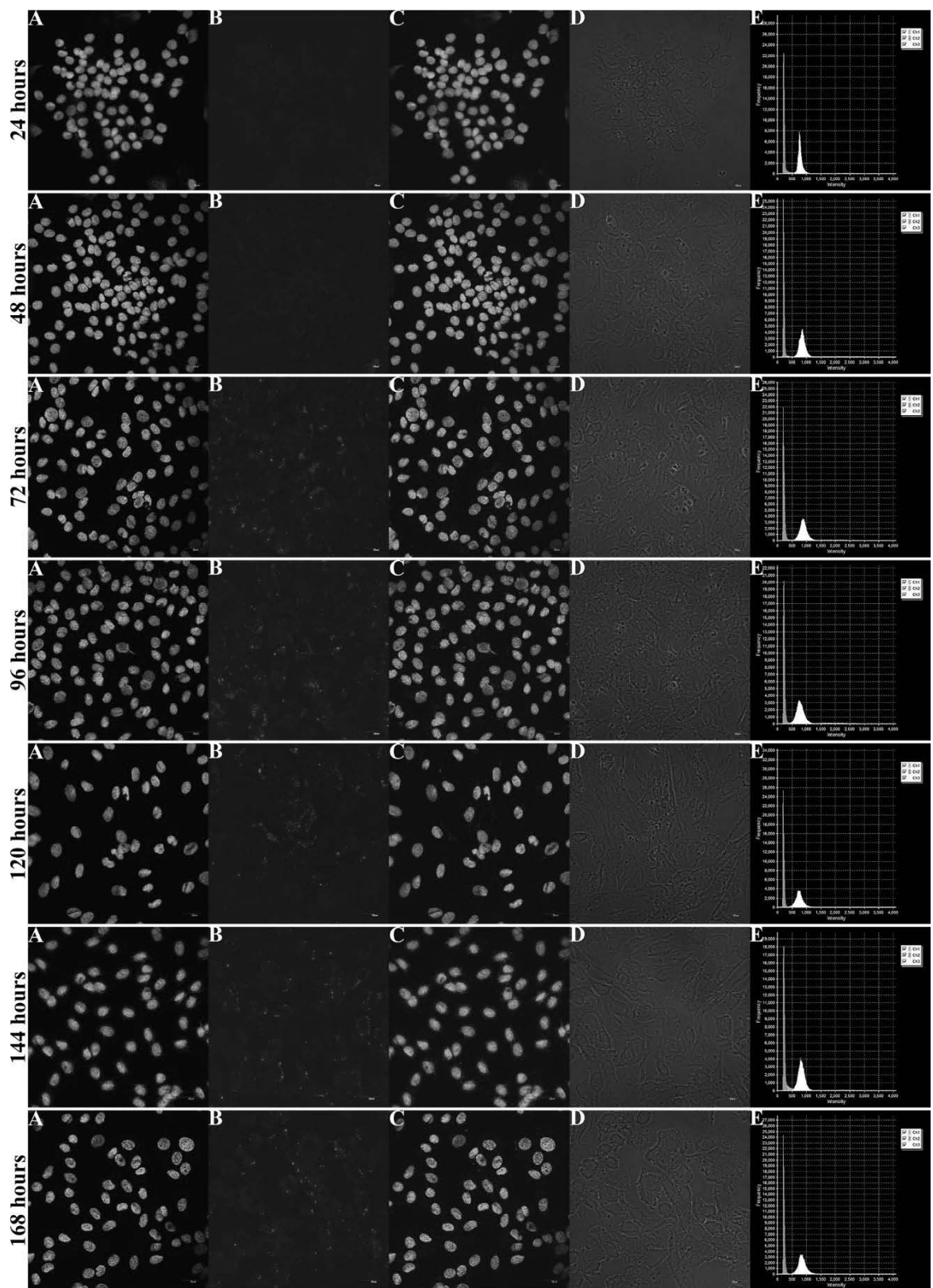
**Table I.** Oligonucleotide sequences used for RT-qPCR analysis.

| Transcript     | Sequence (5'-3' direction)                       | Gene accession no. | Product size (bp) |
|----------------|--------------------------------------------------|--------------------|-------------------|
| COX2           | TGCACTACATACTTACCCACT<br>TTGGTGGGCTATCAATCAGA    | NM_214321.1        | 123 bp            |
| HAS2           | GGCTGAGGACAACCTTTATGAC<br>CCAACGATATAAGCAGCGGTA  | NM_214053.1        | 134 bp            |
| PTX3           | CTGGAGCGCCTAGAGGAAGCT<br>AAAGCCTCAAGTTTCATTGGTGT | NM_001244783.1     | 233 bp            |
| TSG6           | GCTGAACTTCCAGAAGACATC<br>TATTCTTCCCTGACTGGTT     | NM_001159607.1     | 142 bp            |
| PBGD           | GAGAGTGCCCCTATGATGCT<br>ATGATGGCACTGAACTCCT      | NM_001097412.1     | 214 bp            |
| $\beta$ -actin | GGGAGATCGTGCGGGACAT<br>CGTTGCCGATGGTGTATGAC      | DQ845171           | 141 bp            |
| 18S rRNA       | GTGAACTGCGAATGGCTC<br>CCGTCGGCATGTATTAGCT        | AB117609           | 105 bp            |

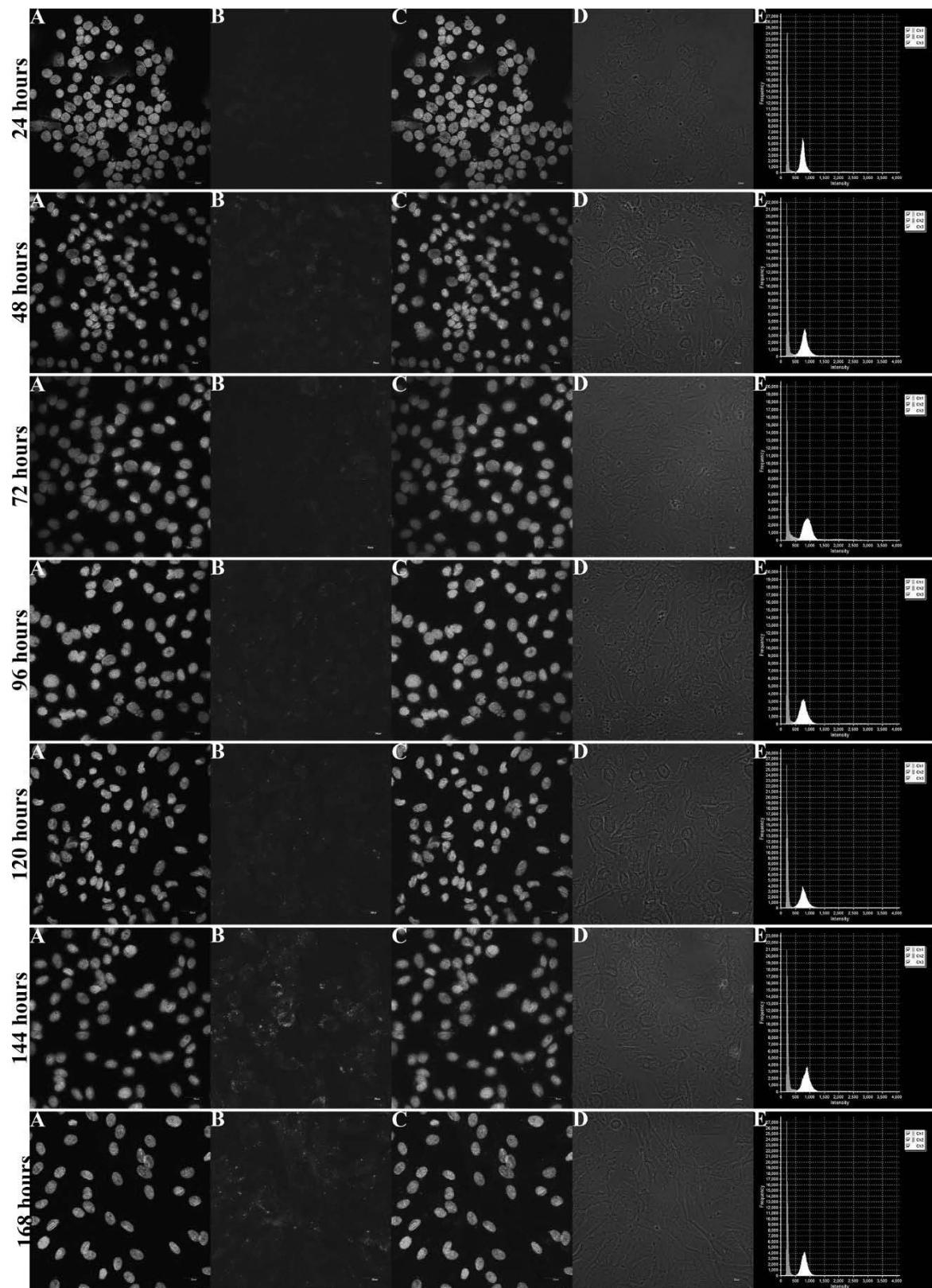


**Fig. 1.** Confocal microscopic observation of COX2 protein and cellular distribution in porcine follicular granulosa cells during 168 h of culture. The scale bars represent 20  $\mu\text{m}$ . A) DAPI; B) antibody stained cells; C) merge; D) Nomarski; E) histogram.



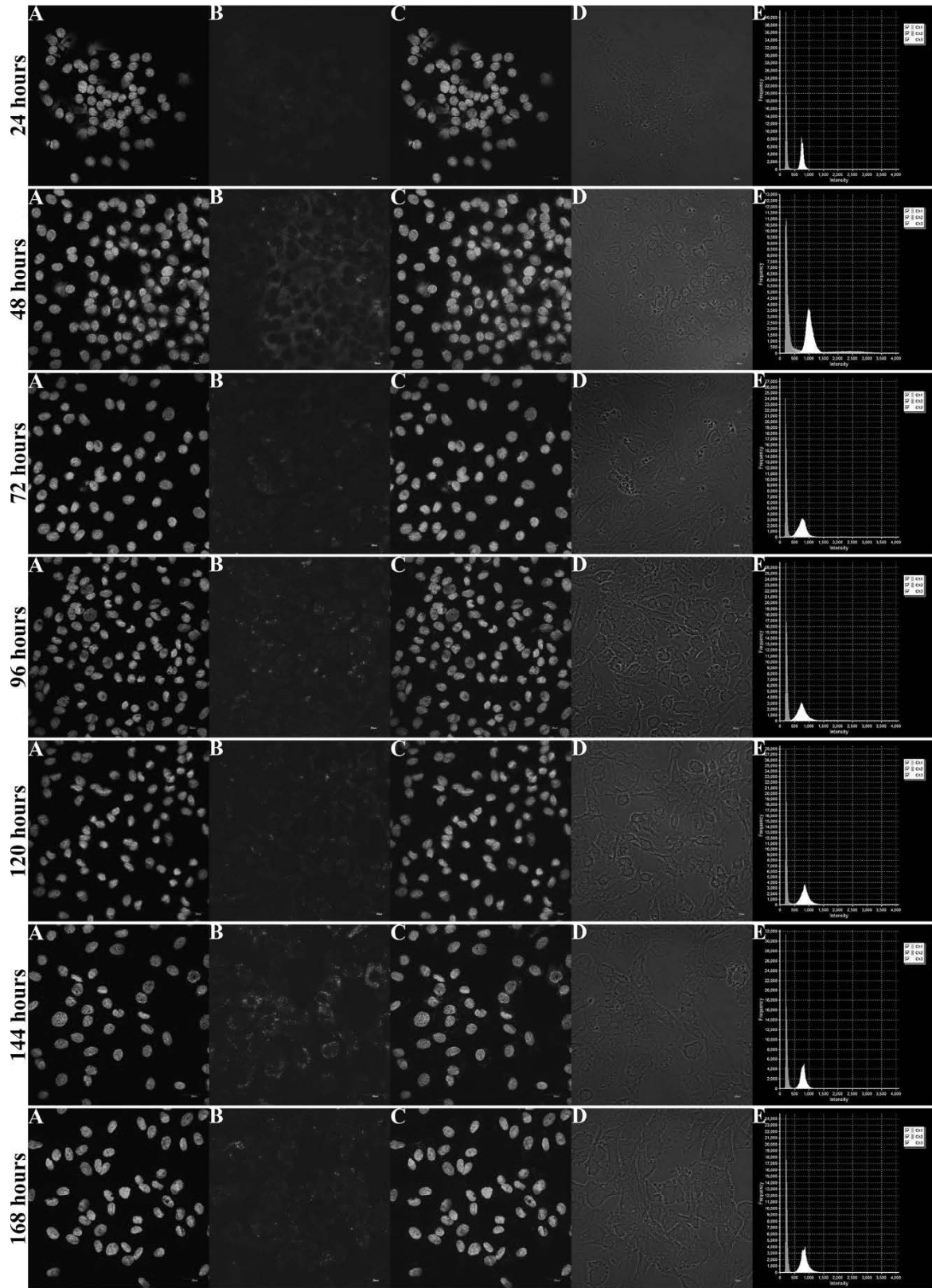


**Fig. 2.** Confocal microscopic observation of HAS2 protein and cellular distribution in porcine follicular granulosa cells during 168 h of culture. The scale bars represent 20 μm. **A)** DAPI; **B)** antibody stained cells; **C)** merge; **D)** Nomarski; **E)** histogram.

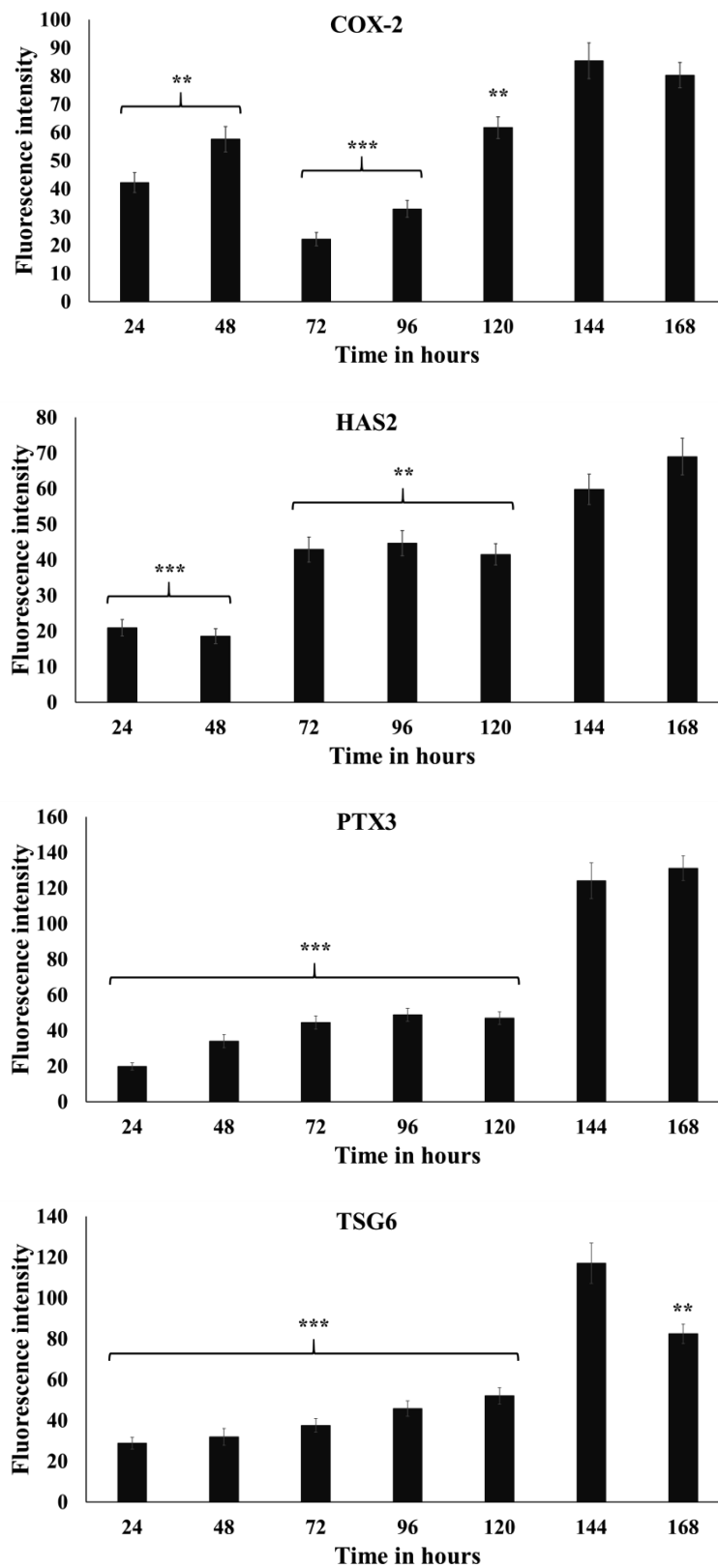


**Fig. 3.** Confocal microscopic observation of PTX3 protein and cellular distribution in porcine buccal pouch oral mucosal cells during 168 h of culture. The scale bars represent 20  $\mu\text{m}$ . **A)** DAPI; **B)** antibody stained cells; **C)** merge; **D)** Nomarski; **E)** histogram.

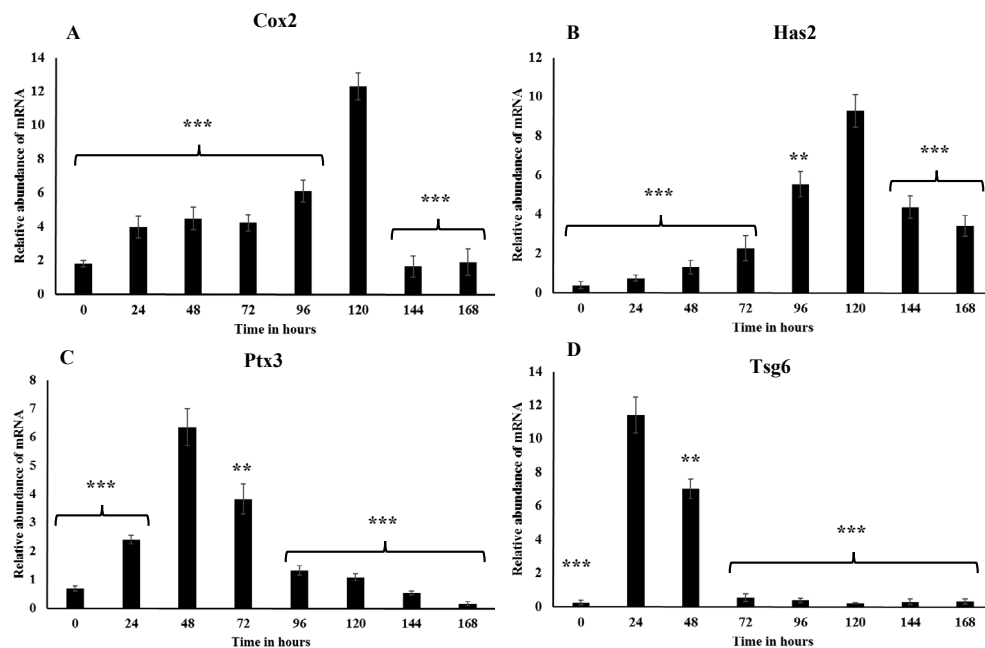




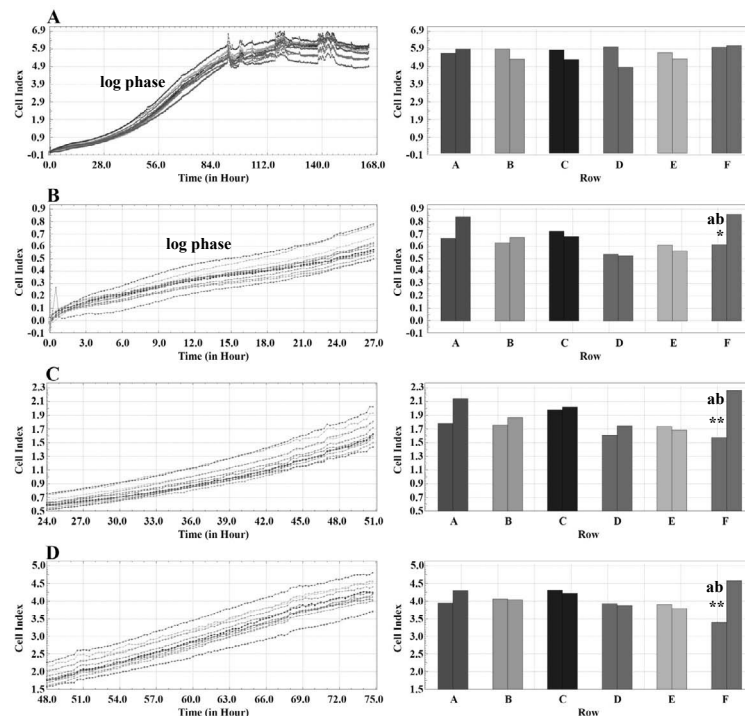
**Fig. 4.** Confocal microscopic observation of TSG6 protein and cellular distribution in porcine buccal pouch oral mucosal cells during 168 h of culture. The scale bars represent 20 μm. A) DAPI; B) antibody stained cells; C) merge; D) Nomarski; E) histogram.



**Fig. 5.** Comparison of fluorescence intensity of COX2, HAS2, PTX3 and TSG6 proteins during 168 h of culture. The differences were considered to be significant at the level of \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .



**Fig. 6.** Relative abundance of COX2, HAS2, PTX3 and TSG6 transcripts in porcine GCs analyzed during 168 h of IVC. The relative abundance of COX2 (A), HAS2 (B), PTX3 (C), and TSG6 (D) mRNAs was evaluated by RT-qPCR analysis before and after each 24 h of IVC. The results are presented as the mean  $\pm$  SEM with the level of significance shown as \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .



**Fig. 7.** Cell proliferation index of porcine GCs cultivated for 168 hours. Experiment performed to depict log and lag phase of GCs proliferation during short-term cell culture. The experiment consisted of six groups (2 replicates in each group) involving the cultivation of the same population of collected cells. At each step of the experiment, the cell proliferation index was assessed for real-time in vitro cultivation for the time periods of 0-168 h, (for 0-24 h, 24-48 h, 48-72 h, 72-96 h, and 96-120 h, 120-144 h, 144-168 h, respectively), (A, B, C, D, E, F, G, H). The differences were considered to be significant at the level of \*  $P < 0.05$ , \*\*  $P < 0.01$  and \*\*\*  $P < 0.001$ . The different superscripts described significant differences (ab).

to other cultivation periods ( $P < 0.01$ ,  $P < 0.001$ ), (Fig. 6A, B). The PTX3 was highly expressed after 48 h of culture, whereas the TSG6 mRNA level increased after 24 h of IVC, ( $P < 0.01$ ,  $P < 0.001$ ), (Fig. 6C, D). After using RTCA assays, the cell proliferation index during short-term GC *in vitro* culture was analyzed (Fig. 7). RTCA assay was performed to depict lag and log phase of GCs proliferation during short term IVC. We observed an increase in cell proliferation between 0-96 h of culture (Fig. 7 A, B, C, D, E). The lag phase of proliferation was reached between 96-168 h of GCs IVC (Fig.s 7F, G, H). After 0-24 h, 24-48 h, 48-72 h, 72-96 h, and 96-120 h, statistical differences in the proliferation index were observed ( $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.01$ ,  $P < 0.05$ ,  $P < 0.01$ , respectively). Between 120-144 h, differences were found in the proliferation index in three of six groups analyzed ( $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.001$ , respectively). Similarly, between 144 h and 168 h, differences at the level  $P < 0.01$ ,  $P < 0.01$ , and  $P < 0.001$  were detected.

## DISCUSSION

A spindle-shaped porcine granulosa cell morphology was observed during the *in vitro* cultivation period. However, it was well investigated by Kossowska, et al. that, in the human model, during long-term, *in vitro* cultivation, the GCs substantially changed their morphology from spindle-shaped to fibroblast-like granulosa cells (18). In addition, both cell populations may exist in the primary culture at the same time but in different proportions. The observations from this study indicate that the porcine GCs, during short-term cultivation (168 hours), changed their morphology from spindle-shaped to mainly fibroblast-like after reaching confluence. It can be suggested that *in vitro* cultivation of GCs may lead not only to cell luteinization after 72 hours, as was shown in several studies, but may be a crucial step in morphological and biochemical differentiation. Published data indicates various possibilities of human and mammalian GC differentiation, de-differentiation, and/or transdifferentiation, which finally lead to the formation of highly specialized cells (19-21). This phenomenon, described as GCs significant plasticity, resulted in the usage of these

differentiated cell populations as grafts in pre-clinical and clinical research (22).

The highly specialized cumulus cells, ones that tightly touch the oocyte *zona pellucida*, pass through the expansion process. It has been observed that this unique process is associated with increased expression of several genes, known as cumulus expansion markers, such as PTX3, COX2, HAS2 and TSG6 (11, 12). However, it has yet to be determined whether these markers are expressed during primary cell culture. Moreover, the expression of these markers in the population of undifferentiated GCs isolated from porcine antral follicles during short-term, real-time *in vitro* cell cultivation has never been investigated. We hypothesized that the common origin of fully specialized and differentiated cumulus cells and GCs may be a reason that both of these cell populations will express all of these markers. However, several other functional assays are needed to check the role of these genes in the population of porcine GCs.

It was recently demonstrated that mammalian GCs undergo several morphological and biochemical modifications both *in vivo* and during *in vitro* culture (23). The primary culture of GCs isolated from antral porcine follicles is also significantly associated with the luteinization process, which mostly occurs after the first 48 h and/or 72 h of IVC (14). However, during folliculogenesis and antral follicle formation, primary GCs differentiated into a subpopulation of specialized cells, known as cumulus cells (CCs), that surround the oocytes and form the cumulus-oocyte complex (COCs). Since the expansion of specialized CCs, which has granulosa origins, undergoes also *in vitro* in pigs, the expression levels of expansion markers in undifferentiated porcine follicular GCs, was investigated in this study.

Utilizing RT-qPCR and protein fluorescence assays, we observed similar expression patterns between COX2 and HAS2 mRNA and protein, which were increased after later periods of IVC (120 h, 144 h, and 168 h) as compared to the first days of culture. Contrary, PTX3 and TSG6 mRNA expression was higher after the first 24 h and 48 h of IVC as compared to the significantly higher expression of encoded proteins after 144 h and 168 h of IVC. The differential expression pattern of the mRNA and proteins may be



the result of different regulatory mechanism induced during folliculogenesis and/or activation of post-transcriptional modifications of mRNA. The higher expression of PTX3 and TSG6 during first 24 h and 48 h of IVC suggests that the mRNA expression of cumulus-related expansion markers may be associated with luteinization of porcine GCs during primary cell culture. The expression of COX2, HAS2, PTX3, and TSG6 in follicular GCs and expanded CCs may indicate a common genetic origin, and denote common biochemical regulations during early stages of folliculogenesis and antrum formation.

Recently, Romero et al. (24) investigated the ability of mouse oocytes to resume meiosis, and also examined the effect of LH and the EGF signaling pathway on ovulation and the physiological properties of fertilizable female gamete. They used the cumulus cell model for searching the effect of the LHR signaling pathway following ovulation and analyzed the expression of transcripts, e.g. HAS2, involved in antral follicle formation and synthesis of secondary messengers in the follicular wall. As a result, they found that the induction of the EGFR cascade preserved the expression of investigated mRNAs, and this signaling pathway protected the CCs from the effect of the LH surge during ovulation. Our similar observations confirm the hypothesis that expression of HAS2, similar to COX2, is negatively regulated by the LH surge following ovulation. Moreover, we suggest the cumulus expansion process must be temporarily distinct from luteinization *in vitro*. Similarly, both COX2 and HAS2 expression is not related to luteinization of porcine GCs *in vitro*. Several studies have previously shown that, following ovulation, important processes related to developmental capacity of GCs, such as CCs expansion and GCs luteinization, must occur. Tian and Diaz first described both of these processes as common and crucial for formation of a fully mature and fertilizable gamete (25). They observed that zinc administration is required for oocyte maturation, CCs expansion as measured by the expression of COX2, HAS2, PTX3, and TSG6, and the hormonal wave during ovulation. Taking into account both of these observations, we suggest that ovulation is the process associated with both CC expansion and GC

luteinization, which is orchestrated by expression of specific gene markers. By using a porcine GC culture model, we found that only PTX3 and TSG6 mRNA expression may be used as a common marker for both of these processes since they are highly expressed soon after 24 h and/or 48 h of *in vitro* culture.

A recent study by Pomini et al. (26) investigated the effect of fibroblast growth factor 10 (FGF10) on bovine COCs and early embryos. They used Hoechst reagent to determined meiosis progression, TUNEL assay to show apoptosis induction within the cells, as well as RT-PCR for evaluation of the relative abundance of developmental capacity markers such as COX2. They found that COX2, used as developmentally important gene, was up-regulated by the addition of FGF10, suggesting that gene expression may be regulated by synthesis of FGF and/or activation of FGF-related signaling during early embryogenesis in bovines. Our recent study (unpublished data) indicated higher expression of vimentin (Vim), used as the fibroblast cell and stromalization marker during short-term IVC of porcine GCs, as compared to significantly down-regulated expression of cytokeratins (panCK and CK18), which were used as the markers of their epithelial origin. Taking into account both of these results, we suggest that cell stromalization may be significantly associated with porcine GCs since they display fibroblast related gene molecular character. Conversely, COX2, used as the developmental marker under FGF10 influence, was highly expressed in embryos and expanding CCs while downregulated in undifferentiated GCs. It appears that COX2 expression in the porcine ovary is not the result of stromalization caused by the influence of FGF in porcine follicular GCs. In our recent study, LHR expression during short-term porcine GC culture was presented (14). We found that the LHR gene and protein expression was higher after 24 hours of primary culture, which may correspond with the up-regulation of LHR following an LH surge during the induction of the granulosa cell luteinization process. Alternatively, Feuerstein et al. found that COX2 levels are induced by a peak in LH and are highly expressed in human CCs after resumption of meiosis (27). Since it was observed in the current study that COX2 is highly expressed after 120 hours of IVC, we

suggested that the expression of COX2 is independent from the LH surge and GCs luteinization in this *in vitro* primary culture model.

The most recent studies involved expression profiling of PTX3 and TSG6 in relation to the LH surge and GC luteinization in animals and the response of CCs to hormonal treatment as well as specificity of their gene expression in human medicine (28). Huang et al. investigated the expression profile of PTX3 in CCs of patients with polycystic ovary syndrome (PCOS). They found that the expression level of PTX3 was significantly decreased in CCs isolated from oocytes with two normal pronuclei compared to gametes with more than two pronuclei after *in vitro* fertilization (IVF) (29). They concluded that gene expression was related to oocyte maturation, outcome of IVF, and quality of the early embryos of patients with PCOS, and the expression profile of PTX3 gene may be used as a fertilization marker. Results from our study indicated that expression of PTX3 and TSG6 mRNA may be upregulated by the LH surge and accompanied by GCs luteinization, conversely to expression of COX2 and HAS2, whose expression seems to be independent from the LH peak. However, it may be suggested that expression of COX2 and HAS2 is accompanied by follicular GCs *in vitro* proliferation since this the level of mRNA encoding these genes increased from 0h up to 120h of IVC. Conversely, it was observed that PTX3 and TSG6 mRNA expression is independent from porcine GCs *in vitro* proliferation during short-term culture.

We conclude that the expression profile of cumulus expansion markers such as Cox2, Has2, PTX3, and TSG6 in porcine GCs differ significantly and may be accompanied by GC luteinization and/or cell proliferation, which is dependent on gene function. Observation of encoded protein expression suggests that all of these investigated proteins may play a subordinate role in the process of growth and/or regulation of GC differentiation in pigs.

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## A NATURAL FORMULA CONTAINING LACTOFERRIN, *EQUISETUM ARVENSIS*, SOY ISOFLAVONES AND VITAMIN D3 MODULATES BONE REMODELING AND INFLAMMATORY MARKERS IN YOUNG AND AGED RATS

L. MENGHINI<sup>1</sup>, C. FERRANTE<sup>1</sup>, L. LEPORINI<sup>1</sup>, L. RECINELLA<sup>1</sup>, A. CHIAVAROLI<sup>1</sup>,  
S. LEONE<sup>1</sup>, G. PINTORE<sup>2</sup>, M. VACCA<sup>1</sup>, G. ORLANDO<sup>1</sup> and L. BRUNETTI<sup>1</sup>

<sup>1</sup>Department of Pharmacy, “G. d’Annunzio” University, Chieti, Italy; <sup>2</sup>Department of Chemistry and Pharmacy, University of Sassari, Sassari, Italy

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A pivotal role in osteoporosis development is played by radical oxygen species (ROS), the increased production of which is related to inhibited osteoblastic activity and bone formation. A new field of research could involve medicinal plants with antioxidant and protective effects in osteoporosis. Furthermore, considering the multifactorial metabolic aspects of osteoporosis, the pharmacological association of multiple medicinal plants could improve patient response. The aim of the present study is to evaluate *in vitro* and *in vivo* the protective effects of a natural formula containing lactoferrin 12%, *Equisetum arvensis* ES 54%, soy isoflavones 34% and vitamin D3 0.002%, in PBMC and C2C12 cells and in the bone matrix of young (3-month-old) and aged (12-month-old) female Sprague-Dawley rats, following chronic (21 days) administration. In this context, we assayed the activities of several inflammation and bone homeostasis mediators, such as IL-6, TNF $\alpha$ , PGE<sub>2</sub>, osteoprotegerin, RANK, RANKL and NF $\kappa$ B. *In vitro* studies showed that natural formula (5-1000 $\mu$ g/ml) was able to significantly inhibit ROS and PGE<sub>2</sub> production. In the same concentration range, the natural formula inhibited both TNF $\alpha$  and IL-6 gene expression. In the *in vivo* studies, we administered to young and aged female rats the natural formula at 5mg/rat for 21 days, finding a significant reduction in inflammatory PGE<sub>2</sub> and NF $\kappa$ B activity. Nevertheless, we observed a significant increase in osteoprotegerin/RANKL ratio only in aged rats, compared to the respective control group. In conclusion, our findings corroborate the rational use of natural formula in the prevention and management of osteoporotic disease.

Bone remodeling is a physiological process finely modulated by the interplay between osteoclasts and osteoblasts, which involves RANK/RANKL/osteoprotegerin pathway (1). Osteoblast and osteoclast activities are also regulated by several hormones, including parathormone and calcitonin, which increase and reduce calcium mobilization, respectively, and vitamin D, which stimulates calcium absorption and bone mineralization (2-3). An imbalance in this process

could lead to osteoporosis onset, a multifactorial age-related disease, with a major incidence in women, and which is characterized by increased bone fragility (4).

A pivotal role in osteoporosis development is played by radical oxygen species (ROS), whose increased production is related to inhibited osteoblastic activity and bone formation (5). In this context, antioxidant supplementation could inhibit bone resorption, and epidemiological and clinical studies have already

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### Mailing address:

Professor Luigi Menghini,  
Department of Pharmacy, “G. d’Annunzio” University,  
Via dei Vestini 31, 66013 Chieti, Italy  
Tel.: +39 0871 3554655 - Fax.: +39 0871 3554912  
e-mail: luigi.menghini@unich.it

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demonstrated the bone protective effects related to antioxidant administration (6).

A new field of research could involve medicinal plants with antioxidant and protective effects, in osteoporosis. Multiple preliminary studies suggested that antioxidant-rich plants, such as turmeric, *Salvia miltiorrhiza*, *Equisetum arvensis*, *Panax nota* ginseng, *Carthamus tinctorium*, *Atratyloides japonica*, and isolated active principles and extracts, such as lactoferrin, soy isoflavones and kahweol, could regulate osteoblastic and osteoclastic functions, with protective effects on bone matrix (7-13). Despite a minor efficacy compared to synthetic drugs, adverse effects following medicinal plant administration were rare (14).

In this context, the aim of the present study is to evaluate *in vitro* and *in vivo* the protective effects of a natural formula (Zelda®) containing lactoferrin 12%, *Equisetum arvensis* ES 54%, soy isoflavones 34% and vitamin D3 0.002%, in PBMC and C2C12 cells, and in the bone matrix of young (aged 3 months) and aged (aged 12 months) female Sprague-Dawley rats, following chronic (21 days) administration. The natural formula composition was selected on the basis of preliminary studies performed on the single components (7-13, 15). The dosage of the single components was assessed starting from an extrapolative model described in literature (16). In this context, we assayed the activities of several inflammatory and bone homeostasis mediators. In particular, prostaglandin (PG)E<sub>2</sub>, nuclear factor kappa-light-chain-enhancer of activated B cell (NFκB), interleukin (IL)-6 and tumor necrosis factor (TNF) α, in addition to their pro-inflammatory effects, have long been involved in bone turnover, by stimulation of osteoclast and inhibition of osteoblast function (17-19). In addition, we evaluated the effects of the natural formula on osteoprotegerin, receptor activator nuclear factor kappa B (RANK) and receptor activator nuclear factor kappa B ligand (RANKL), which play a key role in bone metabolism, by exerting biological effects on osteoblasts and osteoclasts (1).

## MATERIALS AND METHODS

### *Cell culture, viability and oxidative/inflammatory stress tests*

PBMC and C2C12 cells were cultured in DMEM (Euroclone) supplemented with 10% (v/v) heat-

inactivated fetal bovine serum and 1.2% (v/v) penicillin G/streptomycin in 75 cm<sup>2</sup> tissue culture flask (n = 5 individual culture flasks for each condition). The cultured cells were maintained in a humidified incubator with 5% CO<sub>2</sub> at 37°C. For cell differentiation, HT29, PBMC and C2C12 cell suspensions at a density of 1 × 10<sup>6</sup> cells/ml were treated with various doses (10, 50, and 100 ng/ml) of phorbol myristate acetate (PMA, Fluka) for 24 h or 48 h (induction phase). Thereafter, the PMA-treated cells were washed twice with ice-cold pH 7.4 phosphate buffer solution (PBS) to remove PMA and non-adherent cells, whereas the adherent cells were further maintained for 48 h (recovery phase). Morphology of cells was examined under an inverted phase-contrast microscope (18). To assess the basal cytotoxicity of natural formula, a viability test was performed on 96 microwell plates, using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) test. Cells were incubated with the natural formula (ranging concentration 10-1000 µg/ml) for 24 h. About 10 µL of MTT (5 mg/mL) was added to each well and incubated for 3 h. The formazan dye formed was extracted with dimethyl sulfoxide and absorbance recorded as previously described (20). Effects on cell viability were evaluated in comparison to the untreated control group. With regard to oxidative stress test, ROS generation was assessed using a ROS-sensitive fluorescence indicator, DCFH-DA. When DCFH-DA is introduced to viable cells, it can penetrate the cell and become deacetylated by intracellular esterases to form 2',7'-dichlorodihydrofluorescein (DCFH), which can react quantitatively with ROS within the cell, and be converted to 2',7'-dichlorofluorescein (DCF), which is detected by a fluorescence spectrophotometer. To determine intracellular effects on ROS production, cells were seeded in a black 96-well plate (2.0 × 10<sup>4</sup> cells/well) in medium containing scalar concentration of natural formula (10-1000 µg/ml). Cells were treated with samples for 24 h and then stimulated with (H<sub>2</sub>O<sub>2</sub> 1 mM). After cell incubation with DCFH-DA (20 µM) for 30 min, the fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm, using a fluorescence microplate reader (21). Anti-inflammatory activity of the natural formula (10-1000 µg/ml) was studied by evaluating its effects on PGE<sub>2</sub>, IL-6 and TNFα activities, from both cell lines treated with an inflammatory stimulus (H<sub>2</sub>O<sub>2</sub> 1 mM). Briefly, after a 24-h

incubation, the supernatants were removed and used for quantitative determinations of PGE<sub>2</sub> (analytical method described in 'In vivo studies' paragraph) and cells used for RNA extraction and RT-PCR quantification of IL-6 and TNF $\alpha$  gene expression (analytical method described in paragraph PCR). Cells incubated in culture medium without natural formula and treated with H<sub>2</sub>O<sub>2</sub> 1 mM were used as controls.

#### *In vivo studies*

Twelve young (aged 3 months) and 12 old (aged 12 months) female adult Sprague-Dawley rats were housed in plexiglas cages (40 × 25 × 15 cm), one rat per cage, in climatized colony rooms (22±1°C; 60% humidity), on a 12 h/12 h light/dark cycle (light phase: 07:00 – 19:00 h), with free access to tap water and food, 24 h/day throughout the study, with no fasting periods. Aged rats (starting from 9 months of age) have long been described as a valuable experimental model to study age-related bone loss (22). Rats were fed a standard laboratory diet (3.5% fat, 63% carbohydrate, 14% protein, 19.5% other components without caloric value; 3.20 kcal/g). Housing conditions and experimentation procedures were strictly in accordance with the European Union ethical regulations on the care of animals for scientific research. The study was approved by Local Ethics Committee of University "G. d'Annunzio" and by the Italian Ministry of Health.

Rats were randomized for treatments as follows: (1) vehicle (6 young rats); (2) natural formula 5mg/rat (6 young rats); (3) vehicle (6 old rats); natural formula 5mg/rat (6 old rats). Rats were subjected to single daily intragastric (gavage) administration (at 10 a.m.) for 21 days. The administered dosage (5 mg/rat) was chosen on the basis of the preliminary *in vitro* tests performed on HT29, PBMC and C2C12 cells. Twenty-four hours following the last treatment, animals were sacrificed by (100 % CO<sub>2</sub> at a flow rate of 20 % of the chamber volume per minute). Immediately after sacrifice, tissue specimens were sawn from long bones for biochemical evaluations. For PGE<sub>2</sub> evaluation, wet weighted specimens were immediately collected and maintained in a humidified incubator with 5% CO<sub>2</sub> at 37°C for 4 h, in DMEM buffer (incubation period). After the incubation period, tissue perfusates were collected and PGE<sub>2</sub> levels (ng/mg wet tissue) were measured by radioimmunoassay (RIA), as previously reported (23). Briefly, 100  $\mu$ L of PGE<sub>2</sub> standard or sample

were incubated overnight at 4°C with the <sup>3</sup>H-PGE<sub>2</sub> (3000 cpm/tube; NEN) and antibody (final dilution: 1:120000; kindly provided by prof. G. Ciabattoni), in a volume of 1.5 ml of 0.025 M phosphate buffer. Free and antibody-bound prostaglandins were separated by the addition of 100  $\mu$ L 5% bovine serum albumin and 100  $\mu$ L 3% charcoal suspension, followed by centrifuging for 10 min at 4000x g at 5°C and decanting off the supernatants into scintillation fluid (Ultima Gold™, Perkin Elmer) for  $\beta$  emission counting. Sensitivity of the assay was 2 pg/ml; ED50 was 20 pg/ml. The PGE<sub>2</sub> production was expressed as picograms per ml (pg/ml). On the other hand, separate individual bone specimens were stored at -80°C in RNAlater solution (Ambion, Austin, TX) at -20°C until further gene expression analyses of osteoprotegerin, RANK, RANKL and NF $\kappa$ B.

#### *RNA extraction*

Total RNA was extracted from the individual bone tissues and PBMC cell line using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. Contaminating DNA was removed using 2 units of RNase-free DNase 1 (DNA-free kit, Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. The RNA solution was quantified by spectrophotometer reading (BioPhotometer, Eppendorf, Hamburg, Germany) at 260 nm and its purity was assessed by the ratio at 260 and 280 nm readings. The quality of the extracted RNA samples was also determined by electrophoresis through agarose gels and staining with ethidium bromide, under UV light.

#### *Reverse transcription and real-time reverse transcription polymerase chain reaction (real-time RT PCR)*

High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA) was used to reverse transcribe 1  $\mu$ g of bone- and PBMC cell-derived RNA in a reaction volume of 20  $\mu$ L. Reactions were incubated in a 2720 Thermal Cycler (Life Technologies, Carlsbad, CA, USA) initially at 25°C for 10 min, then at 37°C for 120 min and finally at 85°C for 5 s.

Gene expression was determined by quantitative real-time PCR using TaqMan probe-based chemistry. Reactions were performed in MicroAmp Fast Optic 96-well Reaction Plates (Life Technologies, Carlsbad, CA, USA) on an ABI PRISM 7900 HT Fast Real-Time PCR

System (Life Technologies, Carlsbad, CA, USA). PCR primers and TaqMan probes were obtained from Life Technologies. For the bone tissues, the following Assays-on-Demand Gene Expression Products (20X) were used: Rn00563499\_m1 for osteoprotegerin (OPG), also known as tumor necrosis factor receptor superfamily, member 11b (TNFRSF11B) gene, Rn00589289\_m1 for receptor activator nuclear factor kappa B ligand (RANKL), also known as tumor necrosis factor (ligand) superfamily, member 11 (TNFSF11) gene, Rn04340164\_m1 for receptor activator nuclear factor kappa B (RANK), also known as tumor necrosis factor receptor superfamily, member 11a, NFKB activator (TNFRSF11A) gene, Rn00566386\_g1 for bone gamma-carboxyglutamate (GLA) protein (BGLAP) gene, Rn01399572\_m1 for nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NFKB), Rn00667869\_m1 for  $\beta$ -actin gene. For PBMC, the following Assays-on-Demand Gene Expression Products (20X) were used: Hs00174128\_m1 for tumor necrosis factor (TNF), Hs00985639\_m1 for interleukin 6 (IL6), Hs99999909\_m1 for hypoxanthine phosphoribosyltransferase 1 (HPRT1).  $\beta$ -Actin and HPRT1 were respectively used as housekeeping genes for rat and human target genes. In accordance with the manufacturer's instructions, each amplification reaction was performed with 10  $\mu$ l of TaqMan Fast Universal PCR Master Mix (2X), No AmpErase UNG (Applied Biosystems, Foster City, CA), 1  $\mu$ l of primer probe mixture, 1  $\mu$ l of cDNA, 8  $\mu$ l of nuclease-free water. The thermal cycling conditions (fast operational mode) were: 95°C for 20 s, followed by 40 cycles of amplification at 95°C for 1 s and 60°C for 20 s. The real-time PCR was carried out in triplicate for each cDNA sample in relation to each of the investigated genes. In addition to samples, individual runs included no-template controls (one for each Assay-on-Demand Gene Expression Product) and a reverse transcriptase minus control (for BGLAP gene Assay-on-Demand Gene Expression Product). Data were elaborated with the Sequence Detection System (SDS) software version 2.3 (Life Technologies, Carlsbad, CA, USA).

The comparative  $2^{-\Delta\Delta C_t}$  method was used to quantify the relative abundance of mRNA and then determine the relative changes in individual gene expression (relative quantification) (24). This method uses a calibrator sample to enable a comparison of gene expression levels

in different samples. The values obtained indicate the changes in gene expression in the sample of interest by comparison with the calibrator sample, after normalization to the housekeeping gene. 1.00 (calibrator sample) was obviously considered the theoretical mean for the comparison.

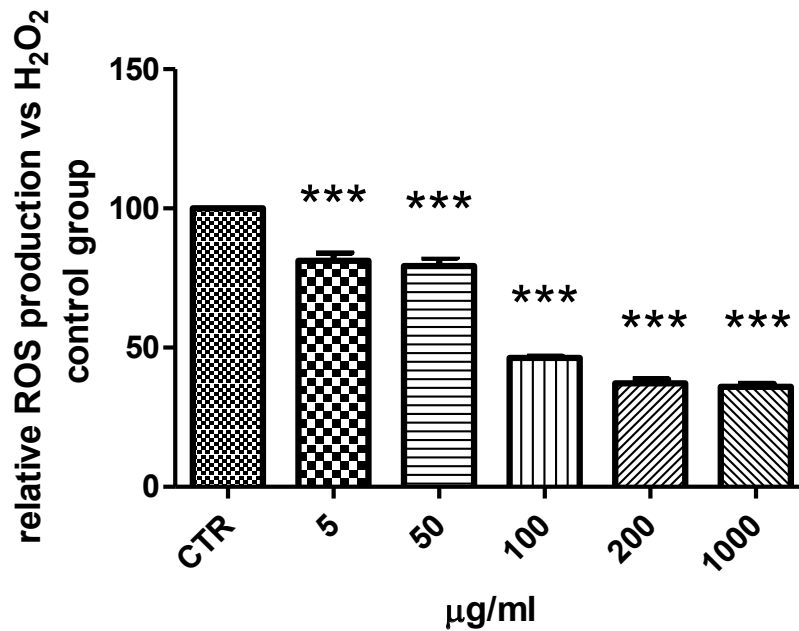
#### Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). *In vitro* data (expressed as means  $\pm$  SE) were analyzed through analysis of variance (ANOVA) followed by Newman-Keuls *post-hoc* test. *In vivo* data were analyzed through unpaired *t*-test. The results of PGE<sub>2</sub> analysis were expressed as means  $\pm$  SE. For the gene expression analysis (relative quantification method), 1.00 $\pm$ 0.00 (calibrator sample) was considered the theoretical mean for the comparison, as previously described (24). Gaussian distribution of data was assessed by D'Agostino and Pearson omnibus normality test. Statistical significance was set at  $P < 0.05$ . The number of the animals randomized for each experimental group was calculated on the basis of the "Resource Equation"  $N=(E+T)/T$  ( $10 \leq E \leq 20$ ) elaborated by the "National Centre for the Replacement, Refinement and Reduction of Animals in Research" (NC3RS) and reported on the following web site: <https://www.nc3rs.org.uk/experimental-designstatistics>.

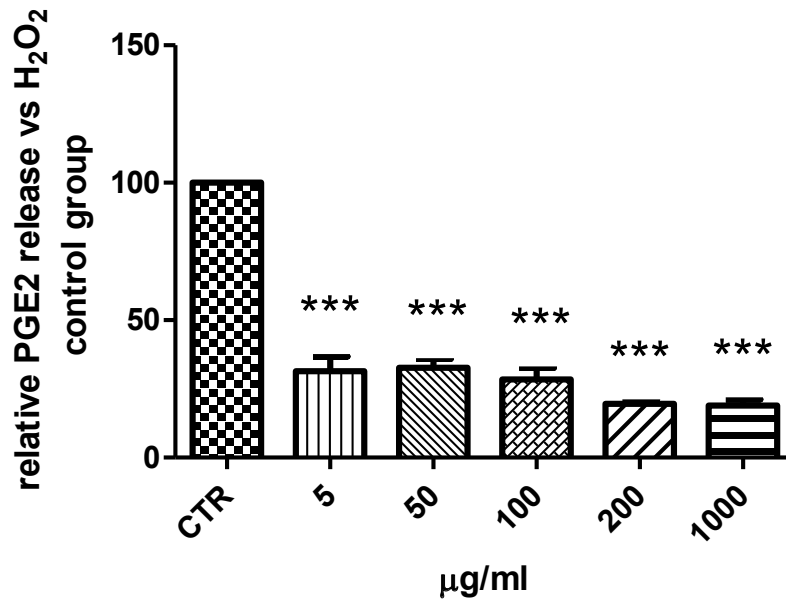
## RESULTS

*In vitro* studies showed that natural formula (10-1000 $\mu$ g/ml) was able to significantly inhibit ROS and PGE<sub>2</sub> production from C2C12 cell lines (Figs. 1-2). In addition, the natural formula (1000 $\mu$ g/ml) inhibited both TNF $\alpha$  and IL-6 gene expression (Fig. 3). Furthermore, the absence of any significant alteration in cell viability (Fig. 4 a-b) was a valuable index of non-toxic and effective concentrations to define the dose for rat treatment.

In the *in vivo* studies, young and old female rats were administered the natural formula at 5mg/rat, in a volume of 5 ml, corresponding to the highest concentration (1000 $\mu$ g/ml) tolerated by the cell lines. Single daily natural formula administration for 21 days led to a significant reduction in inflammatory

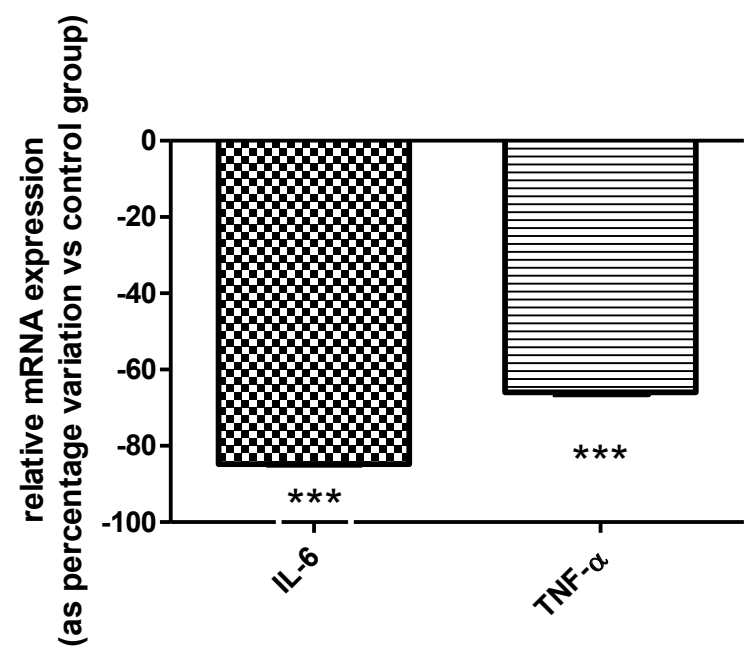


**Fig. 1.** Effect of natural formula (10-1000 µg/ml) on C2C12 cell line ROS production. ANOVA,  $P < 0.0001$ ; \*\*\* $P < 0.001$  vs vehicle.

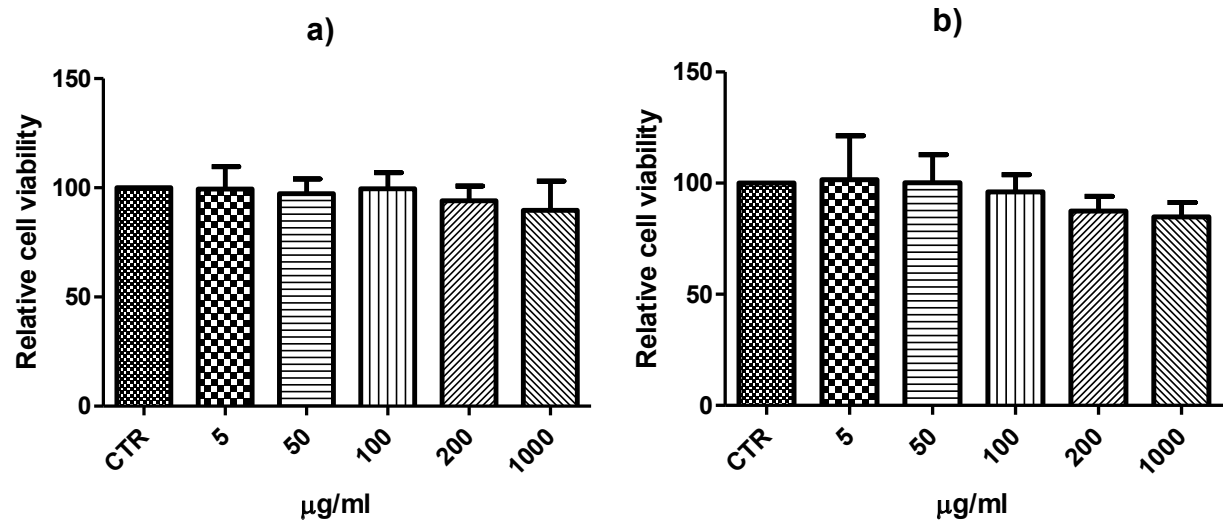


**Fig. 2.** Effect of natural formula (10-1000 µg/ml) on C2C12 cell line PGE<sub>2</sub> production. ANOVA,  $P < 0.0001$ ; \*\*\* $P < 0.001$  vs vehicle.





**Fig. 3.** Effect of natural formula (1000 µg/ml) on PMBC cell line TNFα and IL-6 gene expression. \*\*\**P*<0.001 vs vehicle.



**Fig. 4.** Effect of natural formula (10-1000 µg/ml) on PMBC (a) and C2C12 (b) cell line viability.

PGE<sub>2</sub> and NFκB activity, in bone specimens of both young and aged rats (Figs. 5-7).

Furthermore, the natural formula administration significantly up-regulated RANK, RANKL and osteoprotegerin gene expression in aged rats, with a minor inhibitory effect on young animals (Figs. 6-8). These effects are consistent with the increased NFκB and RANKL and reduced osteoprotegerin and RANK gene expression in aged rats, compared with young animals.

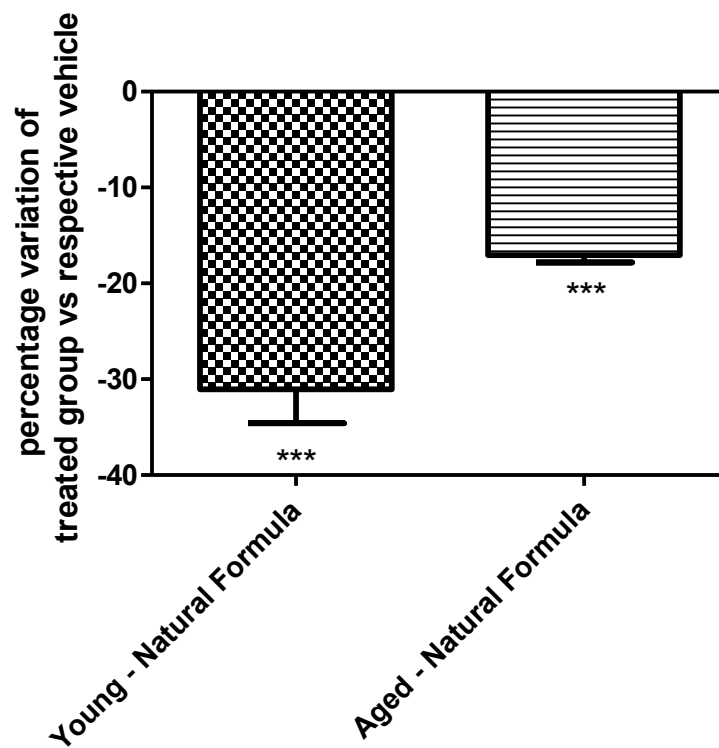
Nevertheless, a significant natural formula-induced increase was observed in osteoprotegerin/RANKL ratio in aged rats, compared to the respective vehicle group ( $1.47 \pm 0.02$ ; \*\*\* $P < 0.001$  vs vehicle). The results could account for a protective effect on bone matrix induced by the natural formula administration.

Finally, the pattern of production of PGE<sub>2</sub> was compared in young and aged rats, finding a significant decrease of PGE<sub>2</sub> level in old rats (Fig. 9).

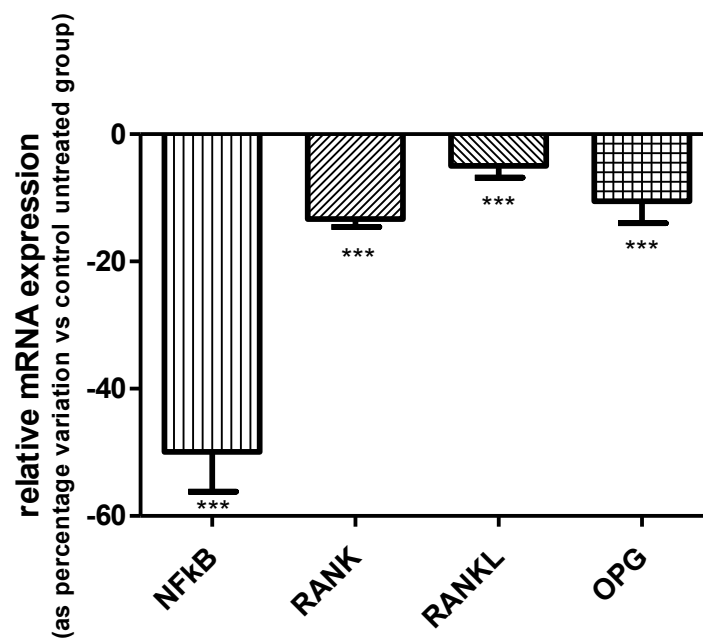
## DISCUSSION

Oxidative stress is an imbalance in the pro-oxidant/antioxidant homeostasis, characterized by excess accumulation of reactive oxygen/nitrogen species (ROS/RNS) and free radicals that can be toxic for cells by initiating disruptive peroxidation reactions on cellular substrates such as proteins, lipids and nucleic acids (25). Oxidative stress has been suggested to play a pivotal role in the imbalance between osteogenesis and bone resorption, through suppression of osteoblast differentiation and induction of osteoclast activity (5). Increased oxidative stress occurs in aging (26), and middle-aged males and females with reduced bone mineral density revealed increased urinary levels of 8-iso-prostaglandin F<sub>2α</sub> level, a stable biomarker of oxidative stress *in vivo* (27). In the present work, we demonstrated that natural formula administration reduced the activity of oxidative stress and inflammatory biomarkers, and modulated the RANK/RANKL/osteoprotegerin system. *In vitro* experiments revealed that natural formula was well tolerated by PBMC and C2C12 cell lines, as indicated by viability and ROS assays. In addition, we found a significant reduction in PGE<sub>2</sub>

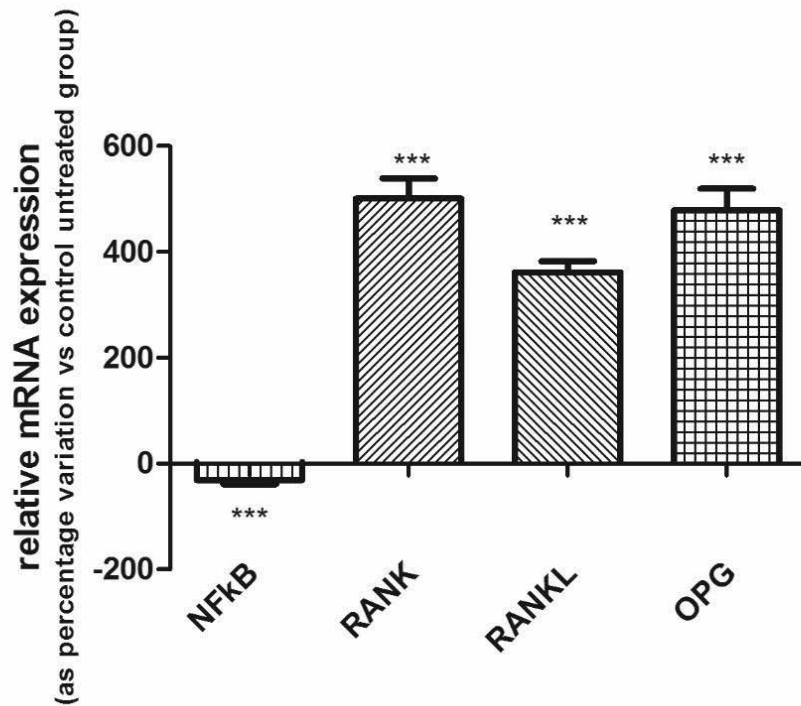
release and in both TNFα and IL-6 gene expression. Furthermore, we observed a reduction in PGE<sub>2</sub> steady state levels and NFκB gene expression in the bone matrix of both young and aged treated-rats, compared to the respective vehicle-treated control group. On the other hand, we observed reduced basal PGE<sub>2</sub> production in old rats, compared with young animals. We cannot exclude that this finding could depend on the dual role of PGE<sub>2</sub> in bone homeostasis, with both stimulatory and inhibitory effects on bone resorption (28). RANK/RANKL/osteoprotegerin axis finely regulates bone homeostasis. In particular, RANKL stimulates osteoclast activation and bone resorption, through selective binding with RANK, while osteoprotegerin, acting as a decoy receptor for RANKL, inhibits osteoclastogenesis (1). With regard to the RANK/RANKL/osteoprotegerin axis, the modulatory effect induced by the administration of the natural formula was stronger in aged rats. On one side, we observed an up-regulated RANK, RANKL and osteoprotegerin gene expression. By contrast, the osteoprotegerin/RANKL ratio, which is inversely related to osteoclastogenesis (1), was significantly stimulated in aged rats, compared to the respective vehicle-treated control group. We could hypothesize that this latter effect is partially related to our findings of reduced RANK and osteoprotegerin gene expression and increased RANKL gene expression in aged rats, compared to young animals. This is consistent with the reduced RANK/RANKL/osteoprotegerin signaling observed in non-ovariectomized aged rats (29). The increased osteoprotegerin/RANKL ratio in aged rats also agrees with the stimulated osteoprotegerin/RANKL ratio in soy isoflavones-treated osteoblasts (30). PGE<sub>2</sub>, NFκB and cytokines TNFα and IL-6, in addition to their well-characterized roles as inflammatory mediators, could also play a role as osteoclastogenic factors (17-19). The modulatory effects induced by natural formula administration, both *in vivo* and *in vitro*, suggest a protective role on bone matrix, supporting a rationale in the prevention and management of osteoporotic disease. Our findings of reduced PGE<sub>2</sub>, TNFα, IL-6 and NFκB activity corroborated previous observations concerning the capability of equisetum and lactoferrin to reduce osteoclastogenesis, by



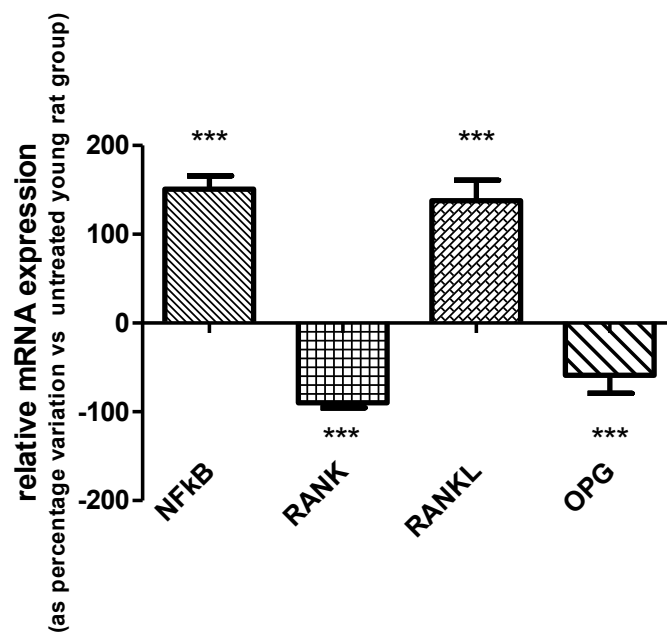
**Fig. 5.** Effect of chronic (21 days) single daily natural formula administration (5mg/rat) on bone  $PGE_2$  production, in both young and old rats. Data are expressed as relative percentage release compared to respective vehicle group. Compared to respective vehicle group, both young and aged-treated rats showed decreased  $PGE_2$  production (pg/mg tissue), following chronic natural formula administration. \*\*\* $P < 0.001$  vs respective vehicle control group.



**Fig. 6.** Effect of chronic (21 days) single daily natural formula administration (5mg/rat) on bone NFkB, RANK, RANKL, Osteoprotegerin (OPG) gene expression, in young rats. Data are expressed as relative percentage release compared to vehicle group. \*\*\* $P < 0.001$  vs respective vehicle control group.

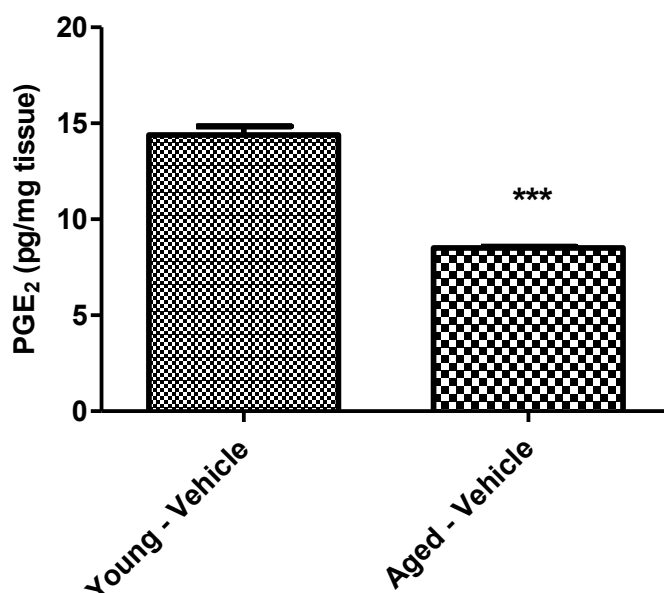


**Fig. 7.** Effect of chronic (21 days) single daily natural formula administration (5mg/rat) on bone NFkB, RANK, RANKL, Osteoprotegerin (OPG) gene expression, in aged rats. Data are expressed as relative percentage release compared to vehicle group. \*\*\* $P < 0.001$  vs respective vehicle control group.



**Fig. 8.** Effect of aging on bone gene expression, in both young and old rats. Data are expressed as relative percentage release compared to young rat group. \* $P < 0.05$  vs respective young rat group.





**Fig. 9.** Effect of aging on bone PGE<sub>2</sub> production (pg/mg tissue), in both young and old rats. Compared to young rats, old rats showed reduced basal PGE<sub>2</sub> level. Data are expressed as mean±SE of. \*\*\**P*<0.001 vs respective young vehicle group.

affecting different signaling pathways, including TNF $\alpha$ , RANKL, NF $\kappa$ B and PGE<sub>2</sub> (31-33). In addition, soy isoflavones resulted able to inhibit NF $\kappa$ B and PGE<sub>2</sub> activity, *in vivo* (34), and to contrast TNF $\alpha$ -induced down-regulation of adiponectin, an anti-inflammatory adipokine, in 3T3-L1 cells (35). It cannot be excluded that the inhibitory effects on TNF $\alpha$ , IL-6 and PGE<sub>2</sub> exerted by the natural formula could be related to the down-regulated NF $\kappa$ B signaling, a transcription factor involved in the regulation of multiple inflammatory genes (36). Neonatal exposure to soy isoflavones also improved bone outcomes in both adult male and female mice, with more significant benefits in females (37). The same authors suggested that soy isoflavones could synergize with folic acid to exert protective effects on bone matrix and reduce the risk of osteoporosis development during aging. Furthermore, considering the multifactorial metabolic aspects of osteoporosis, the pharmacological association of multiple medicinal plants could improve patient response (14). On the other hand, the clinical evidence to support this hypothesis is still inconsistent, and further studies are

needed in this research field to confirm the efficacy of medicinal plants and define therapeutic protocols.

In conclusion, the present study demonstrated that chronic single daily administration of the natural formula (Zelda®) reduced the levels of biomarkers of inflammation such as PGE<sub>2</sub>, ROS and NF $\kappa$ B, TNF $\alpha$  and IL-6 gene expression, both *in vivo* and *in vitro*. In addition, a significant up-regulation was observed of RANK/RANKL/osteoprotegerin axis in aged rats. Finally, the increased osteoprotegerin/RANKL ratio in aged rats corroborates the rational use of natural formula in the prevention and management of osteoporotic disease. These findings are consistent with previous studies concerning the role of the single components of the natural formula on bone homeostasis (28-33, 35). Nevertheless, further investigations, comparing different analytical methods and paradigms, including the experimental induction of osteoporosis *in vivo*, for detection and quantification of oxidative stress, inflammation and immune response biomarkers are required for an accurate evaluation of the protective effects on bone matrix induced by natural formula administration.

## ACKNOWLEDGEMENTS

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## EFFECT OF LOW ENERGY LIGHT IRRADIATION BY LIGHT EMITTING DIODE ON U937 CELLS

G. SPOTO<sup>1</sup>, V. DE IULIIS<sup>2</sup>, M. PETRINI<sup>1</sup>, V. FLATI<sup>3</sup>, J. DI GREGORIO<sup>3</sup>,  
D. VITALE<sup>1</sup>, M. CARUSO<sup>1</sup>, V. DADORANTE<sup>2</sup>, M. CIARMOLI<sup>4</sup>, I. ROBUFFO<sup>4</sup>,  
S. MARTINOTTI<sup>2</sup> and E. TONIATO<sup>1</sup>

<sup>1</sup>Department of Medical, Oral and Biotechnological Sciences, University G. d'Annunzio of Chieti, Italy; <sup>2</sup>Unit of Predictive Medicine and Clinical Molecular biology, SS Annunziata University Hospital, ASL Lanciano-Vasto-Chieti, University G d'Annunzio, Chieti, Italy; <sup>3</sup>Department of Biotechnological and Applied Clinical Sciences, University of L'Aquila, L'Aquila, Italy; <sup>4</sup>CNR, Institute of Molecular Genetics, Section of Chieti, Chieti, Italy

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**Photobiomodulation (PBM) can induce a set of different biological modulators either *in vitro* or *in vivo*. Experimental evidence has highlighted the role of light effects on the mechanisms related to inflammation, apoptosis and autophagy. The goal of this project was the evaluation of PBM on U937, an established cell line of histiocytic lymphoma origin. Several aspects of modulation of proinflammatory pathways were analyzed and autophagic and proapoptotic mechanisms related to low laser light exposure of cells were studied. As a source of low energy light emission, we used an NIR-LED device, characterized by an 880 nm-wavelength as light source. Flow cytometry analysis was performed on supernatants of controls and treated U937 cells to detect inflammatory cytokine levels. In order to evaluate NF- $\kappa$ B and caspase3 expressions, Western blot analysis was performed according to standard procedures. In this report, we show the effect of PBM on a monocyte/macrophage established tumor cell line (U-937). We demonstrate that LED exposure, in the presence or absence of lipopolysaccharide (LPS), activates cell degranulation, increased expression of Interleukin-8 (IL-8) and modulation of beta galactosidase activity. Evidence shows that the well-known pro-inflammatory nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) and the apoptotic marker (caspase3/cleaved-caspase3 ratio) are up-regulated in response to a proinflammatory biochemical pathway.**

Cellular exposure to low energy light activates a set of biochemical pathways. Besides this fact, very little is known concerning the biological effects on cells after the exposure of human and animal cells to electromagnetic radiation. Light-emitting diode (LED) exposure has found a medical application in patients for the treatment of wounds, inflammation and other pathological states. Photobiomodulation (PBM) is used in many therapeutic

conditions: tissue repair, wound healing, relief of inflammation, pain and edema in chronic and neurological diseases (1, 2). To date, no side effects have been reported (3) and the clinical applications are considered safe and non-invasive. PBM actions show a photochemical effect similar to photosynthesis in plants: the light is absorbed and exerts a chemical change (4). Several studies show that an optimal balance between power density and

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### Mailing address:

Dr Elena Toniato,  
Department of Medical, Oral and Biotechnological Sciences,  
University of Chieti, Via dei Vestini 31, 66100 Chieti, Italy  
Tel.: +39 0871 3554161 - Fax: +39 0871 357340  
e-mail: e.toniato@unich.it

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application time must be applied in order to have beneficial effects. The biochemical mechanisms underlying the positive effects of LED therapy are under investigation, thus the rationale for choosing well-defined parameters such as “wavelength”, “fluence”, “power density”, “pulse structure” and “treatment timing” for our experimental setup, were based on several published clinical trials (5, 6).

Cytochrome c oxidase (COX) is the primary photo-acceptor for the red-NIR range in mammalian cells, triggering a cascade of cellular and molecular events that have significant and beneficial biological effects (7, 8). The PBM-induced effects are mediated by several mechanisms: i) the absorption of photons by mitochondria leads to intensification of electron transfer reactions and then to increased formation of ATP, necessary for several cell functions, including cell signaling, contraction or gene expression (9); ii) PBM can also influence the expression of inducible nitric oxide synthase (10-12); iii) the activation of the respiratory chain, by illumination, can result in an alteration of cellular redox state caused by an increase of the superoxide anion  $O_2^-$  (13, 14).

The modification of the mitochondrial redox state affects several intracellular signaling pathways including those mediated by some transcription factors involved in proliferation, differentiation and/or apoptosis such as Fos, Jun, NF- $\kappa$ B, P53, ATF-CREB and HIF-1 (15-17). Furthermore, the modification of ATP and the co-action of Nitrox-Oxide (NO) and Reactive Oxygen species (ROS) cooperate in the activation of a complex network of transcription factors that increase gene expression and regulate cellular growth, extracellular matrix deposition, cell proliferation and motility (15). In particular, most of these pathways act through the nuclear factor NF- $\kappa$ B, which in the inactive state floats in the cytoplasm as a dimeric protein complex, made of p65 and p50 proteins, bound to the inhibitory factor I $\kappa$ B- $\alpha$ . Upon activation, I $\kappa$ B- $\alpha$  is degraded, releasing the p65 member of the NF- $\kappa$ B complex that is phosphorylated and thus can migrate into the nucleus, where it binds to the promoter of target genes (18, 19). Many of these genes encode cytokines and mediators of apoptosis or programmed cell death (PCD), activated under proper stimuli or as a consequence of an active pro-inflammation status. PCD plays a key role in homeostasis of normal tissues (20, 21). In fact, dysregulation of apoptosis is related to many pathologies such as cancer, autoimmune disease

(insufficient apoptosis), acute and chronic degenerative diseases and infertility (accelerated apoptosis) (22). In low stress condition, the cell initiates autophagy, a mechanism activated to maintain homeostasis, but when stress increases and reaches a level too high to be controlled, the cell inhibits autophagy and initiates apoptosis (23). As a consequence, Cytochrome c passes through the mitochondrial membrane in the cytosol, binds the apoptotic protease-activating factor-1 (Apaf-1) and procaspase-9, forming a multi-protein complex, namely aptosome. This complex activates pro-caspase-3, 6 and 7 and initiates apoptosis (24). At the same time, caspases inhibit autophagy by degrading autophagy proteins such as Beclin1, Atg5, and Atg7 (25).

In this study we analyzed the biological effects of Photobiomodulation on U937 cells to investigate the modulation of cytokine release following NF- $\kappa$ B activation and the involvement of the autophagy/apoptosis processes. We show that two cytokines, IL-8 and IL-1 $\beta$ , are induced by treating cells with LED at 880 nm at increasing power density. In addition, a higher and statistically significant expression of IL-8 is associated with an intense cytoplasmic degranulation effect. IL-8 expression was correlated with accumulation and activation of the p65 NF- $\kappa$ B and with increased level of cleaved Caspase-3, suggesting apoptosis activation. Autophagy process seems not to be involved, according to the biological effects induced under our specific conditions of light exposure on U937 cells.

## MATERIALS AND METHODS

### *Light source and irradiation*

An NIR-LED device, characterized by an 880 nm-wavelength was used as light source (Tr-Lux, Errevi, Italy). A LED source was applied on a macrophage line (U937) cultured in standard conditions for a maximum period of 18 min, respectively for 1, 3, 5, 10, 18 min and incubated at 37°C for 48 h. Basing on the different LED irradiation applied to the cells, different groups were distinguished: IR 18 (LED irradiation = 6 min at 2.37 mJ/s, irradiance = 2.1 mW/cm<sup>2</sup>, fluence = 0.756 J/cm<sup>2</sup>; 3 min at 8.15 mJ/s, irradiance = 7.21 mW/cm<sup>2</sup>, fluence = 1.297 J/cm<sup>2</sup>; 3 min at 4.37 mJ/s, irradiance = 3.86 mW/cm<sup>2</sup>, fluence = 0.695 J/cm<sup>2</sup>; 3 min at 4.47 mJ/s, irradiance = 3.95 mW/cm<sup>2</sup>, fluence = 0.711 J/cm<sup>2</sup>; 3 min at 2.46 mJ/s, irradiance = 2.18 mW/



cm<sup>2</sup>, fluence = 0.392 J/cm<sup>2</sup> for a total irradiation of 4354.2 mJ), IR 10 (6 min at 2.37 mJ/s, fluence = 0.756 J/cm<sup>2</sup>; 3 min at 8.15 mJ/s, fluence = 1.297 J/cm<sup>2</sup> and 1 minute at 4.37 mJ/s, fluence=0.232 J/cm<sup>2</sup> for a total of 2582.4 mJ), IR 5 (5 min at 2.37 mJ/s and fluence = 0.63 J/cm<sup>2</sup> for a total of 711 mJ), IR 3 (3 min at 8.15 mJ/s, fluence = 1.297 J/cm<sup>2</sup>) and IR 1 (1 min at 4.37 mJ/s, fluence=0.232 J/cm<sup>2</sup>). In addition, irradiation was also performed twice for 5 and 10 min.

#### Cell culture

U937 cells (ATCC; Manassas, VA, USA, catalogue number: CRL-3253™) were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 1% Pen-Strep at 37°C in 5% CO<sub>2</sub>. Where specified, cells were co-treated with lipopolysaccharide (LPS) at a final concentration of 0.5 µg/ml. In order to better understand the mechanism of Photobiomodulation, U937 cells were irradiated and exposed to a time course application of LED ranging from 1 min up to 18 min w/out LPS; after irradiation the cells were left to recover at 37°C for 48 h. As far microscopic evaluation of degranulation U937 cells, treated for 18 min at LED exposure compared to untreated controls were observed under contrast-phase microscopy. All experiments were performed in triplicate to allow statistical analysis.

#### Cell viability

The cell viability was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Inc.) assay according to standard procedure for suspension cells. At this point, the absorbance (optical density) was measured at 530 nm using a microplate reader (Infinite F50, TECAN). This experiment was performed in triplicate.

#### Flow cytometry

The concentration of cytokines released in the supernatants of U937 was determined using flow cytometry according to supplier's procedure (BD, USA). A series of FITC-mouse anti-human antibodies were used. A panel of 8 different cytokine antibodies (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IFN-γ, and TNFα) was used and different levels of induction were observed among them.

#### Western blots

We evaluated the concentration of p65 NF-kB, LC3BI/

II modulation and cleaved Caspase-3, which represents the activated form of Caspase-3 as a sign of PCD. Total proteins were extracted from lymphomonocyte in lysis buffer (50 mM Tris.Cl pH 7.8, 1% Triton X100, 0.1% SDS, 250 mM NaCl, 5 mM EDTA, 100 mM NaF, 2 mM NaPPi, 2 mM Na3VO4, 1 mM PMSF) as described (26). Cell lysates were separated on 12% SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad). Membranes were incubated overnight at 4°C with specific primary Abs (polyclonal rabbit anti-p65 and anti-caspasi-3, from Santa Cruz Biotechnology, diluted 1/100). Immunocomplexes were visualized using the ECL detection system (Amersham Pharmacia Biotech). Densitometric analysis was performed for the quantification of the immunoblots, using the Molecular Analyst System (Bio-Rad) program.

#### β-galactosidase activity assay

20 µL of U937 supernatant was applied to a paper disc, containing X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside). After 10 min of incubation at room temperature, paper discs were put into a test tube containing 400 µL of N,N-dimethylformamide (DMF, Sigma-Aldrich) for 15 min. The β-galactosidase was carried out spectrophotometrically according to the literature (27, 28). All analyses were carried out at a constant pH 6.00.

#### Statistical analysis

Data are expressed as means ± SEM. Student's *t*-test or ANOVA followed by the appropriate post-hoc test were used (Stat View 4.0 software, Abacus Concepts, Berkeley, CA, USA). P values < 0.05 were considered statistically significant, with a confidence interval of 95%.

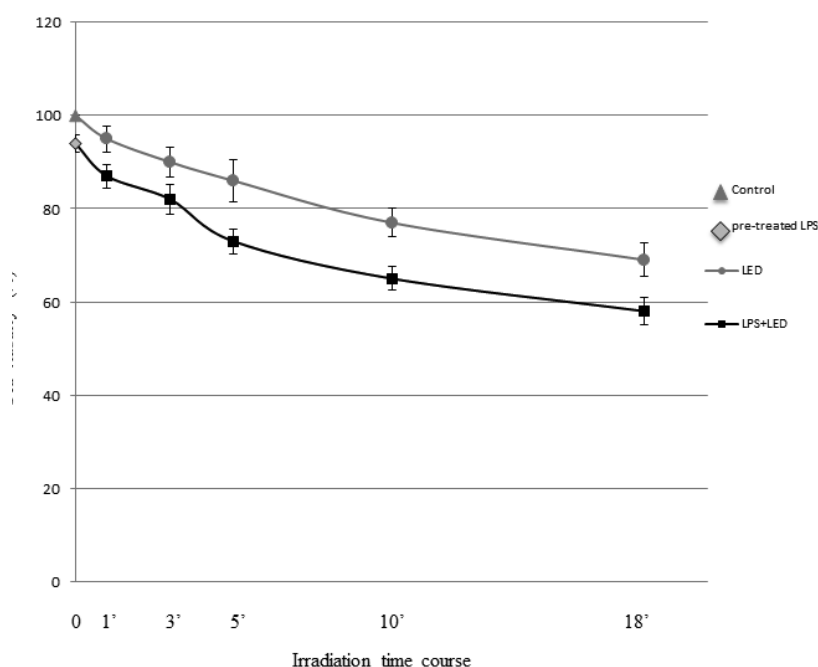
## RESULTS

#### LED w/out LPS reduces cell viability

The proliferation of U937 cells was measured using MTT assay and expressed as a ratio of color intensity from the LED w/out LPS treatment groups to that of the control group. Fig. 1 demonstrates that the viability of cells, undergoing to time course irradiation, was reduced by increasing the time of irradiation.

#### Cytokine release after irradiation of U937 cells at 880 nm in presence or absence of LPS

The cytokine release pattern was analyzed, showing



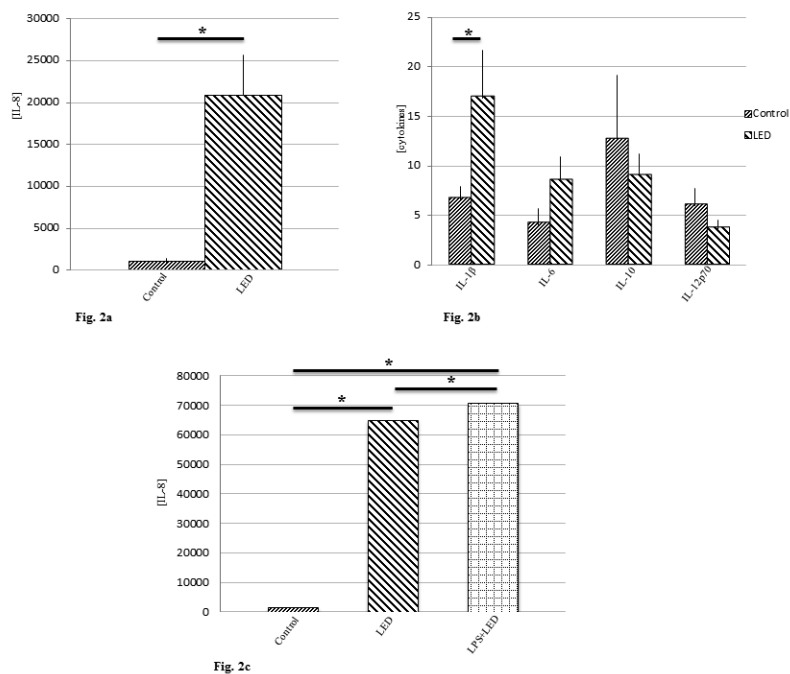
**Fig. 1.** Cell viability of U937 treated with LED w/out LPS. Cell viability of U937 cells with LED w/out LPS was lower compared to the control group. U937 cells were co-treated with lipopolysaccharide (LPS) at a final concentration of 0.5  $\mu\text{g/ml}$  and irradiated for 18 min. Cell viability was determined by MTT assay.

a difference between the two tested groups, control and treated, and that was statistically significant for IL-8 and IL-1 $\beta$  (Fig. 2 a-b). The investigated cytokines did not show statistically significant differences between these two groups [IL-6, IL-10 and IL-12p70 (Fig. 2b) and IL-2, IL-4, IL-5, IFN- $\gamma$ , and TNF $\alpha$  (data not shown)]. Since there was evidence of a selective induction of IL-8 release by LED low energy irradiation, which was statistically significant in respect to control conditions, we analyzed the effect of U937 exposure to LED in the presence or absence of Lipopolysaccharide (LPS) as an inflammatory inducer. As a matter of fact, LPS has been extensively associated to pro-inflammatory induction controlling the steady state level of cytokines, including IL-8 release during degranulation process of immune cells. As shown in Fig. 2c and Table I, in irradiated cells pre-treated with LPS, the cytokine pattern showed a slight increase on IL-8 release, compared to the effect of LED exposure and of LPS treatment alone. The plotting ratio between the controls, the treatments with LED irradiation alone and the treatments with LPS and irradiation was statistically

significant [Table I and Fig. 2c. Anova test:  $P < 0.001$ ; differences between the controls and the two different treatments (with or without LPS) and between the two treatments:  $P < 0.05$ ]. The analysis of the combined data showed evidence of a mild effect of LPS co-treatment with light irradiation on U937 cells and leading to activation of pro-inflammatory pathways.

#### *Modulation of p65 NF-kB expression upon treatment of U937 cells with 880 nm LED irradiation in presence or absence of LPS*

Being a classical marker of the inflammatory response as well as of apoptosis, the NF-kB expression was evaluated. For this purpose a specific antibody raised against the phosphorylated p65 NF-kB protein was used. Western blotting analysis showed a higher effect in terms of p65-NFkB expression on LPS and irradiation treated groups, within 5 min, compared to the only irradiated cells (Fig. 3 a-b). The expression of p65-NFkB was observed in the treated group, but not in the controls (Fig. 3 c-d). The increased phosphorylation of the p65 NF-kB



**Fig. 2.** Evaluation of cytokine expression on cells undergoing LED exposure with or without LPS pretreatment. U937 cells were irradiated and exposed to an application of LED for 18 min. Where specified, cells were co-treated with lipopolysaccharide (LPS) at a final concentration of 0.5  $\mu$ g/ml. After irradiation the cells were left to recover at 37°C for 48 h. a) Evaluation of IL-8 expression on cells undergoing LED exposure. The mean concentration of IL-8 was respectively: in control dishes 963 pg/ml (SD  $\approx$  415 pg/ml) and in treated dishes with irradiation for 18 min 20,836 pg/ml (SD  $\approx$  4,811 pg/ml). The difference between these two groups was statistically significant ( $p = 0.002$ ). b) Evaluation of cytokine release on LED exposed cells. The mean concentration of IL-1 $\beta$  was respectively: in control dishes 6.62 pg/ml (SD  $\approx$  1.28 pg/ml) and in treated dishes 16.88 pg/ml (SD  $\approx$  4.76 pg/ml). The difference between these two groups was statistically significant ( $p = 0.023$ ). There were no statistically significant differences between these two groups for the other cytokines investigated. c) Comparable expression of IL-8 on LED exposed cells with or without LPS pretreatment. The mean concentration of IL-8 was respectively: in controls 1,425 pg/ml (SD  $\approx$  1,291 pg/ml), in treated with 64,783 pg/ml (SD  $\approx$  3,147 pg/ml), and in treated with LPS and irradiation 70,684 pg/ml (SD  $\approx$  2,879 pg/ml). The difference between the control group and the treated group was statistically significant (Anova test:  $p < 0.001$ ; Bonferroni test: Control group vs treated with irradiation  $P < 0.05$ . Control group vs treated with LPS and irradiation  $p < 0.05$  and treated with irradiation vs treated with LPS and irradiation  $p < 0.05$ ).

(Fig. 3c, line 6) was correlated with an increase of cell concentration, prior to LED treatment. In addition, when U937 cells underwent LED exposure for 5 and 10 min with one or two boosts of irradiation, Western blots showed a higher protein expression of p65-NF $\kappa$ B in samples treated with one LED irradiation for 5 min compared to the other groups, depicting the fact that one treatment was sufficient to increase the steady state level of the protein. In fact, Fig. 4 shows a higher intensity of the band in samples treated with one application for 5 min compared to samples treated with one induction for 10 min ( $P = 0.03$ ) and to samples treated with two treatments ( $P = 0.0003$ ). We constantly had evidence that the steady protein level of p65 decreases after

LED exposure, maybe indicating a metabolic consumption of inactive forms in respect to the phospho-activated p65 molecules that accumulate in response to light induction.

#### Modulation of autophagy and apoptosis by cell irradiation

The evaluation of the microtubule-associated LC3BII protein, which is a recognized marker of autophagy, did not show statistically significant differences between controls and treated samples, supporting the induction of apoptosis pathway, but not the activation of autophagy (Fig. 4). The ratio caspase3/cleaved-caspase3 was decreased on irradiated cells and this modulation was statistically significant. Furthermore, a higher amount

was found of the cleaved-caspase3 in samples treated for 5 min with one LED application compared to 10 min (Fig. 5 a-b). As shown for p65 steady state level, short exposure timing seemed to be more effective for early activation of proteins involved in inflammation and/or apoptosis.

#### *Analysis of galactosidase activity after LED irradiation*

As a marker of cytoplasmic vacuole activation,  $\beta$ -galactosidase release was assayed and it was found increased in cells treated with time-different LED applications (Fig. 6). Statistically significant differences between controls and tests irradiated at different time were found for  $\beta$ -galactosidase activity (respectively, control, 5 and 10 min; IR5:  $p < 0.05$ ). There were no significant differences in samples irradiated for 10 min (IR 10). After

LED application a pronounced degranulation effect could be detected by optical microscopy observation (Fig. 7).

## DISCUSSION

It has been shown in clinical dermatologic studies that photobiomodulation may orchestrate biochemical pathways related to inflammation and cellular remodeling (24). In this report, we determined the effect of LED treatment on U937 cell line. We applied LED protocols with several power densities, according to the manufacturer's suggestions. Many *in vitro* studies on pro-inflammatory cell lines, undergoing low energy light exposure, have shown interference on the steady-state biochemical anti-inflammatory signal transduction pathways. As markers of

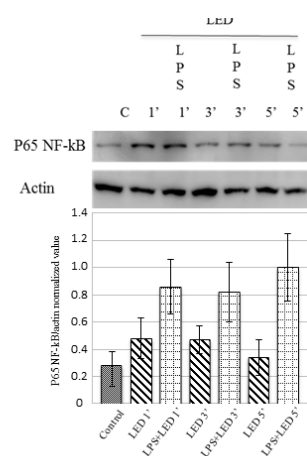


Fig. 3a

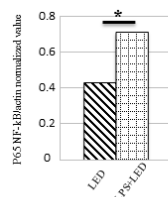


Fig. 3b

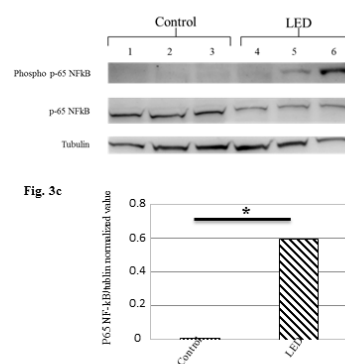
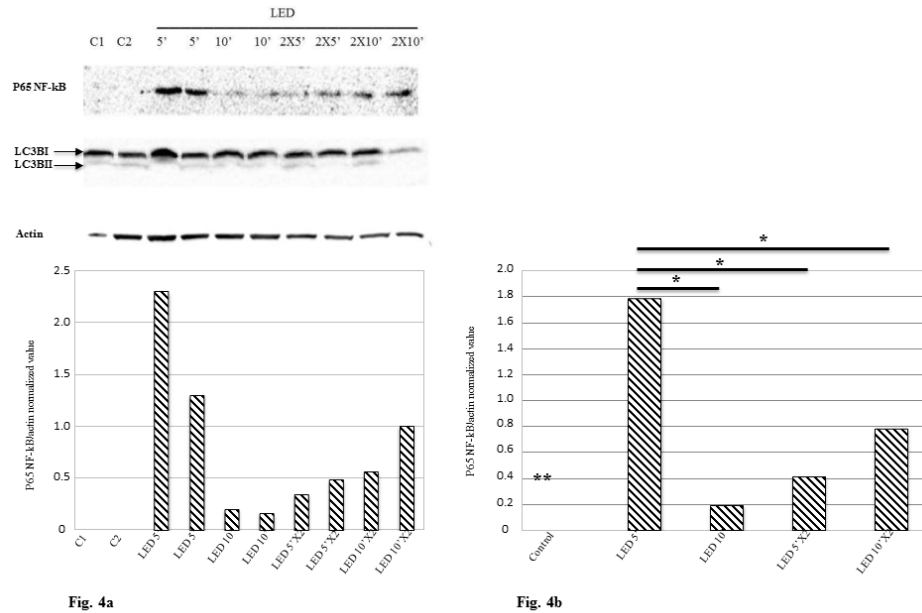


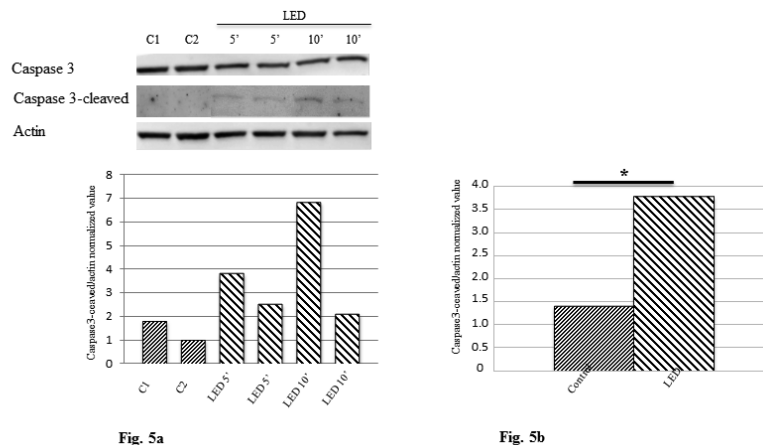
Fig. 3c

Fig. 3d

**Fig. 3. a)** NF- $\kappa$ B activity on U937 cells induced by LED with or without LPS pretreatment. U937 cells were irradiated and exposed to a time course application of LED ranging from 1 min up to 5 min w/out LPS, at a final concentration of 0.5  $\mu$ g/ml; after irradiation the cells were left to recover at 37°C for 48 h. Western blot shows a lower expression of p65 in samples treated with LED compared to samples treated with LPS and LED. **b)** Evaluation of statistical analysis on the p65 activation on LED exposed U937 cells with or without LPS. Statistical analysis shows ratio values of p65/actin respectively on LED with or without LPS groups ( $p = 0.003$ ). **c)** p65 activation on LED-treated cells. Western blot shows the activation of p65-NF $\kappa$ B in treated group, but not in controls. The increased phosphorylation of the p65 NF- $\kappa$ B (lane 6) was correlated with an increase of cell concentration (Plating condition: lanes 1, 4 are  $1 \times 10^5$  cells/ml; lanes 2, 5 are  $2.5 \times 10^5$  cells/ml and lanes 3, 6 are  $5 \times 10^5$  cells/ml). LED treatment was for 18 mins, as specified in materials and methods. **d)** Evaluation of statistical significance on p65 activation after cell exposure to LED. Statistical analysis shows ratio values of P65/actin respectively on LED and control groups ( $p < 0.001$ ).

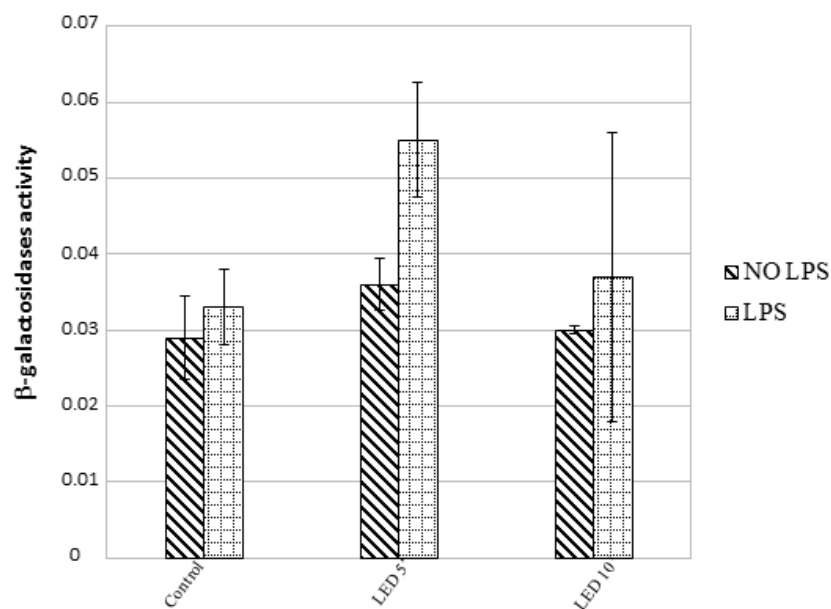


**Fig. 4. a)** Increased steady state level of p65 after early LED treatments of U937 cells and LC3BII modulation of cells undergoing LED exposure. U937 cells undergoing LED exposure for 5 and 10 min with one or two boosts of irradiation. Western blot shows an activation of p65 on treated groups compared to the control group, whereas LC3BII Western blot analysis, did not show any statistically significant modulation. **b)** Evaluation of statistical significance on p65 after early LED treatments of U937 cells. Western blot shows a higher expression of p65 between control and treated groups (\*\*:  $p < 0.001$ ) and in samples treated with one shot of irradiation for 5 min compared to samples treated with one boost for 10 min ( $p = 0.03$ ) and to samples treated with two boosts ( $p = 0.0003$ ).

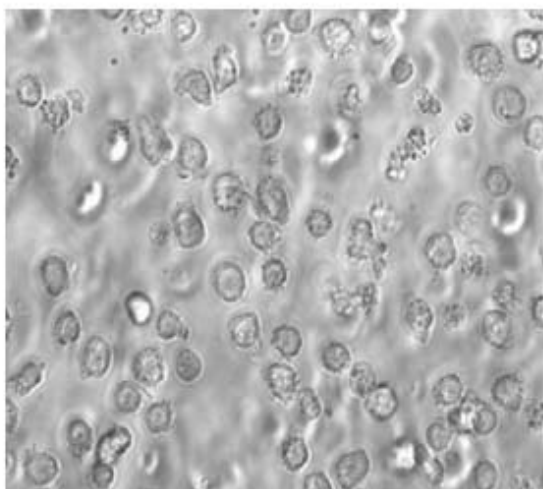


**Fig. 5. a)** Modulation of cleaved-caspase3 on cells exposed to LED. This figure shows a higher expression of cleaved-caspase3 in samples treated with one boost for 5 and 10 min compared to the controls, samples C1 and C2 ( $p = 0.0006$ ). **b)** Statistical evaluation of expression of cleaved-caspase3 on cells after LED exposure. Statistical analysis shows ratio values of cleaved-caspase3/actin on control and treated group with one boost, respectively.

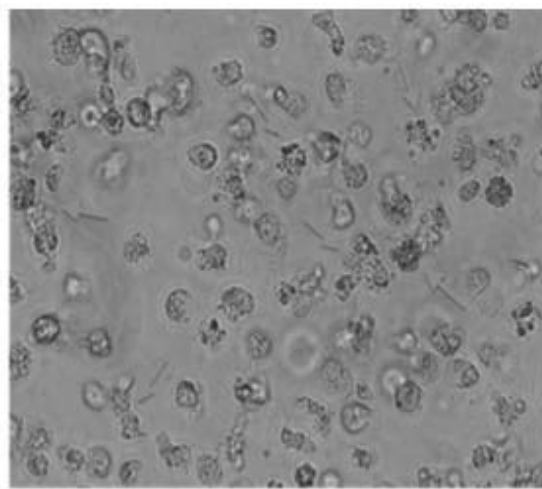




**Fig. 6.** Expression of  $\beta$ -galactosidase on LED treated cells. Statistically significant differences between controls and tests irradiated at different times for  $\beta$ -galactosidase activity (control, 5 and 10 min; IR5:  $p < 0.05$ ). No significant differences in samples irradiated 10 min (IR 10).



**Fig. 7a.** U937-control.



**Fig. 7b.** LED treated U937.

**Fig. 7.** *a)* Optical microscopy evaluation of untreated cells. U937-Control. Phase-contrast microscopy. *b)* Optical microscopy evaluation of cell degranulation after LED treatment. U937-Treated. Phase-contrast microscopy. Red arrows show cell degranulation.

**Table I.** Flow cytometry analysis of IL-8 release on cells after LED exposure. IL-8 concentrations (pg/ml) of controls and samples undergoing a time course LED application ranging from 1 min up to 18 min w/out LPS, at a final concentration of 0.5 µg/ml. In irradiated cells pre-treated with LPS, the cytokine pattern showed slight increase on IL-8 release, compared to the effect of LED exposure.

|        | Control | LED 1' | LED 3' | LED 5' | LED 10' | LED 15' | LED 18' |
|--------|---------|--------|--------|--------|---------|---------|---------|
| [IL-8] | 4,003   | 62,712 | 61,594 | 69,429 | 67,704  | 64,699  | 62,560  |

|        | LPS    | LPS+LED 1' | LPS+LED 3' | LPS+LED 5' | LPS+LED 10' | LPS+LED 15' | LPS+LED 18' |
|--------|--------|------------|------------|------------|-------------|-------------|-------------|
| [IL-8] | 70,463 | 72,430     | 73,752     | 73,335     | 69,038      | 66,700      | 68,847      |

prolonged electromagnetic energy exposure in the visible wavelength spectrum, we analyzed the release of several pro-inflammatory cytokines, in particular IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IFN- $\gamma$ , TNF $\alpha$ , IL-8 and IL-1 $\beta$ . A statistically significant modulation under LED treatment was detected respectively for IL-8 and IL-1 $\beta$  (Fig. 2 a-b). With this observation we point out that a pattern of pro-inflammatory mediators arises on the target cell line, using a standard low energy application of NIR light at 880 nm. In all experiments, IL-8 was the cytokine most induced by low energy light treatment (10 times higher than the basal level). Surprisingly, when we tested LED exposure of U937 cells in the presence or absence of LPS, a known inflammatory stimulus, we observed a slight effect on cytokines release, in particular of IL-8. This strongly supports the idea that IL-8 correlates with degranulation, cytoplasmic remodeling, once related to a functional autocrine transduction pathway and induction of specific pro-inflammatory and proliferative pathways, mostly in terms of induction of VEGF and FGF. Therefore, it could be possible that, when photobiomodulation is applied on tissues *in vivo*, cytoplasmic remodeling of target cells and also neo-capillary sprouting could be activated, leading to remodeling of the tissue undergone to energy. These results may suggest a putative role of LED on promoting apoptosis and on a remodeling process of target cells, including neo-angiogenesis, wound healing and tissue repair mechanisms.

Several studies have shown that a low energy laser therapy, applied *in vitro* on U937 cells treated with LPS and/or free radicals (H<sub>2</sub>O<sub>2</sub>), reduced IL8 and TNF $\alpha$  expression, acting as an inflammatory suppressor (29). Our results are in apparent contrast with them; however, laser application was characterized by a different wavelength

and higher energy (660 nm, 4.5 joule/cm<sup>2</sup>) in respect to that used in our experimental setup. In addition, the different effects could be attributed to the different light source. According to the literature (29), the wavelength of light sources is extremely critical, since it has been demonstrated that photo-acceptor mostly resides in the inner membranes of mitochondria, the energy supplier of which is critical to maintain efficient signaling inside the cells. In addition, by applying photobiomodulation for skin rejuvenation on patients using either a 630 nm or 830 nm wavelength source, increased amounts of mRNA of pro-inflammatory cytokines as well as IL-1 $\beta$ , TNF- $\alpha$  were observed (30). This might suggest a model where an inflammatory-related remodeling mechanism of tissues is effective underlying light exposure. Additionally, a prolonged pretreatment, using different inflammatory stimuli, can hypothetically induce unknown signaling pathways influenced by prolonged photon induction.

To better analyze the inflammatory pathway and the degranulation activity observed under microscopy analysis, we studied the modulation of NF-kB transcription factor and the mediators of autophagy (LC3BII) and apoptosis (caspase3/cleaved-caspase3 ratio). NF-kB seemed to be activated only by LED exposure. Additionally, after early treatment, p65 induction and activation were observed, although maximum p65 activation was detected when cells were plated at a maximum concentration of 5 x 10<sup>5</sup> cells/ml of culture medium. This was probably due to a cell/cell interaction system that might cooperate with low energy light induction in promoting a pro-inflammatory cell phenotype. Furthermore, in order to explain degranulation of cytoplasmic organelles, we investigated either autophagy or apoptosis by analyzing the activation of LC3B and caspase3/cleavedcaspase3 ratio, respectively. As a marker

of cytoplasmic degranulation pathway we measured the level of lysosomal  $\beta$ -galactosidase (SA- $\beta$ -gal).

Lysosomal  $\beta$ -galactosidase is an enzyme that catalyzes the hydrolysis of  $\beta$ -galactosides into monosaccharides. On U937 cells irradiated respectively for 5 and 10 min, we showed a significant increased expression of SA- $\beta$ -gal after early time of exposure (5 min). As IL-8 release is a direct marker of degranulation and having had evidence of an induction of signaling promoting apoptosis, we may suggest that  $\beta$ -galactoside activity correlates with programmed cell death. As described (30), the same linkage has been proposed for human young fibroblasts undergoing apoptosis after TNF $\alpha$ , ceramide and okadaic acid treatment. Whether the early exposure of U937 cells to LED irradiation can induce terminal senescence is an interesting hypothesis that might be further investigated, as U937 is a tumor cell line actively proliferating *in vitro* and tumorigenic *in vivo* (31, 32).

It is important to highlight that SA- $\beta$ -gal is not a specific marker of senescence *per se*, but rather a surrogate marker for increased lysosome number or activity, which has long been associated with replicative senescence and organismal aging (31).

Surprisingly, at least on macrophage cell lines exposed to 880 nm LED emission, no autophagy mechanisms could be observed, or at least no modulation that could be estimated statistically significant. In fact, the ratio between LC3BII and its basal form LC3BI was not modulated on treated *versus* control cells. Inversely, the caspase3/cleaved-caspase3 ratio was mostly affected by light emission and the cleaved activated form of caspase3 was increased on cells treated with one application of LED for 5 and 10 min in respect to basal level on untreated cells. This observation allows some speculation on a putative role for LED light on promoting apoptosis and in new remodeling of target cells, including neo-angiogenesis, wound healing and tissue repair mechanisms. As a matter of fact, the observation that IL-8 is the most induced cytokine after 880 nm LED activity on U937 cells gives strong evidence to a specific role of low energy LED emission in promoting apoptosis and allows cellular regeneration on target organs. In conclusion, we can affirm that LED irradiation at 880 nm of U937 cells induces apoptosis and does not stimulate autophagy.

In summary, the objective of this study was to assess the effects of LED used to stimulate U937 cells in undergoing

apoptosis. In fact, the apoptotic marker (caspase3-cleaved) was mostly expressed on cells treated with one shoot of LED for 5 min in respect to basal level. Additionally, IL-8 is highly increased after PBM exposure, as a hallmark in degranulation process and tissue remodeling, typical phenomena of apoptosis.

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## miR-2861 IS INVOLVED IN OSTEOGENIC COMMITMENT OF HUMAN PERIODONTAL LIGAMENT STEM CELLS GROWN ONTO 3D SCAFFOLD

F. DIOMEDE<sup>1</sup>, I. MERCIARO<sup>1</sup>, S. MARTINOTTI<sup>2</sup>, M.F.X.B. CAVALCANTI<sup>3,4</sup>, S. CAPUTI<sup>5</sup>,  
E. MAZZON<sup>6</sup> and O. TRUBIANI<sup>1</sup>

<sup>1</sup>Laboratory of Stem Cells and Regenerative Medicine, University “G. d’Annunzio”, Chieti-Pescara, Chieti, Italy; <sup>2</sup>Department of Pharmacy, University “G. d’Annunzio”, Chieti-Pescara, Chieti, Italy; <sup>3</sup>UMR 7365 CNRS-UL IMoPA et FR UL-CNRS-INSERM, Faculté de Médecine, Lorraine University Vandoeuvre-les-Nancy, France; <sup>4</sup>Postgraduate Department, Cruzeiro do Sul University, Sao Paulo, SP, Brasil; <sup>5</sup>Department of Medical, Oral and Biotechnological Sciences, University “G. d’Annunzio”, Chieti-Pescara, Chieti, Italy; <sup>6</sup>IRCCS Centro Neurolesi “Bonino-Pulejo”, Messina, Italy

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miR-2861 endorsing osteoblast differentiation through the overexpression of Runt-related transcription factor 2 (RUNX2) protein has been recently described. In this study we evaluated: the performance of living construct, composed by human Periodontal Ligament Stem Cells (hPDLSCs) and 3D scaffold (EXg), and the behaviour of miR-2861/RUNX2 expression pathway on the osteogenic commitment. Human PDLSCs were seeded with and without EXg scaffold and cultured under basal and osteogenic conditions. Morphological features, adhesiveness and differentiation abilities were analysed using scanning electron and confocal laser scanning microscopy. Time-course of RUNX2, ALP, OPN and miR-2861 were evaluated through RT-PCR analysis. Our results highlighted that the osteogenic differentiation was mostly obvious in the hPDLSCs, grown onto 3D scaffold in presence of osteoinductive medium. Moreover, the overexpression of miR-2861 and RUNX2 in hPDLSCs cultured in presence of EXg under osteogenic and standard conditions was demonstrated. In synthesis, the increased expression of miR-2861/RUNX2 provides new insights regarding miRNA signaling network in the presence of scaffold providing an additional method to evaluate the performance of biomaterial in bone regeneration.

Tissue engineering provides novel promising therapeutic strategies to repair bone tissue defects in oral, orthopedic and cranio-facial surgery, given the limited availability and the donor site morbidity of autografts and the high costs and risks of disease transmission as well as adverse immune reactions which characterize allografts. The basic aspects of bone regeneration comprise the importance of the material surface in establishing the types of cells that can attach to biomaterial and modulate the

phenotypic differentiation.

Generally speaking, a suitable scaffold for bone tissue engineering should be biodegradable, biocompatible, with porous structure and interposed connection and, in particular, should be a good mechanical support to load-bearing bone (1).

Currently a promising treatment in bone tissue replacement is represented by suitable appropriate 3-scaffolds from stem or precursor cells (2, 3). Among human mesenchymal stem cells (hMSCs)

**Key words:** miR-2861, RUNX2, hydroxyapatite, human periodontal ligament stem cells, osteogenic differentiation

### Mailing address:

Prof. Oriana Trubiani, Laboratory of Stem Cells and Regenerative Medicine,  
Department of Medical, Oral and Biotechnological Sciences,  
University “G. d’Annunzio” Chieti,  
Via dei Vestini 31, 66100 Chieti, Italy  
Tel.: +3908713554097 - e-mail: oriana.trubiani@unich.it

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we recently reported that those deriving from human periodontal ligament (hPDLSCs) can be an easy and efficient autologous source of stem cells with a high expansion capacity and ability to differentiate into osteogenic cells that can colonize and grow connected to biocompatible scaffold (4-8).

MicroRNAs (miRNAs) are a class of noncoding and single-stranded RNA molecules (9). miRNAs are able to regulate cellular processes such as cell development, differentiation, proliferation, metabolism and protein expression (10). Recent studies have discovered that some miRNAs act as important regulators of bone forming genes, including transcription factors and signaling pathway molecules that are required during the osteoblastogenesis. In particular, bone morphogenic protein 2 (BMP2) and the runt-related transcription factor 2 (RUNX2) possess a central role in bone formation and they are necessary for osteoblast differentiation (11, 12). miRNAs have a key role in bone gene expression, and in particular miR-2861 present a positive regulatory role in osteoblast differentiation by repressing *Hoxa2* and histone deacetylase 5 maintaining the high levels of RUNX2 mRNA and protein (13).

In the present study, we isolated and cultured hPDLSCs and examined their morphology, viability and osteogenic differentiation when seeded onto Endobon® Xenograft (EX) granules, a fully deproteinated and sterilized hydroxyapatite ceramic scaffold derived from cancellous bovine bone. Moreover, the involvement of miR-2861/RUNX2 on the behavior of hPDLSCs/ EXg construct is evaluated, to provide necessary information for the potential use of this bio-complex in bone repair and regeneration.

## MATERIALS AND METHODS

### *Cell culture*

Written approval for the human periodontal ligament collection performed in this study was obtained from the Medical Ethics Committee of the Medical School, "G. d'Annunzio" University, Italy (researcher: Trubiani Oriana, approval number: n°3/14), and written informed consent was obtained from all patients (range age 20–35

years). All volunteers were exempt from systemic and oral diseases. Biopsies were obtained from horizontal fibers of the periodontal ligament via scraping roots of healthy third molar teeth using Gracey's curette. hPDLSCs were obtained and cultured in MSCBM medium (Lonza Walkersville Inc, Walkersville, MD, USA) according to Trubiani et al. (14, 15). Primary cultures of hPDLSCs were constituted of colonies of fibroblast-like cells which, after subcultivation, proliferated with a population-doubling time of 48 h reaching a confluent growth-arrested condition. Single-cell suspensions of the developing adherent layer were prepared by trypsin/ethylenediaminetetraacetic acid (EDTA) and cell growth was evaluated by the trypan blue dye exclusion test.

### *Flow cytometry analysis*

hPDLSCs were washed in PBS and subsequently resuspended in PBS with saturating concentrations (1:100 dilution) of anti-human antibodies fluorescein isothiocyanate conjugated (CD44, CD45, CD73, CD90) and CD14, CD29, CD34, CD105 phycoerythrin conjugated, for 30 min at 4°C. Labelled cells were acquired and analysed using a FACStar-plus flow cytometry system running CellQuest software (Becton-Dickinson, Mountain View, CA, USA) (16).

### *Cell viability assay*

The viability of hPDLSCs seeded with or without EX granules was measured by the quantitative colorimetric MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazoliumbromide test) (Promega, Milan, Italy) as previously reported (17).  $2.5 \times 10^5$  cells/well were seeded into a 96-well culture plate in presence or not of EX granules. After 24, 48, 72 h and 1 week of incubation at 37°C, 15 µl/well of MTT was added to culture medium, and cells were incubated for 3 h at 37°C. The supernatants were read at 650 nm wavelength using an ND-1000 NanoDrop Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). The MTT assay was performed in three independent experiments, six replicate wells for each experimental point.

### *Scanning electron microscopy (SEM) analysis*

Granules after 24 h of culture with hPDLSCs were fixed for 4 h at 4°C in 4% Glutaraldehyde in 0.05 M phosphate buffer (pH 7.4), dehydrated in increasing

ethanol concentrations and then critical point dried. They were then mounted on aluminum stubs and gold-coated in an Emitech K550 (Emitech Ltd. Ashford, UK) sputter-coater before imaging with Zeiss SEM EVO 50 (Zeiss, Jena, Germany).

#### *Immunofluorescence staining and confocal laser scanning microscope (CLSM) analysis*

Cells grown on EX granules were fixed for 10 min at room temperature (RT) with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS), pH 7.4. After washing in PBS, cultures were processed for immunofluorescence labelling. Briefly, hPDLSCs grown on granules were permeabilized with 0.5% Triton X-100 in PBS, followed by blocking with 5% skimmed milk in PBS. Primary monoclonal antibodies to anti human vinculin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used, followed by Alexa Fluor 488 green fluorescence conjugated goat anti-mouse as secondary antibodies (Molecular Probes, Invitrogen, Eugene, OR, USA). Subsequently, the sample was incubated with Alexa Fluor 594 phalloidin red fluorescence conjugate (Molecular Probes), as actin cytoskeleton marker. Nuclei were stained with TOPRO (Molecular Probes). Samples were placed face down on glass slides and mounted with Prolong antifade (Molecular Probes) (18, 19). Staining of samples was visualized using a Zeiss LSM510 META confocal system (Zeiss), connected to an inverted Zeiss Axiovert 200 microscope equipped with a Plan Neofluar oil-immersion objective (40×/1.3 NA). Images were collected using an argon laser beam with excitation lines at 488 nm.

#### *Osteogenic differentiation*

For osteogenic induction, primary cells at 2<sup>nd</sup> passage were seeded at  $4 \times 10^3$  cells/cm<sup>2</sup> in MSCBM culture medium and maintained in culture at 37°C, in a humidified atmosphere of 5% CO<sub>2</sub>. After reaching 80% confluence, cells were incubated with MSCBM medium with the addition of osteogenic supplements, i.e. 100 nM dexamethasone (Applichem GmbH, Darmstadt, Germany), 10 mM  $\beta$ -glycerol-phosphate (Applichem) and 0.05 mM 2-phosphate-ascorbic acid (Sigma-Aldrich, Milan, Italy) (20). Confluent cells with and without EX granules were induced to osteogenic differentiation. Visualization of calcium deposition and extracellular matrix mineralization was obtained by Alizarin red S (ARS) staining assay

performed after 3 weeks of culture. This test was carried out according to the method described by Gregory et al. (21). Cells were washed with PBS, fixed in 10% (v/v) formaldehyde (Sigma-Aldrich) for 30 min and washed twice with abundant dH<sub>2</sub>O prior to adding 0.5% Alizarin red S in H<sub>2</sub>O, pH 4.0, for 1 h at room temperature. After cell incubation under gentle shaking, cells were washed with dH<sub>2</sub>O four times for 5 min. For staining quantification, 800  $\mu$ l 10% (v/v) acetic acid was added to each well. Cells were incubated for 30 min with shaking, and then scraped from the plate, transferred into a 1.5 ml vial and vortexed for 30 s. The obtained suspension, overlaid with 500  $\mu$ l mineral oil (Sigma-Aldrich), was heated to 85°C for 10 min, then transferred to ice for 5 min, carefully avoiding opening of the tubes until fully cooled, and centrifuged at  $20,000 \times g$  for 15 min. Five hundred microliters of the supernatant was placed into a new 1.5-ml vial and 200  $\mu$ l of 10% (v/v) ammonium hydroxide was added to neutralize the acid, assuring a pH between 4.1 and 4.5. 150  $\mu$ l of the supernatant obtained from differentiated and undifferentiated hPDLSCs were read in triplicate at 405 nm by a spectrophotometer (Synergy HT, BioTek Instruments, Swindon, UK). The osteogenic induction was performed in three independent experiments after 1, 2 and 3 weeks of differentiation.

#### *RNA isolation and real time-PCR analysis*

Osteogenic markers were evaluated by real-time PCR after 1 and 3 weeks of culture. To this end, total RNA was isolated using the Total RNA Purification Kit (Norgen Biotek Corp., Ontario, Canada) according to the manufacturer's instructions. The M-MLV Reverse Transcriptase reagents (Applied Biosystems, Foster City, CA, USA) were used to generate cDNA. Real-time PCR was carried out with the Mastercycler ep realplex real-time PCR system (Eppendorf, Hamburg, Germany). hPDLSCs expression of Runt-related transcription factor-2 (RUNX2), Alkaline Phosphatase (ALP) and Osteopontin (OPN) was evaluated after 1 and 3 weeks in culture with EX granules. Expression levels in cells cultured with basal medium and with differentiating medium in presence or in the absence of EX granules were compared. Commercially available TaqMan Gene Expression Assays (RUNX2 Hs00231692\_m1; ALP Hs01029144\_m1; OPN Hs00157093\_m1) and the TaqMan Universal PCR Master Mix (Applied Biosystems) were used according

to standard protocols. Beta-2 microglobulin (B2M Hs99999907\_m1) (Applied Biosystems) was used for template normalization.

#### *miRNA quantization*

miRNA was extracted, after 1 and 3 weeks of culture, using the PureLink RNA mini kit (Life Tech), treated with the RNase-Free DNase Set (Qiagen) according to the instructions of the manufacturer and quantified with Nanodrop2000 (Thermo-Scientific, Waltham, MA, USA). Gene sequences were from NCBI (<http://www.ncbi.nlm.nih.gov/>), RNA sequences for miR-2861, were used into the Universal ProbeLibrary (UPL) Assay Design Center software ([https://www.rockeappliedscience.com/sis/rtpcr/upl/index.jsp?id=uplct\\_030000](https://www.rockeappliedscience.com/sis/rtpcr/upl/index.jsp?id=uplct_030000)) to identify primers and UPL probe. Total RNA (50–200 ng) was retrotranscribed with High Capacity cDNA Reverse Transcription Kit (Life Technologies, Milan, Italy). miRNA quantization was performed using stem loop RT primers designed (22) with a modification to include the UPL #21 sequence binding site (23). UPL probe #21 was from UPL database (Roche Diagnostics, Basel, Switzerland). Total RNA (50 ng) was retrotranscribed with TaqMan MicroRNA Reverse Transcription Kit (Life Technologies). Real-time PCRs were performed with an Applied Biosystems 7900 instrument. miRNA and mRNA levels were measured using Ct (threshold cycle). Target amount, normalized to an endogenous references 18S/RNU44 and relative to a calibrator, was given by 2<sup>-DDCt</sup> and/or 2<sup>-DCt</sup> methods (Life Technologies).

#### *Data and statistical analysis*

The Statistical Package for Social Science (SPSS, v.21.0, Inc. Chicago, IL, USA) was used for data analysis. Parametrical methods were used after having verified the existence of the required assumptions. In particular, the normality of the distribution and the equality of variances were assessed by the Shapiro-Wilk and Levene's tests, respectively. The factors under investigation were the time elapsed and the presence of granules for MTT assay, osteogenic differentiation. Data were expressed as means and standard deviation of the recorded optical density values. The differences among the levels of the two factors under investigation were evaluated by performing three distinct two-way-ANOVA tests, one for each experiment. Tukey tests were applied for pairwise comparisons. A

value of  $p < 0.05$  was considered statistically significant in all tests.

## RESULTS

#### *Phenotype of hPDLSCs*

The expression of surface molecules (CD29, CD44, CD73, CD90 and CD105), and pluripotency-associated markers (OCT3/4, SSEA4, SOX2) was evident in human PDLSCs cultured as reported in material and methods. While cells were negative for the following markers: CD14, CD34, and CD45 (Fig. 1, a).

#### *Cell viability assay*

Data from MTT assays indicated that the proliferation rate of hPDLSCs seeded along with EX granules was similar to the control ones seeded without biomaterials from 24 h up to 1 week, as shown in Fig. 1, b.

#### *Scanning electron and confocal laser microscopy analysis*

The behavior and morphology of the hPDLSCs were examined by SEM analysis. Adhesion ability of hPDLSCs on the scaffold was evident after 1 week of culture. Morphological analysis showed extending cytoplasmic processes and filopodia, which enabled the anchorage of cells (Fig. 2, a, b). Cells proliferated on the uneven surface and in granule holes to create bridges across the particles, organizing a multilayer covering the 3D substrate (Fig. 2, c, d).

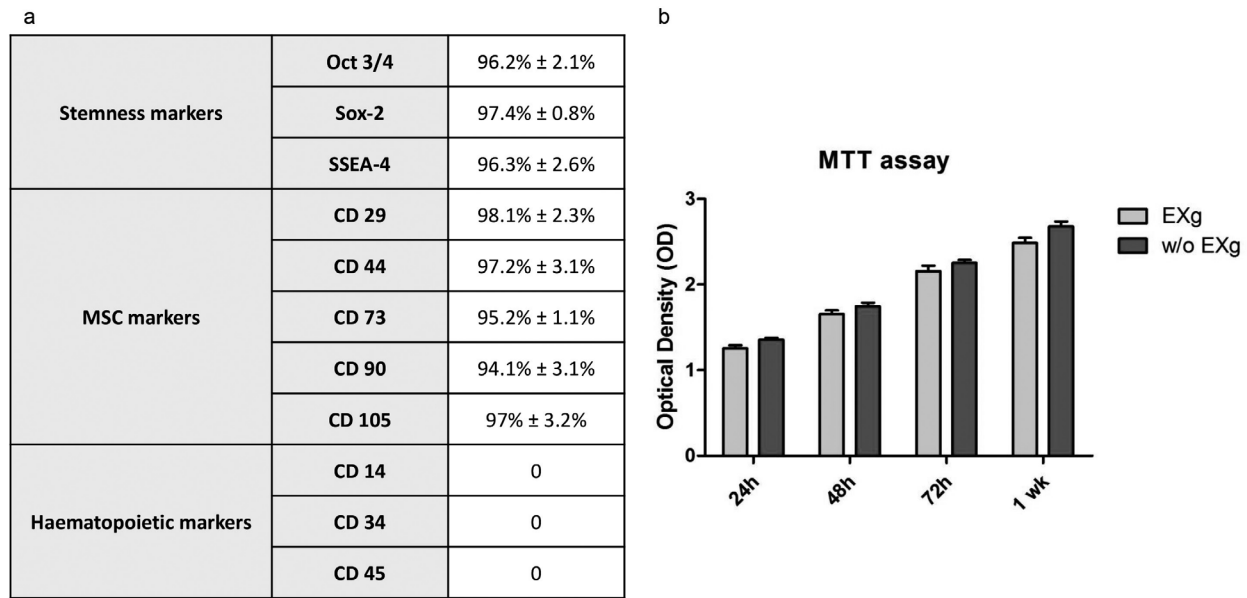
#### *Immunohistochemistry analysis*

Immunohistochemistry results displayed the high performance of viable hPDLSCs cultured onto EXg after 1 week of culture. The fluorescent micrograph showed a specific positive response to vinculin, and cells and biomaterial established an intimate contact (Fig. 3). Actin filaments, stained with phalloidin-rhodamine, revealed the cellular cytoskeleton arrangement.

#### *Osteogenic differentiation*

The osteogenic differentiation process induced in primary culture, with and without scaffold,





**Fig. 1.** Cell growth and characterization. **a)** Cytofluorimetric analysis of hPDLSCs cultures. Table is representative of five separate experiments performed using cells at 2<sup>nd</sup> passage. **b)** Cell proliferation analysis of hPDLSCs cultured with and without EX granules (EXg). The average of three separate experiments of the measured optical density (OD) is shown. Error bars represent standard deviation.

was evaluated by ARS staining (Fig. 4, c, d) and colorimetric detection (Fig. 4, g). A progressive increased extracellular deposition was evident in cells grown under osteogenic conditions in comparison with control cells (Fig. 4, a, b). Differentiation induction was more evident in cells seeded onto EX granules cultured with osteoinductive medium (Fig. 4, g), showing the scaffold ability to improve the new bone deposition in presence of osteogenic inducers.

#### RT-PCR of h PDLSCs seeded onto EXG scaffold

RT-PCR of osteogenesis-related markers showed an upregulation of RUNX2, ALP and OPN, and were particularly more evident in differentiated hPDLSCs cultured onto EX granules (Fig. 4, f; Fig. 5, a). In control samples no significant differences were present (Fig. 4, e; Fig. 5, a).

#### miRNA expression

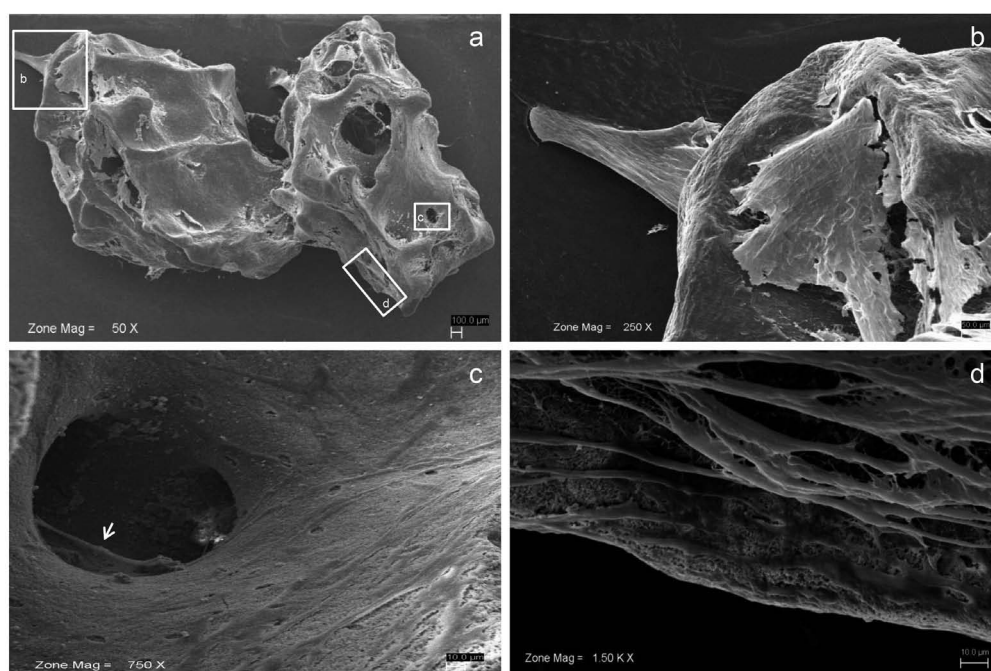
Expression level of miR-2861 was measured in presence or not of EXg during osteoblast differentiation and under standard conditions. MiR-2861 was up-regulated in hPDLSCs cultured or not onto biomaterial under osteogenic conditions (Fig. 5, b).

## DISCUSSION

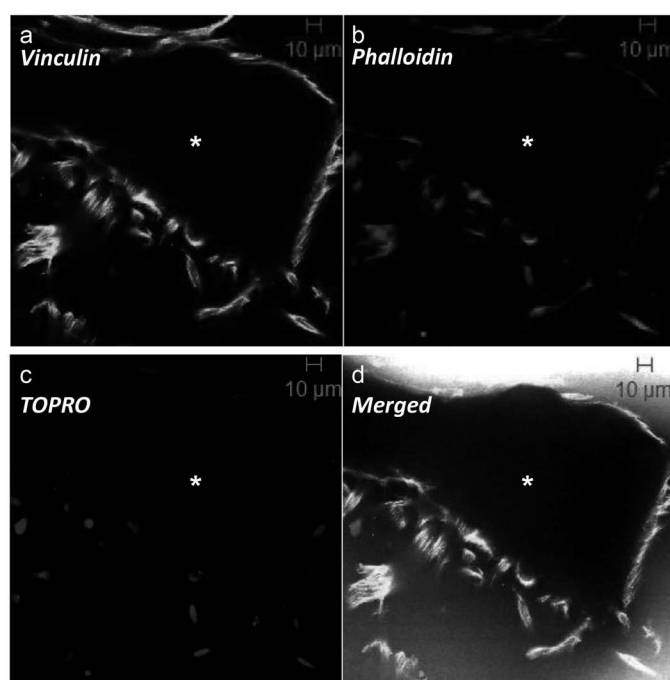
The cellular fate in bone tissue engineering can be profoundly influenced by the chemical composition, roughness, and geometry of the scaffold design. Most studies regarding the performance of biomaterials have been carried out considering the signaling involved in growth and differentiation (24, 25). In this paper we evaluated the aspect linked to cell growth, morphological features and osteogenic differentiation ability of the living construct hPDLSCs/EXgranules. Previously, we described a stem cell population deriving from periodontal ligament (hPDLSCs) with similar features of bone marrow stem cells having lower donor-site morbidity, available in larger numbers, expressing stemness markers and able to undergo multilineage differentiation, in particular towards osteogenic differentiation (14, 26).

Our data highlighted that hPDLSCs seeded onto the biomaterial grow and proliferate without signs of cell death or growth inhibition. Cells, in very close contact with the uppermost scaffold surface, build cellular bridges between themselves and into

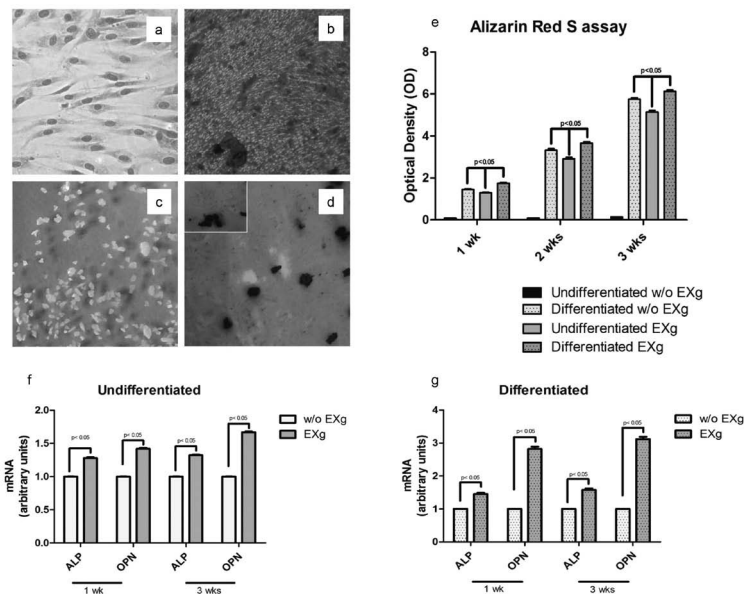




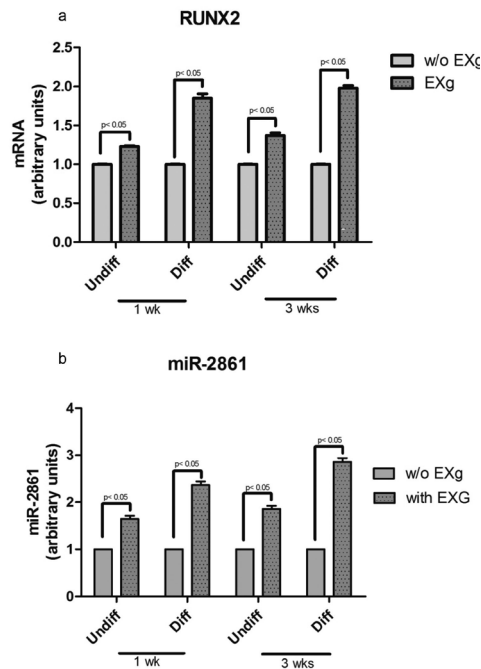
**Fig. 2.** Scanning electron microscopy analysis. Scanning electron micrographs of hPDLSCs cultured in the presence of EX granules after 1 week of incubation. **a)** Picture at low magnification showed hPDLSCs seeded onto granules (Mag: 50X). **b)** Cells, adhering on the bone substitute, made contact and covered the biomaterial surface (Mag: 250X). **c)** High interactions between cells and granule surface, in particular cellular bridge was evident in the cancellous space (arrow) (Mag: 750X). **d)** hPDLSCs adhered on the biomaterial showing a morphological homogeneous fibroblast-like appearance and long cytoplasmic processes (Mag: 1500X)



**Fig. 3.** Confocal laser scanning microscopy analysis. Confocal laser scanning micrographs of hPDLSCs seeded on bone substitutes expressing vinculin and actin. **a)** Vinculin labeling in cells adhered onto the granule. **b)** Phalloidin staining showed actin labeling indicating the spatial cytoskeleton arrangement of hPDLSCs. **c)** Nuclei stained with TOPRO. **d)** Merged image of the triple staining of hPDLSCs seeded on bone substitute. Original magnification: 20X. \*: EX granule.



**Fig. 4.** Osteogenic differentiation. **a)** Undifferentiated hPDLSCs, stained with toluidine blue, show fibroblast-like morphology with elliptical nuclei and long cytoplasmatic processes. **b)** Alizarin red S staining (ARS) was evident in cell cultures grown under osteogenic conditions. **c)** EX granules without cells at light microscopy. **d,** inset) hPDLSCs grown onto EX granules (EXg) under standard and **(d)** osteogenic conditions (Mag: 10X). **e)** The mineralization of hPDLSCs, evaluated by the quantification of ARS staining via extraction with ammonium hydroxide at different commitment times (1, 2 and 3 weeks) in osteogenic medium and in basal medium with and without EXg. The amount of released dye is measured by a microplate reader at 405 nm. Values, expressed as units of optical density (OD), are the mean  $\pm$  SEM of three independent experiments. **f** and **g)** ALP and OPN expression were measured, after 1 and 3 weeks of culture, by means real-time PCR for all conditions. Data are the mean  $\pm$  SEM of three separate experiments.  $p < 0.05$  was considered statistically significant.



**Fig. 5.** Osteogenic-related marker expressions. Graphs show the up-regulation of **(a)** RUNX2 and **(b)** miR-2861 in presence or not of EXg under osteogenic and standard conditions, after 1 and 3 weeks of culture. Data were quantified by means of real-time PCR and presented as mean  $\pm$  SEM of three separate experiments.

the biomaterial. The CLSM analysis of vinculin, a protein known to crosslink actin filament to the talin molecule at focal adhesion (27, 28), demonstrated that several focal adhesion areas between cells and biomaterial can be evidenced, strengthening data obtained at scanning electron microscopy level. RT-PCR carried out at 1 and 3 weeks of culture time showed an upregulation of different gene expression of osteogenic markers, such as RUNX2, ALP and OPN, in cells seeded onto the scaffold under standard conditions. A more significant increase of upper mentioned markers was present when hPDLSCs were cultured onto granules in the presence of osteoinductive medium.

Bone tissue development and regeneration is strictly dependent on the complex interactions between bone-making osteoblasts, bone-degrading osteoclasts and other cell types that constitute the bone microenvironment. The activity of these cell populations is regulated by several signaling pathways and currently an emerging role of miRNAs controlling multiple layers of gene regulation for bone development and postnatal functions has been assumed (29).

Although numerous microRNAs have been tested to be up or down regulated during bone formation, only a few of them are significant and are involved in skeletal cell development and differentiation, in any case the exact regulatory mechanism has yet to be determined. In particular, some miRNAs have been reported to act as negative regulators of osteoblast differentiation, such as miR-26a and miR-125b (30). On the contrary, miR-29b promotes osteogenesis by way of down-regulation of various inhibitors of osteoblast differentiation (31), also miR-210 positively regulates osteogenesis by inhibiting the TGF- $\beta$ /activin signaling pathway (32).

Recently, miRNAs (miR-3960 and miR-2861) which represent an autoregulatory loop in osteoblast differentiation have been cloned and identified (32). Validation experiments indicate that miR-3960 and miR-2861 target *Hoxa2* and HDACs, respectively, indirectly increase the expression of RUNX2 promoting osteoblast differentiation. RUNX2 binds to the promoter and activates transcription of the miR-3960/miR-2861 cluster (33). In fact, the silencing of

miR-2861 expression resulted in a loss of bone density in mice, supporting the positive regulatory role of miR-2861 in bone formation (34). Furthermore, the indication that miR-2861 mutation causes primary osteoporosis in humans, suggests its key role in the pathogenesis of this disease. MiR-2861 plays a positive role in regulating osteoblast differentiation and indirectly increases the expression of RUNX2. RUNX2, also known as core binding factor alpha 1 (Cbfa1), osteoblast specific transcription factor 2 (Osf2) and acute myeloid leukemia 3 protein (AML3), is a bone restricted transcription factor essential for osteoblast differentiation and bone formation. Their mutations are found in 65%–80% of individuals with cleidocranial dysplasia (35, 36), and RUNX2-deficient mice were found to show a complete lack of skeletogenesis owing to the maturational arrest of osteoblasts (37). RUNX2 induces osteogenic differentiation process and subsequently the up regulation of osteocalcin, osteopontin, collagen type I, bone sialoprotein, and alkaline phosphatase expression. hPDLSCs/EXg living construct, in basal and osteogenic conditions, induced an overexpression of ALP, Osteocalcin and RUNX2, demonstrating that cells go towards a bone mineralization already at 1-3 weeks of culture, and this differentiation process was more pronounced in presence of differentiation medium.

Moreover, miR-2861/RUNX2 upregulation was identified in hPDLSCs in the presence of biomaterial, indicating that they played a regulatory role in the osteoblast commitment. These data contribute to demonstrating that miRNAs, and in particular miR-2861, are involved in osteogenic differentiation and open a new scenario in the study of biomaterial performance.

In conclusion, the identification of miR-2861 interacting with RUNX2 tissue-specific transcription factors offers a standpoint on the influence of signaling pathways and regulatory molecule networks driving bone formation in presence of bone substitutes.

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## LETTER TO THE EDITOR

## REAL-TIME SHEAR WAVE ELASTOGRAPHY AND APRI INDEX FOR EVALUATING AUTOIMMUNE HEPATITIS FIBROSIS

LL. SUN<sup>1</sup>, G. DONG<sup>1</sup>, B. WANG<sup>2</sup>, Q. ZHENG<sup>1</sup>, S. WANG<sup>1</sup> and RF. ZHANG<sup>1</sup><sup>1</sup>Department of Ultrasonography, The First Affiliated Hospital of Zhengzhou University, Henan, China;<sup>2</sup>Department of Radiology, The First Affiliated Hospital of Zhengzhou University, Henan, China*Received January 28, 2016 – Accepted May 26, 2016*

The objective of this study was to investigate the significance of real-time supersonic shear imaging (SSI) and serum biochemical index in evaluating the degree of autoimmune hepatitis fibrosis. Retrospective analysis was carried out to study 291 cases of patients with autoimmune hepatitis, and discuss the value of SSI application, APRI index and bilirubin on autoimmune hepatitis. The area under the receiver operating characteristic curve (AUROC) was taken for statistical analysis to determine its diagnostic accuracy. In the high degree of hepatic fibrosis, the hepatic SSI measured value of autoimmune hepatitis positively correlated to ALT, AST, APRI ratio, AST and AST/ALT. The SSI measured value significantly and positively correlated to the degree of hepatic fibrosis ( $r=0.598$ ,  $p=0<0.01$ ). In chronic hepatic fibrosis, the elasticity values of AIH, PBC, AIH-PBC overlap syndrome (OS) were in a rising trend, and the difference in the elasticity value of each fibrosis stage was statistically significant ( $P<0.01$ ). Hepatic SSI measured value was employed to respectively detect the AUROC of S3 stage and S4 stage for AIH, PBC, and AIR-PBC OS groups, which resulted as being higher than the APRI index detected in S3 stage and S4 stage. SSI measured index had better diagnostic significance than APRI index on hepatic fibrosis for AIH, PBC and AIH-PBC OS groups.

To the Editor,

Autoimmune hepatitis (AIH) is an immune disease which mainly affects the liver, causing primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) (1). Most patients ultimately develop cirrhosis. In recent years, the incidence of AIH has been on the rise. The reversible intervention method on hepatic fibrosis progress could be a promising measure (2-3).

Supersonic shear imaging (SSI) could measure the elasticity value of the tissue of a specific area, and quickly measure the degree of hepatic fibrosis (4).

This study aims to evaluate the diagnosis of the fibrosis stage in AIH, PBC, and AIH-PBC overlap syndrome (OS) patients.

## MATERIALS AND METHODS

Two hundred and ninety-one cases of patients suffering from AIH who underwent hepatic aspiration biopsy in our hospital between May 2011 and September 2015 were included in the study. This total included 125 cases of AIH, 55 of PBC, and 111 of AIH-PBC OS. The study was

*Key words: real-time shear wave elastography, hepatic fibrosis, autoimmune hepatitis, primary biliary cirrhosis, overlap syndrome*

Mailing address:

Dr Ruifang Zhang,  
Department of Ultrasonography,  
The First Affiliated Hospital,  
Zhengzhou University, No.1, Jianshe East Road,  
Zhengzhou City, 450000, PR China  
e-mail: Rui\_high20151223@hotmail.com

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approved by the ethics committee of our hospital. Informed consent was received from the patients to participate in the study. All cases of patients were confirmed according to the standard diagnosis of Autoimmune Hepatic Guide.

### Methods

All patients were subjected to AixPlover Supersonic shear imaging with 4C convex array probe and a probe frequency of 1.0-6.0 MHz. The hepatic anterior right area was chosen for detection. After the elasticity imaging mode was set, patients were required to hold their breath for 3 ~ 5 seconds. Images were stored when they became stable without shaking; a round detection area of 2 cm diameter in the imaging area was chosen to measure the elasticity value, and Kpa was set as the unit. The measurement detection was repeated 5 times, the average value of the analysis was calculated. The measured elasticity value was calculated using Young's modulus. A higher Young's modulus value indicates tissue hardness (2).

All the patients were subjected to percutaneous liver biopsy using ultrasound within a week after SSI detection. According to the pathology classification stage of chronic hepatitis in Viral Hepatitis Prevention Plan, 2001, the hepatic inflammatory activities were divided into G0, G1, G2, G3 and G4. The degrees of fibrosis were divided into S0, S1, S2, S3 and S4. Fasting venous blood samples were taken for clinical biochemical detection and routine examination.

### Statistical analysis

SPASS 17.0 statistical software was applied to analyze the data. The measurement data that met Gaussian distribution were marked as mean $\pm$ SD. One-way ANOVA was applied for comparison between groups. Kruskal-

Wallis detection was employed to analyze the differences between groups. Spearman rank correlation analysis was adopted to study the correlation between two variables. ROC curve was utilized to analyze the accuracy of SSI diagnostic hepatic fibrosis, with a  $P < 0.05$  marked statistical significant difference. The different AUROCs are to detect fibrosis stage  $\geq S2$ ,  $\geq S3$  and S4.

## RESULTS

From Table I, we can see that with hepatic fibrosis aggravation, Spearman rank correlation analysis detected that hepatic SSI value was positively correlated to ALT, fibrosis degree, APRI index, AST, AST/ALT ratio, and bilirubin level,  $P < 0.05$ .

The correlation of SSI measured value and fibrosis degree in AIH, PBC, and AIH-PBC OS groups is shown in Table II. Table I shows the SSI index has high diagnostic accuracy on moderate and high fibrosis degrees ( $S \geq 2$ ). The AUROC, sensitivity and specificity of SSI index and APRI index in the present investigation showed high diagnostic efficiency.

## DISCUSSION

This study investigated the significance of applying real-time SSI and serum biochemical index to evaluate the degree of autoimmune hepatitis fibrosis. Using retrospective analysis, we found that the SSI measured index had better diagnostic significance than APRI index on hepatic fibrosis for AIH, PBC and AIH-PBC OS groups.

SWE is the latest clinical elasticity imaging

**Table I.** Comparison of results of SSI value of AIH different fibrosis degrees and APRI index.

|           |   | Fibrosis | APRI  | Bilirubin               | AST   | ALT   | AST/ALT |
|-----------|---|----------|-------|-------------------------|-------|-------|---------|
|           |   | degree   | index | level $\mu\text{mol/L}$ | (U/L) | (U/L) | ratio   |
| SSI value | r | 0.598    | 0.348 | 0.302                   | 0.235 | 0.18  | 0.151   |
| (Kpa)     |   |          |       |                         |       |       |         |
|           | P | 0        | 0     | 0                       | 0     | 0.02  | 0.01    |

**Table II.** SSI values and fibrosis degree in AIH, PBC, AIH-PBC overlap syndrome group.

| cases     | Disease<br>classification      | SSI measured value      |                            |                          |                           |                           | P     |
|-----------|--------------------------------|-------------------------|----------------------------|--------------------------|---------------------------|---------------------------|-------|
|           |                                | S0                      | S1                         | S2                       | S3                        | S4                        |       |
| 55 cases  | PBC                            | 9.346±2.7<br>(7 cases)  | 9.241±2.9<br>(20 cases)    | 14.826±5.1<br>(19 cases) | 17.924±11.3<br>(5 cases)  | 29.849±3.1<br>(4 cases)   | <0.05 |
| 125 cases | AIH                            | 8.724±2.1<br>(11 cases) | 10.770±5.9<br>(39 cases)   | 14.303±6.5<br>(41 cases) | 16.623±5.3<br>(15 cases)  | 22.774±9.0<br>(19 cases)  | <0.05 |
| 111 cases | AIH-PBC<br>overlap<br>syndrome | 10.716±4.<br>3(5 cases) | 11.037±5.1<br>4 (27 cases) | 11.809±4.3<br>(46 cases) | 19.572±8.02<br>(41 cases) | 28.179±10.8<br>(12 cases) | <0.05 |
|           | P*                             | >0.05                   | >0.05                      | >0.05                    | >0.05                     | >0.05                     |       |

technology, and can measure the absolute value of the hardness in tissues of certain areas. Many studies showed its significance in the assessment of hepatic fibrosis (5-6). The study of Feld et al. (7) applied the SSI index for hepatitis hardness to judge the fibrosis stage, and draw the ROC curve. Although all the AUROC were above 0.90, the cases were mainly chronic hepatitis B patients.

At present, there are few reports related to SWE application on evaluating AIH fibrosis, and there are even fewer with regard to SWE on AIH, PBC, AIH-PBC OS classification. Wang et al. (1) applied SWE detection on 71 cases of patients with AIH, and concluded that the AUROC of middle fibrosis stage (S2 and S3), severe hepatic fibrosis and early hepatic cirrhosis (S4) were respectively 0.77 and 0.97. In this study, the SSI measured AUROC of AIH S2 stage, S3 stage and S4 stage of the 291 patients were respectively 0.516, 0.712, and 0.877, the results showing certain differences. SSI index may be related to the differences in specimen quantities. The overall samples in that study were less, just 70 cases.

Compared to the APRI index, the SSI index had higher diagnostic efficiency on each fibrosis stage of AIH, PBC and AIH-PBC OS. SSI index had significance on patients with high risk of liver fibrosis of S3 stage and S4 stage.

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## LETTER TO THE EDITOR

**CLINICAL EFFECTS OF THERMOTHERAPY IN COMBINATION WITH INTRACAVITARY INFUSION OF TRADITIONAL CHINESE MEDICINE IN THE TREATMENT OF MALIGNANT PLEURAL EFFUSION**Y. GU<sup>1</sup>, L. JIANG<sup>2</sup>, J.H. MIAO<sup>3</sup>, T.S. LIANG<sup>1</sup>, Q.C. KAN<sup>4</sup> and D.K. YANG<sup>5</sup><sup>1</sup>Radiotherapy Ward III, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China;<sup>2</sup>Radiotherapy Ward II, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China;<sup>3</sup>Integrated Medical Department, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China; <sup>4</sup>Dean's Office, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China;<sup>5</sup>Compositive Thermotherapy Center, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, China*Received February 7, 2016 – Accepted September 9, 2016*

The first two authors contributed equally to this study

**Malignant fluid, a commonly seen tumor associated complication, mainly includes peritoneal effusion, malignant pleural effusion and pericardial effusion. It can produce huge negative influence on the quality of life of patients and even lead to death. Treatment of malignant effusion is one of the effective measures for improving life expectancy of patients. To evaluate the effect of thermotherapy in combination with intracavitary infusion of Kangai injection in treating malignant pleural effusion, 195 patients who received treatment from April 2010 to October 2014 in the First Affiliated Hospital of Zhengzhou University were selected and divided into an observation group and two control groups (group A and B). The observation group was treated by thermotherapy in combination with intracavitary infusion of kangai injection. Control group A was treated by intracavitary infusion of kangai injection and control group B was treated by hyperthermal perfusion in combination with intracavity chemotherapy. Clinical effects, quality of life, treatment safety and untoward reactions were compared between the groups. It was found that differences of WBC, RBC and PLT levels before and after treatment had no statistical significance comparisons within group and comparisons between groups ( $P>0.05$ ); hepatic and renal functions of the groups had no remarkable difference before or after treatment ( $P>0.05$ ). The clinical effect of the observation group was superior to that of control groups A and B ( $P<0.05$ ); the Karnofsky performance status (KPS) score of the observation group was much higher than that of control groups A and B ( $79.34\pm10.58$  vs  $71.11\pm9.64$ ), but the difference of the ZPS score between groups had no statistical significance ( $P>0.05$ ). It can be concluded that thermotherapy in combination with intracavitary infusion of traditional Chinese medicine can be safely applied as it has positive effects and remarkably improves quality of life, therefore it is clinically worth promoting.**

To the Editor,

Malignant pleural fluid, a complication in middle

and late-stage malignant tumors, is induced by the

exosmosis of serous fluid when tumors transfer to

*Key words: malignant pleural effusion, thermotherapy, intracavitary infusion, kangai injection**Mailing address:*

Dr Daoke Yang,  
No.1 Jianshe Road,  
The First Affiliated Hospital of Zhengzhou University,  
Zhengzhou, Henan, 450052, China  
e-mail: dkyangqckan@163.com

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the pleural cavity; an excess of pleural effusion can oppress pulmonary tissue and induce respiratory dysfunction, finally leading to pulmonary atelectasis and infection. The clinical manifestation of malignant pleural effusion includes dyspnea, unable to lie on the back and shortness of breath (1~3). Currently, malignant fluid can be treated in various ways, however, the clinical use of chemotherapeutics and surgery have been gradually restrained (4, 5). Therefore, it is necessary to find a new approach for the treatment of malignant pleural effusion.

Intracavitary injection of a Chinese herbal preparation in combination with chemotherapeutic drugs can improve the curative effect and can also strengthen immunity and enhance the quality of life (QoL) (6). Kangai injection composed of the effective components of *Radix sophorae flavescentis* (Sophora root flavescent), *Astragalus membranaceus* (membranous milk-vetch root) and ginseng can benefit vital energy, strengthen body resistance, and resist tumors. Furthermore, thermotherapy has gradually become a new anti-tumor method after surgery, chemotherapy, radiotherapy and biological treatment (7, 8). The theoretical basis for why intracavity thermotherapy is effective for treating malignant effusion is the difference of temperature tolerance between tumor tissue cells and normal tissue cells. Normal tissue cells can not be killed at 47°C in one hour, while a temperature of 43°C can produce irreversible damage to tumor cells in one hour. This study adopted the intracavitary injection of a Chinese herbal preparation combined with thermotherapy to treat malignant pleural fluid, aiming to find a safe and effective treatment.

## MATERIALS AND METHODS

### General data

One hundred and ninety-five patients with malignant pleural effusion who received treatment in the Department of Radiotherapy of the First Affiliated Hospital of Zhengzhou University from April 2010 to October 2014 were selected. All the patients participating in the study conformed to the inclusion criteria. All subjects had been pathologically proved as having a malignant tumor and pleural effusion by imaging and clinical

examinations. The patient met the following criteria: Karnofsky performance score (KPS)  $\geq 50$  points, Zubrod-ECOG-WHO score (ZPS)  $\leq 3$  points, white blood cell count (WBC)  $\geq 4.0 \times 10^9/L$ , absolute neutrophil count (ANC)  $\geq 2.0 \times 10^9/L$ , platelet count (PLT) count  $\geq 100 \times 10^9/L$ , Haemoglobin B (Hb)  $\geq 9$  g/L, total bilirubin level  $\leq 1.5$ -fold higher than the normal value, transaminase level  $\leq 1.5$ -fold higher than the normal value, and levels of urea nitrogen and creatinine  $\leq$  normal value. They had no obvious infection or bleeding tendency and had signed informed consent before surgery. Those who did not meet the above criteria, who had developed heart, liver, kidney or blood diseases, rejected treatment, did not follow the treatment scheme, had predicted survival period less than three months or developed no tumor-associated diseases were excluded. Of 195 patients, 104 were males and 92 were females, with ages ranging from 31 to 56 years (average  $45.62 \pm 4.86$  years) and disease course ranging from 3 months to 4 years (average  $3.58 \pm 1.26$  years); there were 56 cases of primary lung cancer, 36 cases of breast cancer and 12 cases of other tumors. All patients were examined with 1000~1500 mL of pleural effusion by ultrasound and computed tomography (CT) and had not undergone local drug perfusion or systemic chemotherapy in the previous 30 days. The subjects were randomly divided into an observation group and two control groups (group A and B), 65 in each group. Difference of baseline data such as gender, age and disease course between the three groups had no statistical significance ( $p > 0.05$ ); hence, the results were comparable.

### Therapeutic method

After puncture location under the guidance of B ultrasound, the site with the largest amount of fluid or the juncture of scapular line, posterior axillary line and 7<sup>th</sup>~9<sup>th</sup> rib was punctured after local anesthesia, followed by closed drainage of the thoracic cavity and the connection of a drainage pack. The drainage volume was lower than 600 mL on the first day and was lower than 1000 mL after the first day. The fluid was completely expelled in 2-3 days. Thermophysical treatment was adopted on the day following that when there was no pleural fluid or only little pleural fluid observed by B ultrasound or chest X-ray examination; silicone tubes were connected to a hyperthermic perfusion machine; thermophysical treatment using normal saline was performed according

to operation procedures, for 60~90 min; four sequences of treatment was considered as one treatment course; initially there was an interval of 24 h between treatments, but gradually the interval became longer. After thermophysical treatment, 60 mL of Kangai injection (Batch No.: Z20026868; Changbaishan Pharmaceutical Inc., China) was injected into the cavity. After drug injection, the patients were asked to change from supine position to lateral position or prone position to make the drug be evenly distributed in the pleura. For control group A, the chests of the patients were injected with Kangai injection after puncture and drainage and thermophysical treatment was not performed. In control group B, 60~80 mL of cisplatin dissolved with normal saline was injected into

the thoracic cavity following thermophysical treatment after puncture and drainage.

#### Observation index

Levels of WBC, red blood cells (RBC) and PLT as well as changes of liver and renal functions were compared before treatment and one month after treatment. The curative effects were evaluated one month after the treatment taking WHO criteria as reference: complete response (CR): pleural effusion disappeared for 4 weeks; partial remission (PR): pleural effusion reduced for more than 50% and the condition lasted for at least 4 weeks; stable disease (SD): effusion reduced for less than 50% and showed no increasing tendency, and clinical symptoms

**Table I.** Comparison of count of blood cells between the groups.

| Group             | WBC ( $10^9/L$ ) |           | ANC ( $10^{12}/L$ ) |           | PLT ( $10^9/L$ ) |               |
|-------------------|------------------|-----------|---------------------|-----------|------------------|---------------|
|                   | Before           | After     | Before              | After     | Before           | After         |
| Observation group | 8.12±3.11        | 8.93±3.34 | 3.86±0.84           | 3.72±0.54 | 322.83±131.18    | 331.49±130.57 |
| Control group A   | 8.07±3.03        | 8.09±2.92 | 3.93±0.7            | 3.88±0.71 | 320.47±132.46    | 319.41±143.65 |
| Control group B   | 9.14±3.04        | 7.84±3.61 | 3.84±0.53           | 3.87±0.52 | 321.45±129.42    | 299.50±145.32 |

(mean ± SD)

**Table II.** Comparison of liver and renal functions between groups.

| Group           | Urea (mmol/L) |           | Creatinine (umol/L) |             | Transaminase (U/L) |             |
|-----------------|---------------|-----------|---------------------|-------------|--------------------|-------------|
|                 | Before        | After     | Before              | After       | Before             | After       |
| Control group   | 6.34±2.34     | 6.56±2.11 | 77.04±35.82         | 84.33±46.81 | 29.14±15.96        | 33.86±19.28 |
| Control group A | 5.50±1.98     | 5.53±2.11 | 68.75±19.04         | 68.14±20.08 | 32.88±16.18        | 32.39±12.4  |
| Control group B | 7.15±1.77     | 7.42±2.3  | 70.37±21.24         | 69.67±18.94 | 36.72±21.13        | 36.26±17.42 |

(mean ± SD)

**Table III.** Comparison of KPS and ZPS between two groups.

| Group             | KPS (point) |                            | ZPS (point) |                        |
|-------------------|-------------|----------------------------|-------------|------------------------|
|                   | Before      | After                      | Before      | After                  |
| Observation group | 72.63±9.58  | 79.34±10.58 <sup>*ab</sup> | 2.14±0.62   | 1.64±0.25 <sup>b</sup> |
| Control group A   | 71.34±1.24  | 71.11±9.64                 | 2.25±0.74   | 1.64±0.25 <sup>b</sup> |
| Control group B   | 74.12±9.68  | 72.02±9.74                 | 2.17±0.79   | 1.76±0.62 <sup>b</sup> |

(mean ± SD) <sup>\*</sup>*p* < 0.05, compared to control group A; <sup>a</sup>*p* < 0.05, compared to control group B; <sup>b</sup>*p* < 0.05, compared to before treatment.

significantly relieved; progressive disease (PD): effusion had no significant reduction, or even increased (CR+PR as effective). KPS and ZPS scored in a twelve-month follow-up were compared between two groups. Incidence of untoward reactions was compared between two groups taking gastrointestinal reaction as the reference.

#### Statistical analysis

SPSS19.0 was used for analyzing data. Enumeration data were expressed as rate (%). Measurement data were expressed as mean±SD. Comparison between groups was performed using *Chi*-squared test and *t*-test. Difference was considered to be statistically significant if *P*<0.05.

## RESULTS

#### Comparison of count of blood cells before and after treatment

No significant differences were observed in WBC, RBC and PLT before or after treatment between groups (*P*>0.05) (Table I).

#### Changes of liver and renal functions before and after treatment

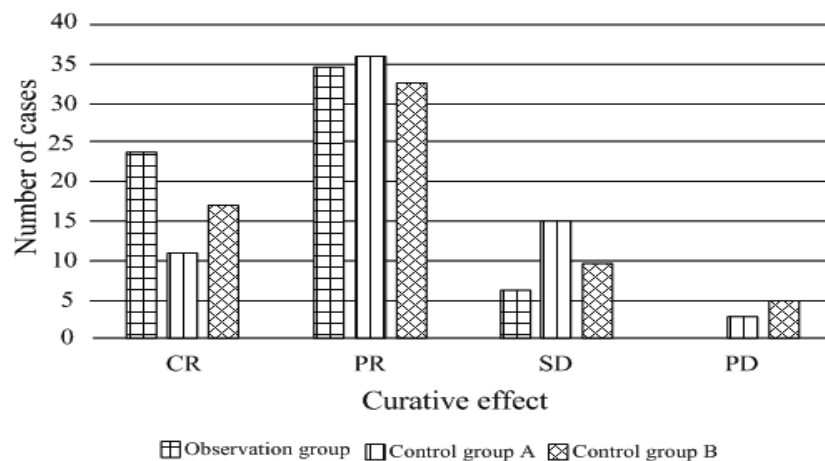
Indexes of liver and renal functions of patients in the groups are shown in Table II. Statistical analysis resulted that there was no significant difference in levels of urea, creatinine and transaminase of the groups before or after treatment (*P*>0.05).

#### Comparison of incidence of untoward reactions between groups

In the observation group, 1 case had intracavity infection-induced fever, but it was cured after undergoing anti-infection treatment. In control group A, there were 3 cases of nausea and vomiting and 1 case of intracavity infection-induced fever. In control group B, there were 7 cases of adverse reactions, 4 cases of gastrointestinal symptoms (nausea and vomiting), 2 cases of bone marrow suppression and 1 case of hemothorax. The differences of the incidence of adverse reactions between the three groups had no statistical significance (*P*>0.05).

#### Comparison of clinical effect between two groups

Curative effects were observed after four-week treatment. There were 24 cases of CR, 35 cases of PR, 6 cases of SD and no cases of PD in the observation group, with an overall response rate of 90.77%; there were 11 cases of CR, 36 cases of PR, 15 cases of SD and 3 cases of PD in the control group A, with an overall response rate of 72.31%; there were 17 cases of CR, 33 cases of PR, 10 cases of SD and 5 cases of PD in the control group B, with an overall response rate of 76.92%; the overall response rate of the observation group was much higher than that of the control group A and B, and the differences had statistical significance (*P*<0.05) (Fig. 1).



**Fig. 1.** Comparison of curative effect between groups.

#### *Comparison of KPS and ZPS between groups before and after treatment*

ZPS of the observation group was much higher than that of the control groups A and B after treatment ( $P < 0.05$ ); ZPS were observed with a significant decrease in the three groups, but the difference between the groups had no statistical significance ( $P > 0.05$ ) (Table III).

### DISCUSSION

Malignant pleural fluid, which is difficult to treat due to its rapid development and recurrence in late stage, can induce compression atelectasis and restrictive ventilatory dysfunction, lower mediastinal displacement, and restrain respiratory and circulatory functions (9, 10). Positive treatment of malignant pleural effusion can improve the prognosis and benefit the treatment of primary tumors.

This study treated malignant pleural fluid with intracavity thermotherapy in combination with the perfusion of Chinese traditional medicine and obtained a certain curative effect. The research results demonstrated that the overall remission rate of the treatment group was much higher than that of the control group A and B ( $P < 0.05$ ), indicating thermotherapy could improve the treatment effect of malignant pleural fluid in combination with Kangai injection (12, 13).

Furthermore, the hemocyte and hepatic and renal function of the patients before and after treatment had no statistically significant difference in the three groups. But the incidence of adverse reaction of the treatment group was lower than that of the other two groups, indicating thermotherapy combined with Chinese traditional medicine perfusion had no obvious influence on gastrointestinal reactions. Additionally, all patients had improved QoL after treatment. The treatment group had the most remarkable improvement in KPS score, which suggested thermotherapy combined with Kangai injection could effectively enhance QoL.

To sum up, thermotherapy combined with intracavitary injection of Chinese herbal preparation is significantly effective in treating malignant pleural fluid, therefore it is worth clinical application.

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## LETTER TO THE EDITOR

**EFFECTS OF CARBOMER EYE DROPS IN COMBINATION WITH ORTHOKERATOLOGY LENS IN TREATING ADOLESCENT MYOPIA**

Y.H. SHI<sup>1</sup>, L.T. ZHOU<sup>2</sup>, C.X. ZHANG<sup>3</sup>, Y.Z. LI<sup>4</sup>, J.Z. ZHANG<sup>1</sup>, H.M. ZHOU<sup>1</sup>,  
Y.G. LI<sup>1</sup>, T. LIU<sup>1</sup>, L.L. ZHANG<sup>1</sup>, L.N. SUN<sup>1</sup> and Z. CHEN<sup>1</sup>

<sup>1</sup>Department of Ophthalmology, Henan Provincial People's Hospital, Zhengzhou, China; <sup>2</sup>Teaching and Research Section of Optometry, Department of Medical Technology, Zhengzhou Railway Vocational and Technical College, Zhengzhou, China; <sup>3</sup>Department of Ophthalmology, Kaifeng Central Hospital, Kaifeng, China; <sup>4</sup>Luoyang Boai Ophthalmology Hospital, Luoyang, China

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To investigate the effects of carbomer eye drops (CED) during long-time wearing of overnight orthokeratology lens of adolescents with myopia, 260 teenagers with myopia treated in the Henan Provincial People's Hospital from June 2012 to August 2014 and followed-up for more than 2 years were enrolled. All the patients underwent regular fitting of orthokeratology lens. They were divided into a CED (Vidisc) group (130 cases, 260 eyes treated with CED) and rewetting drops (RD) (Baushe and Lomb) group (130 cases, 260 eyes treated with RD). The effects in the two groups were observed. The incidence of corneal epithelial defects one day, one week and one month after treatment of the CED group was lower than that of the RD group, and the difference was statistically significant ( $P < 0.05$ ); the tear break up time (TBUT) of the CED group was higher than that of the RD group at different time points, and the difference had statistical significance ( $P < 0.05$ ); the difference of the value of Schirmer I test between the two groups had no statistical significance ( $P > 0.05$ ). It is concluded that carbomer eye drops can stabilize tear film and protect and repair corneal epithelium during the wearing of orthokeratology lens.

To the Editor,

Orthokeratology, a myopia correction method, has been extensively applied as it can temporarily and reversely lower the degree of myopia (1, 2). Among the prevention and control strategies for myopia, orthokeratology has attracted extensive attention as it can control the increase of myopia degree (3-5). However, mild corneal epithelial wounds, such as cornea epithelial halftones and corneal epithelium edema or corneal epithelial defects, may occur in the initial stage of lens wearing, with an incidence of 15%~40% (6). Symptoms such as foreign body sensation, phengophobia and

unaided vision decline are the most common causes in early stage for the dissatisfaction of patients who wear orthokeratology lens.

Carbomer eye drops, a hydrophilic gel containing 0.2% polyacrylic acid, is composed of solid substrate and water phase dispersion layer which is similar to the double-layer structure of tear film, i.e., mucous layer and water layer, can be adhered to the surface of cornea and form a liquid storage cavern on the surface of eyeball. The pharmacological property of carbomer eye drops lies in protecting the corneal and conjunctival epithelium by increasing the adhesion and retention time of substrate on the surface of

*Key words: orthokeratology lens, myopia, carbomer eye drops*

Mailing address:

Dr Yinghui Shi,  
Department of Ophthalmology,  
Henan Provincial People's Hospital,  
No.7 Weiwu Road,  
Zhengzhou, 450003, China  
e-mail: syhshiyh@163.com

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eyeballs through improving the viscosity of substrate (7). This study enrolled 130 myopia orthokeratology patients who used carbomer eye drops, aiming to observe the protective effect of carbomer eye drops on cornea.

## MATERIALS AND METHODS

### *Research subjects*

Two hundred and sixty patients with myopia who received treatment in the Department of Ophthalmology in the Henan Provincial People's Hospital, Henan, China were enrolled, and a total of 520 eyes were studied. There were 172 males and 88 females, aged 7~20 years old (average  $13.3 \pm 3.4$  years). All patients signed informed consent before examination. Before wearing the lens, the average spherical equivalent refraction was ( $-3.46 \pm 1.75$ ) D and the average curvature of central cornea was ( $43.04 \pm 1.93$ ) D. All the subjects had no history of other eye diseases, eye surgery or trauma, had never worn any corneal contact lens, and had no contraindications to the wearing of corneal contact lens. They were divided into two groups, i.e., a carbomer eye drops (CED) group and a rewetting drops (RD) group, 130 cases (260 eyes) in each group. The differences of general data between the two groups had no statistical significance; therefore, the results were comparable.

### *Methods*

The material for the lens was Boston XO with an oxygen permeation coefficient (DK) of  $100 \times 10^{-11} (\text{cm}^2/\text{s}) \cdot \text{mL} (\text{O}_2)/\text{mL} \cdot \text{mm-Hg}$  and an optic central thickness of 0.22 mm. The internal surface was designed into reversal geometric four curves. The diameter of the lens was 10.6 mm. The patients in the CED group used carbomer eye drops (Dr. Gerhard Mann, Germany) before wearing or removing lens, while the patients in the control group used rewetting drops (Baushe and Lomb Co., Ltd., Batch No.: CA2017).

The patients in the CED group were trained how to wear, remove and take care of the lens. They were required to wear the lens after washing with care solution and cold boiled water before sleeping and drop CED onto the lens. They wore the lens for 8-10 hours every night. The following morning, CED were dropped into both eyes, then the lens were removed using a lens remover after blinking several times. The lens were stored in a lens box after being

washed. The RD group patients used RD and the procedure was the same as the CED group.

### *Observation indicators*

The observation of corneal epithelial wound: the patients went for reexamination one day, one week and one month after the first day of lens wearing. Corneal fluorescein staining was observed under the cobalt-blue light of a slit lamp. Corneal epithelial wound severity was divided into different grades: grade 0: no dotted staining on corneal epithelium or several dotted stains; grade I: mild scratch or a little scattered dotted staining; grade II: mild discomfort and relatively intensive dotted staining on corneal epithelial; grade III: obvious simulative symptoms and defects of small pieces of epithelium; grade IV: severe simulative symptoms and large pieces of corneal epithelium.

Detection of TBUT: sodium fluorescein ( $10 \text{ g} \cdot \text{L}^{-1}$ ) was dropped into the conjunctival sac. The examination was performed under the cobalt-blue light of the slit lamp. The patients were asked to blink for 2 or 3 times to make tear films distribute evenly and then look straight ahead. The time between the last time of blinking and the appearance of the first black spot was recorded; each patient was measured three times and the average value was taken as the final value.

Schirmer I test (SIT): one end of a piece of test strip was gently put at the position 1/3 below the conjunctival sac outside the lower eyelid. The length of wetting part of the test paper was observed after 5 min of eye closing.

### *Statistical analysis*

Data were processed using SPSS 19.0 software. Measurement data were expressed as mean  $\pm$  standard deviation (SD). TBUT and SIT values were processed by independent sample *t*-test. Ranked data were compared using rank sum test. *P* values  $< 0.05$  were taken as indicating statistical significance.

## RESULTS

### *Changes of corneal fluorescein staining of the two groups after wearing the lens*

The corneal epithelial wounds of the CED group were milder than those of the RD group ( $P < 0.05$ ), as shown in Table I. Patients who were observed

with corneal central epithelial defects were advised to stopping wearing lens and use corneal repairing adhesive; the defects healed within one or two days. As the corneal central epithelial defects were not the adverse reaction of lens fitting, the patients continued to wear the lens after the healing of cornea.

#### *Comparison of TBUT between the two groups*

The TBUT of the CED group and the RD group

had no statistical significance before lens wearing ( $P>0.05$ ); the TBUT of the CED group was longer than that of the RD group at different time points after lens wearing, and the difference had statistical significance ( $P<0.05$ ); the TBUT of the two groups at different time points after lens wearing was remarkably lower than that before lens wearing, and the difference had statistical significance ( $P<0.05$ ), as shown in Table II.

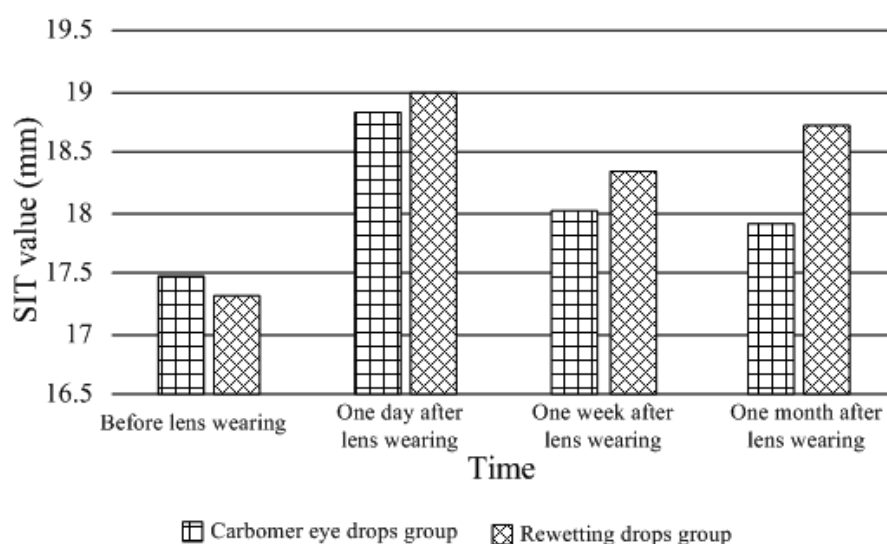
**Table I.** Comparison of corneal epithelial injury severity between the two groups after lens wearing

|                            | One day after lens wearing |                       | One week after lens wearing |                       | One month after lens wearing |                       |
|----------------------------|----------------------------|-----------------------|-----------------------------|-----------------------|------------------------------|-----------------------|
|                            | Carbomer eye drops group   | Rewetting drops group | Carbomer eye drops group    | Rewetting drops group | Carbomer eye drops group     | Rewetting drops group |
| Corneal staining grade 0   | 238                        | 102                   | 242                         | 142                   | 249                          | 189                   |
| Corneal staining grade I   | 18                         | 136                   | 15                          | 104                   | 11                           | 67                    |
| Corneal staining grade II  | 4                          | 18                    | 3                           | 11                    | 0                            | 4                     |
| Corneal staining grade III | 0                          | 4                     | 0                           | 3                     | 0                            | 0                     |
| u value                    | 6.901                      |                       | 5.241                       |                       | 2.795                        |                       |
| P-value                    | <0.05                      |                       | <0.05                       |                       | <0.05                        |                       |

**Table II.** The comparison of TBUT between the two groups.

| Group                    | Before lens wearing | One day after lens wearing | One week after lens wearing | One month after lens wearing |
|--------------------------|---------------------|----------------------------|-----------------------------|------------------------------|
| Carbomer eye drops group | 10.22±2.61          | 6.88±1.80*                 | 6.03±1.56*                  | 6.37±1.42*                   |
| Rewetting drops group    | 10.57±2.21          | 5.49±1.71*                 | 5.36±1.43*                  | 5.43±1.04*                   |
| t                        | -0.673              | 4.114                      | 2.509                       | 3.054                        |
| P-value                  | >0.05               | <0.05                      | <0.05                       | <0.05                        |

(mean±SD, t/s) \*  $P<0.05$ , compared to before lens wearing



**Fig. 1.** Changes of SIT values of the two groups.

#### *Comparison of SIT values between the two groups*

The SIT values of the two groups had no statistically significant differences before lens wearing ( $P>0.05$ ); the TBUT of the CED group at different time points after lens wearing and before lens wearing had no statistical significance ( $P>0.05$ ), as shown in Fig. 1.

### DISCUSSION

Orthokeratology corrects vision by flattening the cornea through the special design of the lens and the interaction of hydrostatic pressure of tear layer and film pressure (8, 9), indicating the important role of the quality of tear film for moulding. The research results demonstrated that the TBUT of the CED group was longer than that of the RD group at different time points, but the difference of SIT value was not significant. The preservative ingredients of the rewetting drops in the RD group can induce the toxic reactions of ocular surface including the effects on the stability of tear film and toxic and immune reactions of corneal and conjunctival epithelial cell (10). CED is a thixotropic gel whose consistence can be changed through shear stress (blinking). Water in the gel can be released partially to supplement tears at each blink (11). Studies concerning acute and long-

term toxicity and local toxicity demonstrated that CED had no toxicity and was highly tolerable (12). Therefore, CED can effectively maintain the quality of tears. In addition, this study suggested that the corneal staining grade of the CED group was lower than that of the RD group at different time points, indicating that CED could promote repair of corneal epithelium, lower the incidence of corneal epithelial dotted staining or corneal epithelial edema induced by wearing orthokeratology lens, and protect cornea during lens wearing.

In conclusion, CED can stabilize tear films and protect and repair corneal epithelium during the wearing of orthokeratology lens. The effect of CED was observed for one month only, therefore its long-term effects remain to be deeply investigated.

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## LETTER TO THE EDITOR

## ACUPUNCTURE IN ENDOCRINE DISORDERS: A CRITICAL APPRAISAL

Y-Z. DENG<sup>1</sup>, L-B. LI<sup>2</sup>, L-G. XU<sup>1</sup>, D. ZHOU<sup>3</sup>, L-J. WEI<sup>3</sup> and Y. LIU<sup>1</sup>

<sup>1</sup>Department of Acupuncture, The affiliated Hospital to Changchun University of Chinese Medicine, Changchun, China; <sup>2</sup>Department of Radiology, The affiliated Hospital to Changchun University of Chinese Medicine, Changchun, China; <sup>3</sup>Department of Ophthalmology, The affiliated Hospital to Changchun University of Chinese Medicine, Changchun, China

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Acupuncture is an integral part of ancient Chinese medical practice. The technique has been used extensively in pain relief and is being tried for many other chronic conditions. Industrial development and affluence lead to the increase in the prevalence of many endocrine disorders such as diabetes, obesity, and polycystic ovarian disease. The rising prevalence of the endocrine morbidity is observed in both the developing and developed nations. The management of these disorders involves major lifestyle modification coupled with a long-term drug intake. In such situations, patients often look at alternative therapeutic options existing in complementary and alternative medicine. The globalization of the world medical practice has led to the spread of acupuncture beyond China to other parts of the world. Acupuncture has been tried extensively in the management of various endocrine disorders with inconsistent results. In this review, we highlight the principles of acupuncture and its role in the management of various endocrine disorders.

To the Editor,

Acupuncture originated in China about 5,000 years ago and is an integral part of traditional Chinese medicine (TCM). The word ‘acupuncture’ means puncturing of the skin using a sharp- pointed object, leading to stimulation of certain points in the body. The major research increase on the use of acupuncture is in the areas of chronic pain, cardiovascular disorders, and neuropsychiatric conditions (1-3). However, use of acupuncture did not spread beyond China for many centuries due to lack of knowledge, technical expertise and the language barrier in understanding the technique. Moreover, the Westerners did not believe in the

therapy in the absence of pathophysiological basis and lack of randomized controlled trials (4).

Research on acupuncture has been growing rapidly in the last few decades. Though most of it originated from China, other countries have contributed to its exponential growth. The use of acupuncture spread initially to other Asian countries, followed by Europe and America. The use of acupuncture in the Western world was appreciated for the first time in the consensus statement of the U.S. National Institute of Health (NIH), in 1997 (5). The National Institute of Health stated that there is sufficient scientific evidence to incorporate acupuncture in modern medicine.

*Key words: acupuncture, endocrinology, obesity, traditional medicine, diabetes*

*Mailing address:*

Dr Yang Liu,  
Training Centre, The affiliated Hospital  
Changchun University of Chinese Medicine, Changchun, China,  
No. 1478 Gongnong Road, Changchun, China  
e-mail: changchun\_ctm@yahoo.com

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Acupuncture has been shown to have beneficial effects beyond that of pain in combating metabolic and reproductive disorders (3). Endocrine disorders are also increasing in their prevalence due to changing lifestyle, and diabetes is one of the major disorders that affect the entire world population (6, 7).

In this review, we highlight the basic principles of practice involved in acupuncture and the effects of this technique in the management of various endocrine disorders.

#### *Traditional medicine and acupuncture*

Metabolic and endocrine disorders often run a relentlessly progressive course and are not amenable to allopathic treatments. Both patients and practitioners look at complementary and alternative forms of medicine (CAM) in the management of metabolic disorders. The practice of yoga in India has shown to be of immense benefit in the management of diabetes and its complications (8). These CAM therapies use innate immunity, neuroendocrine responses and anti-stress mediators to improve the metabolic condition. The importance of these traditional systems of medicine in India can be gauged by the existence of a separate ministry as part of the health system. However, certain problems, such as lack of standardized methods of assessment and treatment protocols, lead to the absence of widespread use of these forms of therapy (6, 8).

The TCM considers that body is in an equilibrium state due to the presence of two opposing forces, Yin and Yang. The former represents the weak, dark, passive aspects, whereas the latter (9) accounts for those strong, bright and active. Qi is the energy flow, which represents the underlying physiological process that flows along the specific channels in the body, known as meridians. The human body contains about 400 acupuncture points (acupoints) and 20 meridians that connect these points. The energy flow along the meridians ensures proper functioning of the underlying systems and organs. The technique of acupuncture involves manual insertion of needles into the particular acupoints and their further stimulation. This stimulation is carried out using manual pressure or by applying a small electric current, which is also known as electro-acupuncture

(3, 4, 10). Another modification consists in the use of a cigar-shaped stick made of dried mugwort to warm the local regions known as moxibustion (11). This results in improving the blood flow and also the energy flow of the underlying tissue.

#### *Acupuncture and endocrinology—pathophysiological basis*

The Chinese traditionally believe that any disease is caused by the imbalance between Yin and Yang and a disruption to the movement of Qi. Acupuncture is thought to restore the energy flow Qi through proper meridians and improve the functioning of the underlying organs. Acupoints act as the nodal controls in the pathway of energy and regulate the flow of Qi for the desired outcome. The basic understanding is that the stimulation of the nerve endings results in a cascade of changes leading to the release of certain neurotransmitters at a distant location. The pathophysiological mechanisms have been clearly demonstrated in cases of acupuncture analgesia. Acupuncture analgesia is mediated by the stimulation of the sensory fibers that convey the impulses to the central nervous system via the spinal cord. This results in the release of neurotransmitters, such as enkephalins and endorphins, which help in blocking the pain perception (3). The release of certain other neurotransmitters has been postulated to mediate other beneficial effects of acupuncture. These effects are mediated by the neural, endocrine, immunological signals to the specific areas of the central nervous system resulting in physiological alterations in the body. The suggested beneficial effects of acupuncture in various organs are illustrated in Fig. 1.

#### *Acupuncture and obesity*

The surplus intake of calories is the main culprit responsible for the pandemic of obesity in the current generation. The positive calorie balance is seen even in children and adolescents leading to an alarming rise in childhood obesity (1). The medical management of obesity is ineffective, and bariatric surgery is considered as the last resort for many patients. Hence, obese individuals look at CAM that helps in the weight loss. These include the use of

products, such as apple cider vinegar, ginseng, and green tea, which are utilized for the weight loss strategies. Acupuncture also has been studied in the management of obesity that is resistant to lifestyle modification. Although the results on the efficacy of acupuncture in weight reduction are inconsistent, they do establish the safety, tolerability, and durability of this procedure. A recent meta-analysis suggested that acupuncture has shown beneficial effects in tackling obesity (1). The benefits are mediated by appetite suppression, improvement in the insulin sensitivity, effective fat disposal, and perceived psychological benefits. A recent study showed that 20 weeks of regular acupuncture therapy resulted in mean weight loss of about 7 kg coupled with a 0.2% reduction in the glycosylated hemoglobin (12).

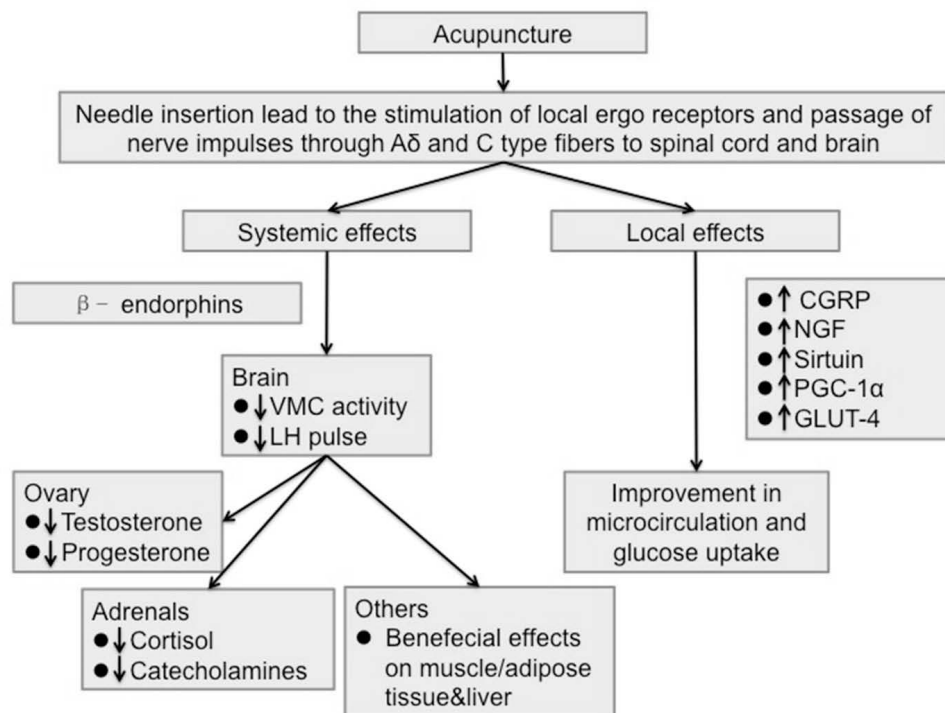
#### *Acupuncture and diabetes*

Acupuncture has been widely studied in a spectrum of clinical conditions related to insulin resistance (2). They include obesity, type 2 diabetes mellitus (T2DM), polycystic ovarian disease (PCOD)

and dyslipidemia (1, 2). The beneficial effects of acupuncture in the amelioration of IR and obesity are shown in Fig. 2.

Electro-acupuncture has been demonstrated to release the endogenous peptides which may stimulate the release of insulin from the pancreas. The majority of the studies showing beneficial effects in diabetes were carried out on rats, with only a limited number involving humans (6). Acupuncture has been used at various levels for the prevention of metabolic disorders. It has been shown to delay the onset of diabetes, serve as an adjunct in the management of diabetes and also reduce the rate of complications. The acupuncture meridians of Zusanli and Sanyinjiao gave better control of postprandial hyperglycemia over that of the Shousanli meridian. Acupuncture and exercise have shown to exert beneficial effects over those of the placebo in the management of diabetes along with the background therapy (6, 8).

The use of CAM therapies is very common in chronic disabling conditions such as diabetic motor sensory neuropathy (DMSN) (13). DMSN



**Fig. 1.** Effects of acupuncture – systematic and local effects of stimulation by needle, on various (not only endocrine) organs.

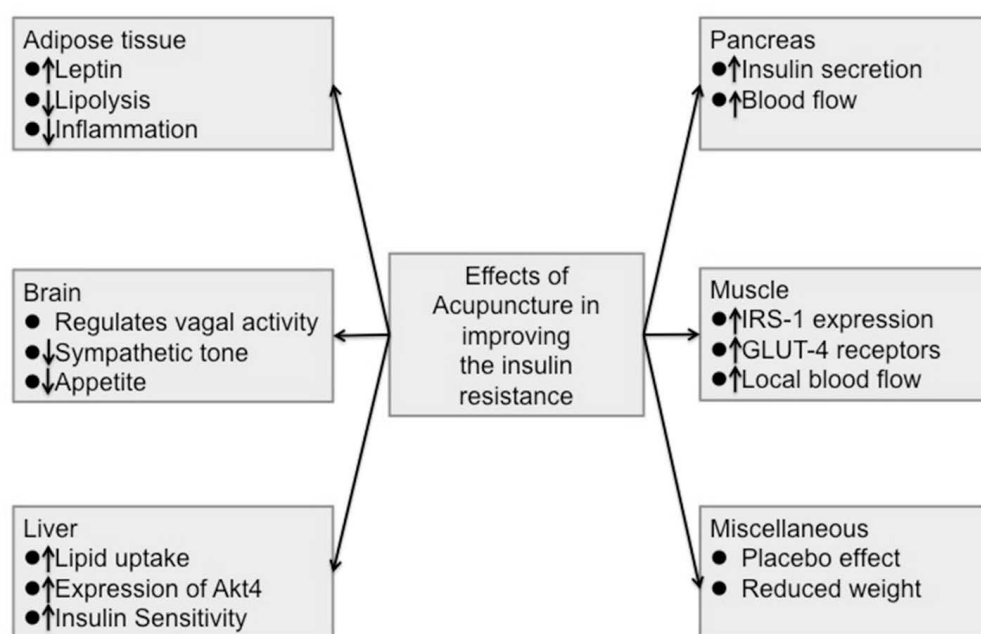
is often resistant to a variety of therapeutic options, prompting the patients to look for alternative modes of therapy. Acupuncture analgesia has been tried on DMSN patients, with reasonable success rates. Researchers have used specific points of Ganshu, Pishu, Shenshu and Feishu that help in reducing the pain associated with the this pathology (3). Few authors have shown the long-term effects of acupuncture, to nullify the impact of placebo effect. The benefit of acupuncture lies in its wide availability, low cost and absence of significant side effects. The preferences of the patients and the practitioners regarding the use of complementary medicines in the long-term management of diabetes were studied. The patients may opt for such therapies in order to meet their self-perceived needs despite a worsening clinical condition (3, 4, 13). The advantages of these therapies include the ease of availability at the community level and perception of being harmless to the body.

#### *Acupuncture and gynecological endocrinology*

Acupuncture has been evaluated extensively in the field of gynecological endocrinology. The specific

disorders where acupuncture has been used include infertility, menstrual irregularity, PCOD, hirsutism and menopausal hot flashes. Stress is common among women undergoing assisted reproduction techniques and is a common cause for the poor responders undergoing *in vitro* fertilization (IVF) (14). Stress and inadequate coping result in the alterations in uterine blood flow and also alter the contraction rate hindering the successful implantation rate (2). Prolonged stress also alters the immune response by altering the T cells in the peripheral blood. Acupuncture has been shown to reduce the stress and improve the rate of successful pregnancies in IVF. However, these findings were questioned by a researcher for the heterogeneity in the trials and the lack of a proper control group (4). Few authors have shown that the use of acupuncture was safe during pregnancy. The benefits of acupuncture are mediated by the change in the levels of cortisol and prolactin, which are the stress hormones of the body. Elevated prolactin levels suppress the immune response and increases the decidualization of the uterine cavity, making it more receptive to the embryo.

PCOD is characterized by a triad (menstrual



**Fig. 2.** Pathophysiological mechanisms of improving insulin resistance due to acupuncture release the endogenous peptides which may stimulate the release of insulin from the pancreas.



irregularity, hyperandrogenism and ovarian cysts) along with IR (2). Acupuncture therapy showed amelioration of IR in PCOD using an insulin clamp in one study. The study used the placement of needles in the somatic segments corresponding to the innervation of ovaries and pancreas and used both electrical and mechanical stimulation (1, 2). Acupuncture has been shown to improve various metabolic abnormalities such as IR, hyperandrogenism, ovulation frequency and hypercoagulable state in PCOD patients (4, 13). The effects are mediated by modulating the sympathetic nervous system activity and sex steroid synthesis. However, a recent Cochrane review highlighted the lack of evidence to recommend the use of acupuncture as a therapeutic option in the management of PCOD (2). This is due to the low quality of evidence and also failure to report potential important outcomes relevant to PCOD like infertility (2).

Endocrine disturbances involving the hypothalamo-pituitary-gonadal (HPG) axis presents commonly with menstrual irregularities and dysfunctional uterine bleeding. Previous studies have shown that the mind tranquilizing and menstruation regulating acupuncture method is superior to the simple acupuncture for these conditions. Menopausal transition is a phase of many hormonal alterations in the body. Hot flush is a debilitating symptom during peri-menopausal stage with unclear etiology and treatment. Acupuncture has been shown to reduce the severity of nocturnal hot flushes and also improve the quality of sleep. The effects are mediated by the increase in melatonin, estrogen receptor mRNA, calcitonin gene-related peptide and the choline acetyl transferase enzyme levels (38).

#### *Acupuncture in other endocrine disorders*

Moxibustion is a modified form of acupuncture that is used in male infertility and erectile dysfunction (11). Different types of acupuncture treatment have been tried based on the etiology of the erectile dysfunction. The acupuncture points for the psychogenic, vascular, hormonal erectile dysfunction are stratified and are used to strengthen the Yang Qi. Acupuncture and moxibustion have been shown to improve height in adult patients of short stature (11).

The authors proposed that a rise in the testosterone level could be responsible for the height gain. The effects of acupuncture during puberty have only been studied in animal models (15). Repeated electro-acupuncture over the arcuate nucleus results in the down-regulation of the HPG axis, mediated by the reduced expression of the neuropeptide Y (4). However, our search did not reveal any human studies regarding the effects of acupuncture during puberty.

#### *Future of acupuncture research*

The lack of knowledge and paucity of studies carried out on the methods of acupuncture limits the widespread use of this technique. It is difficult to carry out placebo-controlled, double-blind trials using acupuncture methods. The only solution is in the performing of a sham acupuncture using minimal surface stimulation at incorrect points (4). A placebo comparison in the acupuncture involves using Streitberger needles. However, these needles do exert pressure on the deeper tissues questioning the validity of the placebo effect as it is loosely termed as acupressure versus acupuncture. Future studies should be designed to minimize the above-mentioned errors and also to limit the placebo effect in evaluating the beneficial consequences of acupuncture.

## CONCLUSION

In conclusion, the use of acupuncture has come a long way in modern medicine. The beneficial effects extend well beyond the analgesic effect in the improvement of chronic metabolic, endocrine disorders. Future well-designed, large-scale studies are required to realize the potential of this mode of therapy in the management of endocrine disorders.

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## LETTER TO THE EDITOR

MECHANISM OF SERUM miR-21 IN THE PATHOGENESIS OF FAMILIAL AND TRIPLE  
NEGATIVE BREAST CANCER

L. YANG, Y. FENG, P. QI, S. XU and Y. ZHOU

*Surgery Department of Oncology, The Central Hospital of Xinxiang City, Xinxiang City, Henan  
Province, PR China**Received November 25, 2015 - Accepted May 25, 2016*

The aim of this study was to clarify the mechanism of miR-21 in familial and triple-negative breast cancer (TNBC) by exploring the expression of serum miR-21. The sera were collected from healthy women at high risk of breast cancer. miR-39 was employed as the external reference, and real-time fluorescence quantitative PCR was used to detect the expression of serum miR-21 in 77 subjects. The miR-21 expression of the familial breast cancer group, TNBC group, and breast cancer high risk group were significantly higher than those in the normal control group and other breast cancer groups ( $P < 0.01$ ). A high serum miR-21 expression level was associated with lymph node metastasis and Ki67 expression ( $P < 0.01$ ). Serum miR-21 was closely associated with TNBC and familial breast cancer, and its expression was associated with genetic expression, degree of malignancy, and prognosis.

To the Editor,

Breast cancer has a high incidence rate and can be caused by many factors, including environmental and genetic factors (1). Triple-negative breast cancer (TNBC) with negative estrogen, progesterone receptors, and human epidermal growth factor receptor-2 (HER-2) accounts for 15%~20% of all cases of breast cancers, and it has a higher recurrence rate and worse prognosis than other types (1-2).

MicroRNA (miRNA) is an endogenous, non-coding small RNA. MiRNA is closely associated with clinical pathological characteristics of tumors, and its expression can be used in the diagnosis, prognosis, and treatment of malignant tumors. Thus, microRNAs can serve as biomarkers in detecting cancer and its prognosis. The miR-21 expression level in breast cancer tissue is significantly higher than in normal breast tissue. The objective of this study was to uncover the role of miR-21 in the

pathogenesis of familial and triple negative breast cancer, and provide a possible biomarker for the diagnosis of breast cancer.

## MATERIALS AND METHODS

*Research subjects*

The research subjects were patients of The First Affiliated Hospital of Henan University between January, 2013 and May, 2014. The patients were divided into four groups: Group A consisted of 20 cases of TNBC patients aged  $53 \pm 1.33$  years, Group B was 8 cases of familial breast cancer, excluding TNBC patients, aged  $49 \pm 1.98$  years, Group B2 contained 9 cases of healthy first-degree relatives of familial breast cancer patients with a high risk of breast cancers and were aged  $37 \pm 4.12$  years, Group C was composed of 20 cases of other breast cancer patients, excluding TNBC and familial breast cancer patients, aged  $56 \pm 2.43$  years, and Group D was 20 cases of healthy

*Key words: miR-21, familial breast cancer, TNBC, serum, real-time fluorescent quantitative PCR detection*

*Mailing address:*

Dr Yueqing Feng,  
The Central Hospital, Xinxiang City,  
No.5 6, Jinsui Avenue, Xinxiang City, 453000, PR China  
e-mail: yangliqin1117@sina.com

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control group patients aged  $45 \pm 3.24$  years. To be included in the control group, subjects had to be healthy females without a tumor history or clinical signs of cancer (Table I). The collected sera were stored in a refrigerator at  $-80^{\circ}\text{C}$ . The study was approved by the ethics committee of the hospital. All the participants gave informed consent.

### Materials

Light Cycler 1.5 Instrument was from Roche, German; Applied Biosystems 7000 Real-Time PCR System was from ABI, USA; High-speed Low Temperature Centrifuge was from USTC ZONKIA, China; Class II Type B2 Biosafety Cabinet was from ES-CO, Singapore; Pipettes and Biophotometer was from Eppendorf, Germany.

Trizol reagent was from Invitrogen, USA; Isopropyl alcohol, chloroform and anhydrous ethanol were from DAHUI, China; HmiR-21 Hairpin-it<sup>TM</sup> Real-Time PCR Kit and Cel-miR-39 Hairpin-it<sup>TM</sup> Real-Time PCR Kit were from GenePharma, China, and RNA enzyme inhibitors were from Thermo Fermentas, Canada.

### Sample preparation

4 mL of peripheral venous blood samples were taken from all the study subjects. Then, the cells were pelleted using centrifugation at 3000 r/min for 10 min and the supernatant was discarded.

### Extraction of total RNA

250  $\mu\text{L}$  of each sample was treated with 1 mL of Trizol and the samples were placed at room temperature for 5 min. Then, 200  $\mu\text{L}$  of chloroform was added to the blood sample and the sample was oscillated for 15 s. followed by a centrifugation at 12000 r/min for 15 min. The supernatant (around 600  $\mu\text{L}$ ) was collected, and 500  $\mu\text{L}$  of isopropyl alcohol was added and left at room temperature for 10 min. After centrifugation at 12,000 r/min for 10 min at  $4^{\circ}\text{C}$ , the liquid was removed. 1 ml of fraction was treated with 750  $\mu\text{L}$  of andydrous ethanol and 250  $\mu\text{L}$  of DEPC-treated water. Finally, RNA was prepared for real time PCR.

### Reverse transcription reaction

The reaction volume was 20  $\mu\text{L}$ , consisting 4  $\mu\text{L}$  of  $5\times$  buffer, 0.75  $\mu\text{L}$  of dNTP (10 mmol/L), 1.2  $\mu\text{L}$  of miR-21 retrovirus primers (1  $\mu\text{mol/L}$ ), 25  $\mu\text{L}$  of RNA enzyme inhibitors (40 U/ $\mu\text{L}$ ), 2  $\mu\text{L}$  of MMLV reverse transcriptase (200 U/ $\mu\text{L}$ ), 5  $\mu\text{L}$  of total RNA sample, and 8.6  $\mu\text{L}$  of

DEPC-treated water. Reaction conditions were as follows:  $25^{\circ}\text{C}$  for 30 min,  $42^{\circ}\text{C}$  for 30 min, and  $85^{\circ}\text{C}$  for 5 min.

### Real-time fluorescent quantitative PCR

The reaction volume was 20  $\mu\text{L}$ , consisting 10  $\mu\text{L}$  of  $2\times$  Master Mix,  $\text{MgCl}_2$ , dNTPs, SYBR green fluorescent dye, 0.32  $\mu\text{L}$  of RT-PCR primers (5 mol/ $\mu\text{L}$ ), 0.2  $\mu\text{L}$  of Taq DNA polymerase ( $5\times 10^6$  U/L), 2  $\mu\text{L}$  reverse transcription product, and 8.6  $\mu\text{L}$  of DEPC-treated water. Reaction conditions were as follows:  $95^{\circ}\text{C}$  degeneration for 12 s and  $62^{\circ}\text{C}$  for 40 s for 40 cycles.

### Quantification of miR-21 expression level in serum

In the qPCR data analysis, the  $C_t$  values obtained from two different experimental RNA samples were directly normalized to a housekeeping gene and then compared. The difference between the  $C_t$  values ( $\Delta C_t$ ) of  $C_{t_{\text{miR-21}}}$  and  $C_{t_{\text{cel-mir-39}}}$  in was calculated for each experimental sample, and the difference in the  $\Delta C_t$  values between the experimental and control samples  $\Delta\Delta C_t$  was then calculated. The fold-change in expression of miR-21 between the two samples is equal to  $2^{-(\Delta\Delta C_t)}$  (5).

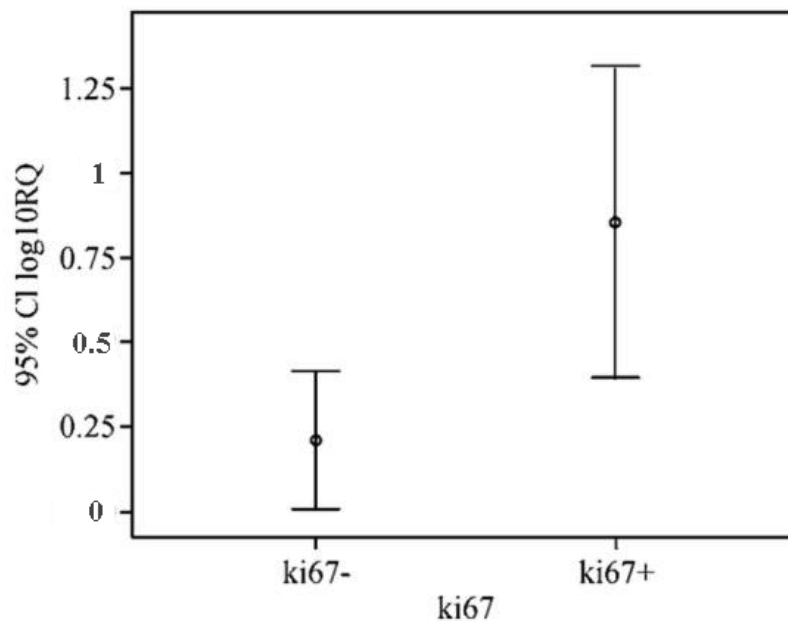
### Statistical analysis

All the data were analyzed by SPSS17.0 software. RQ  $2^{-\Delta\Delta C_t}$  was variance and uneven variance. The RQ was processed by data, and the simple sample  $K-S$  detection was carried out, which meets the normal distribution ( $Z=0.643$ ,  $P=0.803>0.05$ ). The Levene test results proved the equality of variance ( $P=0.007<0.05$ ). Single factor ANOVA analysis was applied to compare the mean sample values. Tamhane's T2 method was applied to discern between two samples. The parameters for the comparison of serum miR-21 expression levels between different breast cancers were set using the Wilcoxon rank sum test.  $P<0.05$  was considered as statistically significant.

## RESULTS

### Dissolution curves of miR-21 and Cel-miR-39

The miR-21 dissolution curve showed that the unimodal curve was at  $79^{\circ}\text{C}$ . The Cel-miR-39 dissolution curve showed that the unimodal curve was at  $81^{\circ}\text{C}$ , which proved that the PCR reaction parameters can apply to this research, and the amplification target product specificity was accurate.



**Fig. 1.** Association of miR-21 expression with ki67. TNBC: triple negative breast cancer; HER-2: human epidermal growth factor receptor-2; RQ: relative expression

#### *Real-time PCR analysis of serum miR-21 expression levels*

The PCR results of the serum miR-21 of each group and Cel-miR-39 showed an 'S' shape response curve, and the curve of the negative control group showed an irregular shape. The blank control group showed a non-amplification curve.

$\Delta C_t$  was applied to quantify the expression levels of miR-21 in different samples. If  $\Delta C_t$  decreased, it indicated that the miR-21 expression level was up-regulated.  $M(P_{25}, P_{75})$  is marked if data was not in a normal distribution.

The relative expression levels of miR-21 in the 5 groups were compared using a single factor ANOVA variance analysis, which showed a statistical significance ( $F = 17.11$ ,  $P < 0.01$ ). The Tamhane's T2 method was applied to compare each group. The miR-21 expression level in the TNBC and the familial breast cancer groups was significantly higher than that of the normal control group and other breast cancer groups ( $P < 0.01$ ). The miR-21 expression level in the serum of the breast

cancer high-risk group was significantly higher than that of the normal control group and other breast cancer groups ( $P < 0.01$ ). The comparison of the TNBC group and the familial breast cancer group combined with the breast cancer high-risk group had no statistical significance ( $P < 0.05$ ). The normal control group had no statistically significant difference compared with the other breast cancer groups.

#### *Relationship between the expression of serum miR-21 and clinicopathological parameters of the breast cancer*

The relative expression quantity of the miR-21 in serum was not obviously associated with the unilateral or bilateral tumor occurrence ( $P = 0.679$ ), age ( $P = 0.245$ ), CA153 ( $P = 0.893$ ), or menstrual status ( $P = 0.717$ ). There was no statistical significance between groups ( $P > 0.05$ ). The relative expression quantity of miR-21 in the breast cancer serum was positively associated with lymph node metastasis ( $P < 0.01$ ) and Ki67 ( $P = 0.006$ ), as shown in Table I, Fig. 1.



**Table I.** Association of miR-21 expression with clinical and pathological features

| Observe Index              | Tumor feature              |                             | age                    |                            | CA153     |           |
|----------------------------|----------------------------|-----------------------------|------------------------|----------------------------|-----------|-----------|
|                            | bilateral breast cancer    | unilateral breast cancer    | Less than 45 years old | Not less than 45 years old | Positive  | Negative  |
| C log 10 <sup>2-ΔΔCt</sup> | 0.40±0.26                  | 0.62±0.17                   | 0.77±1.01              | 0.30±0.61                  | 0.35±0.60 | 0.44±0.78 |
| P value                    | 0.679                      |                             | 0.245                  |                            | 0.893     |           |
| Observe Index              | menstrual status           |                             | lymph node metastasis  |                            | Ki67      |           |
|                            | Premenopause <sup>1)</sup> | Postmenopause <sup>1)</sup> | yes                    | no                         | ≥30%      | <30%      |
| log 10 <sup>2-ΔΔCt</sup>   | 0.47±0.76                  | 0.24±0.52                   | 1.50±0.36              | 0.13±0.46                  | 0.86±0.76 | 0.21±0.59 |
| P value                    | 0.717                      |                             | p<0.01                 |                            | 0.006     |           |

## DISCUSSION

miR-21 is abnormally expressed in colon cancer (6), lung cancer (7), gastric cancer (8), breast cancer (9) and other tumors. This study investigated the expression of miR-21 in the sera of the breast cancer patients.

Our data showed that the miR-21 expression levels of the familial breast cancer group, the TNBC group, and the breast cancer high risk group were significantly higher than those in the normal control group and other breast cancer groups. The serum miR-21 expression level was associated with lymph node metastasis and Ki67 expression.

The over-expression of Ki67 was closely associated with malignant biological behavior and the prognosis of breast cancer, and can be identified as a precancerous biological marker for people at a high risk of breast cancer. Ki67 and miR-21 were positively associated with cancer. The prediction of miR-21 plays a role in judging the degree of malignancy in breast cancers, tumor proliferation, and high-risk prediction.

Serum miR-21 was closely associated with familial breast cancer and TNBC, and its increased expression was associated with hereditary breast

cancer, degree of malignancy, and prognosis. Whether miR-21 in serum can be used in breast cancer diagnosis remains to be investigated.

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## LETTER TO THE EDITOR

**CELL-BASED THERAPIES FOR EXPERIMENTAL DIABETIC NEPHROPATHY:  
A SYSTEMATIC REVIEW AND META-ANALYSIS**HJ. TANG<sup>1</sup>, ZG. TIAN<sup>2</sup>, X. YANG<sup>1</sup>, Y. CAO<sup>3</sup> and WG. LI<sup>3</sup>

<sup>1</sup>*School of Software, Beijing Institute of Technology, Beijing, China;* <sup>2</sup>*Beijing GuangdalongtaiPolytron Technologies Inc., Beijing, China;* <sup>3</sup>*Department of Nephrology, China-Japan Friendship Hospital, Beijing, China*

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The first two authors contributed equally to this study

Study has shown that stem cell-based therapies are promising strategies in the treatment of several chronic diseases, but their overall benefit in the treatment of diabetic nephropathy (DN) remains controversial. The purpose of this study is to summarize the evidence of the effect of cell-based therapy in the treatment of DN to guide future clinical trials. We searched PubMed, EmBase, and the Cochrane Library for studies from the inception of cell-based therapies up to July 2015. We included animal trials that reported the effects of cell-based therapy on kidney function, cardiovascular risk factors, and body factors. A random-effects model was used to process the data, and the standard mean difference (SMD) was used to evaluate the efficacy of cell-based therapy. We included eight studies that reported data on 159 mice. Overall, we noted that cell-based therapies were associated with significantly reduced plasma creatinine level ( $P = 0.003$ ), glomerular filtration rate ( $P < 0.001$ ), plasma glucose level ( $P = 0.004$ ), serum cholesterol level ( $P = 0.010$ ), serum triglyceride level ( $P = 0.032$ ), plasma urea level ( $P < 0.001$ ), proteinuria ( $P = 0.008$ ), and  $\text{Cl}^-$  fractional excretion ( $P = 0.023$ ). Furthermore, cell-based therapies were associated with lower kidney weight ( $P = 0.003$ ), and kidney/body weight ( $P = 0.004$ ). A sensitivity analysis suggested that cell-based therapy might play an important role in increased body weight. In conclusion, cell-based therapies significantly improve kidney function, cardiovascular risk factors, and body factors in the treatment of DN.

To the Editor,

Globally, an increasing number of patients are living with diabetes mellitus (DM), and it is estimated that more than 430 million people will suffer from DM by 2030 (1). Subsequently, the development and progression of diabetic nephropathy (DN) has been and will be affected by the increasing number of persons developing DM, and it is the major cause of

end-stage renal disease (ESRD) and major diabetic microvascular complications in developing countries (2). Furthermore, patients with DN tend to have high cardiovascular morbidity and mortality rates (3).

Despite the increase in the number of patients with DN worldwide, no effective drugs are available for its treatment, which requires exploration and identification (4). As a result, a safe and effective

*Key words: cell-based; diabetic nephropathy, systematic review, meta-analysis*

Mailing address:

Dr Yali Cao,  
Department of Nephrology,  
China-Japan Friendship Hospital, Beijing 100029, China  
Tel.: +86 13240081645 - Fax: +86 21 64085875  
e-mail: caoyali2015@163.com

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treatment for DN is needed. Previous studies have stated that bone marrow–mesenchymal stem cells (BM-MSC) as adult stem cells not only have self-renewal capacity but can maintain their function and phenotype *in vitro* and promote tissue repair *in vivo* (5). Experiments with diabetic mice subjected to BM-MSC transplantation have shown that hyperglycemia and the symptoms of DN can be improved. Furthermore, BM-MSC fused nuclei are reprogrammed in response to intracellular cytoplasmic factors and extracellular signals in the microenvironment. However, not all stem cells can be used to repair tissues (5). Therefore, we conducted a systemic review and meta-analysis to evaluate whether stem cell–based therapies could have the best curative effect for DN.

#### *Data sources, search strategy, and selection criteria*

This review was conducted and reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analysis Statement issued in 2009. PubMed, EMBASE, and the Cochrane Library were searched for articles published since the inception of cell-based therapies up to July 2015 using “diabetic nephropathy” and “cell therapy” as the core keywords. Abstracts and virtual meeting presentations were also searched to identify relevant unpublished studies. We also hand-searched journals known to publish data relevant to our search as well as the reference lists of all retrieved articles and those of relevant review articles. The literature retrieval process was performed in duplicate by two independent reviewers. Studies were included if they met the following criteria: i) animals with DN were used to evaluate the efficacy and safety of cell-based therapy; ii) therapy included or consisted of cells or cell-derived products (conditioned medium or exosomes/microvesicles); iii) at least one of the following outcomes was reported: plasma creatinine, creatinine clearance, proteinuria, glomerular filtration rate (GFR), plasma glucose, serum triglyceride level, serum cholesterol level, plasma insulin, plasma urea, urinary flow, Na, Cl, K, body weight, kidney weight, and kidney/body weight ratio. For the studies without adequate data, we contacted the authors to obtain the unpublished results; if the author could not provide

the necessary data, these studies were excluded from the analysis.

#### *Data collection*

The data extractions and assessments were conducted independently by two authors. Publication information (first author’s name, publication year), animals’ characteristics (species, sex, injury model, administered cell or cell-derived product type, number of cells, administration route, time at administration, follow-up duration), and reported outcomes were extracted. Other data retrieved included the study question, experimental design, and presence or absence of individual data. Disagreement was resolved by consensus with a third reviewer.

#### *Statistical analysis*

We examined the effect of cell-based therapy in the treatment of DN using mean, standard deviation, and sample size in the intervention and control groups. Standard mean difference (SMD) was used as a summary statistic. The SMD was significant when the 95% confidence interval (CI) did not include 0. The pooled SMD values for the effect of cell-based therapy were analyzed using random-effects models (6). Sensitivity analyses were conducted to explore the influence of a single study on each outcome, and the heterogeneity evaluation was conducted using the Q statistic (7). All reported P values are two-sided, and those <0.05 were considered statistically significant. Data analyses were performed using STATA software (version 12.0; Stata Corporation, College Station, TX, USA).

## RESULTS

The primary search produced 698 records. After scanning titles and abstracts, we excluded 665 irrelevant articles. A total of 33 full-text articles were reviewed, and eight animal experiments were ultimately included in this meta-analysis with a total of 159 mice. A manual search of the reference lists of these studies did not yield any new eligible studies.

In these eight studies, the dose of diabetes induced by streptozotocin was 40–200 mg/kg, the effect of



cell-based therapy in male mice was reported, and all body weights were 150–250 g except for one (19–23 g). Furthermore, six studies used adipose-derived stem cells to treat DN, one study used mononuclear cell therapy, and one used autologous bone marrow transplantation to treat DN. In addition, the duration of the follow-up periods ranged from 4 weeks to 4 months.

A meta-analysis of the included studies showed that the use of cell-based therapies was associated with significantly lower plasma creatinine level (SMD, -3.68; 95% CI, -6.12 to -1.24;  $P = 0.003$ ), GFR (SMD, -1.95; 95% CI, -3.03 to -0.86;  $P < 0.001$ ), plasma glucose level (SMD, -1.97; 95% CI, -3.31 to -0.63;  $P = 0.004$ ), serum cholesterol level (SMD, -1.85; 95% CI, -3.26 to -0.44;  $P = 0.010$ ), serum triglyceride level (SMD: -2.03; 95% CI, -3.88 to -0.18;  $P = 0.032$ ), plasma urea level (SMD, -4.96; 95% CI, -7.55 to -2.37;  $P < 0.001$ ), proteinuria (SMD, -3.46; 95% CI, -6.01 to -0.92;  $P = 0.008$ ), and  $\text{Cl}^-$  fractional excretion (SMD, -1.09; 95% CI, -2.04 to -0.15;  $P = 0.023$ ). Furthermore, cell-based therapies had little or no significant effects on creatinine clearance, plasma insulin level, urinary flow,  $\text{Na}^+$  fractional excretion, or  $\text{K}^+$  fractional excretion. The  $Q$  statistic showed high heterogeneity for plasma creatinine level, creatinine clearance, proteinuria, plasma glucose level, plasma insulin level, plasma urea level, serum triglyceride level, and serum cholesterol level (Fig. 1).

The summary RR showed that cell-based therapy was not associated with body weight (SMD, 0.96; 95% CI, -1.00 to 2.92;  $P = 0.339$ ), but potential evidence of significant heterogeneity was detected ( $P < 0.001$ ). As a result, a sensitivity analysis was conducted for body weight, and after excluding the Jian et al. study, we noted that cell-based therapy was associated with elevated body weight (SMD, 2.02; 95% CI, 0.27–3.76;  $P = 0.024$ ). Furthermore, cell-based therapies were associated with lower kidney weight (SMD, 2.88; 95% CI, -4.79 to -0.97;  $P = 0.003$ ) and kidney/body weight ratio (SMD, -2.35; 95% CI, -3.96 to -0.73;  $P = 0.004$ ). Although substantial heterogeneity was detected across the included studies, after sequential exclusion of each study from the pooled analyses showed that the

conclusion was not affected by the exclusion of any specific study.

## DISCUSSION

The objective of the present meta-analysis was to determine the effect of cell-based therapy in the treatment of DN. The results showed that cell-based therapy was associated with reduced plasma creatinine level, GFR, plasma glucose level, serum cholesterol level, serum triglyceride level, plasma urea level, proteinuria,  $\text{Cl}^-$  fractional excretion, kidney weight, and kidney/body weight ratio. These results will help researchers better define the efficacy of cell-based therapy in the treatment of DN.

Previous study has demonstrated a significant role of inflammation in the pathogenesis of DN (8). In the treatment of DN, interventional regimens based on the targeting of a single pathway or network of the inflammatory cascade failed to prevent the progression to ESRD. The reason could be the use of immunosuppressive and anti-inflammatory drugs was not associated with a decreased contribution of inflammation to DN progression or even worsening kidney function (9). Furthermore, stem cells, characterized by pluripotency and self-renewal, are immunoprivileged cells that have the ability to regenerate virtually all adult tissues and organs, and modulate the immune response and exert anti-inflammatory functions (10).

The findings of this study suggest that cell-based therapy was associated with beneficial impact in treatment of DN. The possible reason for this could be that cell-based therapeutic effects through complex paracrine and endocrine actions (11). Stem cells localized in glomeruli could express CD31, which suggested that stem cells that differentiate into endothelial cells may be involved in kidney repair (12). Cell-based therapy might play an important role on urine protein excretion and improve renal pathological changes, including glomerular sclerosis, mesangial proliferation, tubule dilatation, and protein casts.

In conclusion, the present meta-analysis suggested that cell-based therapies might play an important role in renal function and weight. Since

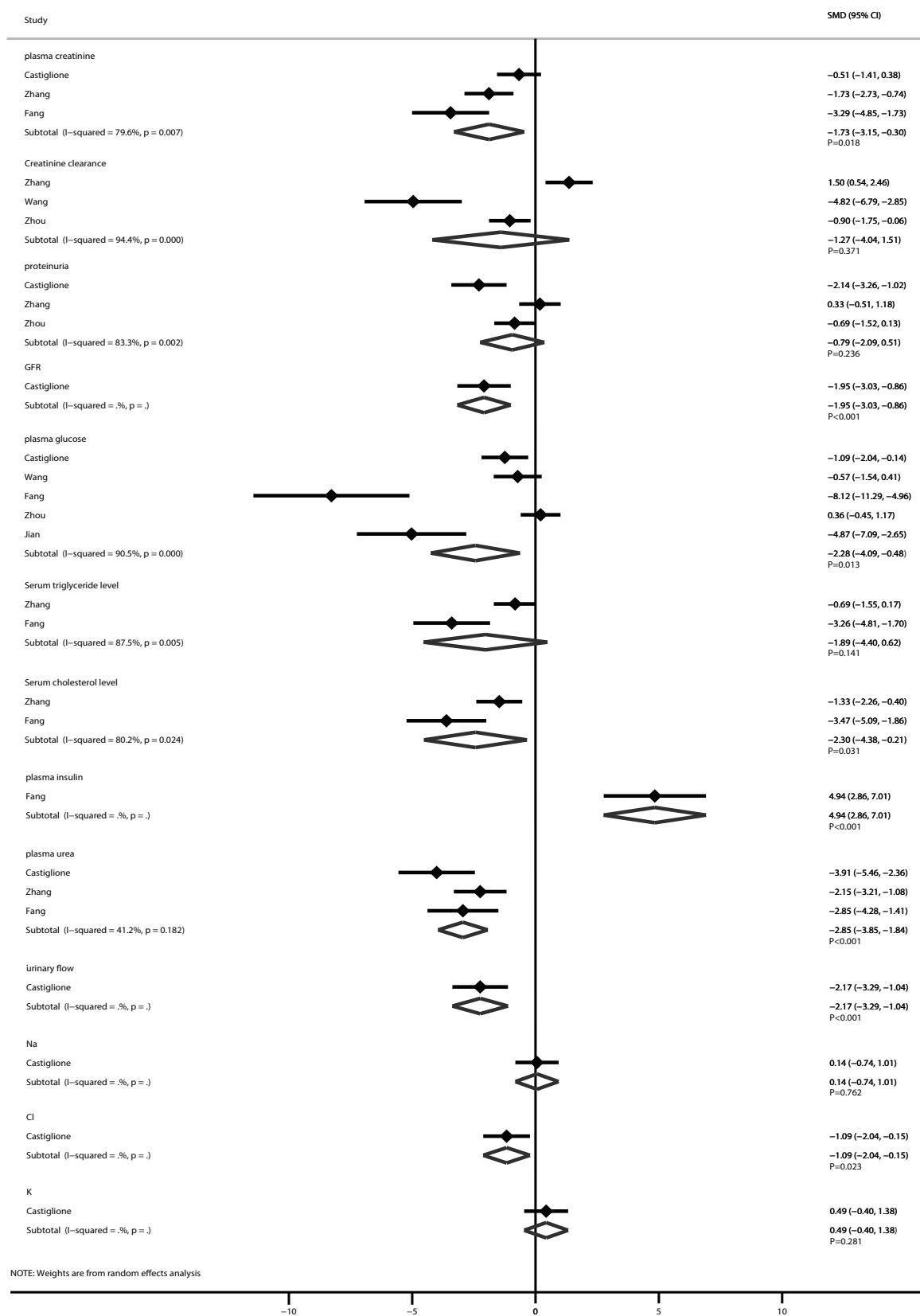


Fig. 1. Summary of the standard mean differences for indexes of hematology.

the goal of DN treatment is to maximize therapeutic outcomes while slowing the progression of ESRD, additional studies on the effect of each stem cell or cell-derived product for DN may be required.

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## LETTER TO THE EDITOR

**OXIDATIVE STRESS IN ASTHMA:  
A DISTINCT CLINICAL AND PATHOLOGIC FEATURE?**Y. LI<sup>1</sup> and GP. LI<sup>2</sup><sup>1</sup>*The First Clinic College of Chongqing Medical University, Chongqing, China;*<sup>2</sup>*Inflammation and Allergic Diseases Research Unit in Respiratory Department, Affiliated Hospital of Southwest Medical University, Luzhou, China**Received December 14, 2015 – Accepted July 22, 2016*

**Asthma is a type of chronic airway inflammation. Corticosteroids are inadequate for asthma therapy. However, it remains unclear whether oxidative stress is a distinct clinical and pathologic feature in asthma. We reviewed the articles on asthma-associated oxidative stress. The exposures to airborne allergens, such as house dust mite (HDM) and birch pollen, may not only trigger innate and adaptive immune responses but also cause oxidative stress damage in the airways. Allergen-induced reactive oxygen species (ROS) is involved in p38 MAPK, phosphoinositide-3-kinase (PI3K)/Akt and nuclear factor erythroid 2-related factor (Nrf2) kinase pathway signaling. Airborne particulate matter (PM) is an important environmental contaminant and is related to asthma development through increasing oxidative stress in the airways. Whether oxidative stress status is associated with the degree of asthma is needed to be further studied. Oxidative stress-induced corticosteroid insensitivity was associated with p38 MAPK, PI3K/Akt and Nrf2 signaling, and inhibited histone deacetylase 2 (HDAC2) activity and corticosteroid receptor (GR) function. Antioxidant treatments may be useful for oxidative stress in asthma.**

To the Editor,

Asthma is a type of chronic airway inflammation that includes airway remodeling and hyper-responses, and is a global health problem that affects an estimated 300 million people worldwide. Airborne allergen and environmental contaminant exposure causes the oxidative stress status in the lung to change. Eosinophils and T helper 2 (Th2) cytokines have long been credited with a central role in pathophysiology mechanisms. Asthmatic patients exhibit different inflammatory phenotypes. Although inhaled corticosteroids have been used extensively, the present clinical strategies are inadequate for asthma therapy. Severe asthma

occurs in approximately 10% of asthma cases and is defined as asthma that requires treatment with high-dose inhaled corticosteroids plus a second controller and/or systemic corticosteroid. The severe asthma inflammatory phenotypes have been described as severe eosinophilic asthma, noneosinophilic severe asthma with increased neutrophils, and severe paucigranulocytic asthma (1).

Oxidative stress is the result of an imbalance between the production of reactive oxygen species (ROS) and antioxidant mechanisms, and has been credited as a key mechanism in chronic obstructive pulmonary disease (COPD). COPD patients show a poor response to corticosteroids and this is

*Key words: oxidative stress, corticosteroids insensitivity, asthma**Mailing address:*

Prof. Guoping Li M.D., Ph.D.  
Inflammations and Allergic Diseases Research Unit,  
Respiratory Department, Affiliated Hospital,  
Southwest Medical University, 25N Taiping Street,  
Luzhou, Sichuan, 646000. P. R. China  
Tel.: +868303165321 - Fax: +868303165324  
e-mail: lzlzp@163.com

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associated with oxidative stress. Oxidative stress is increased in severe asthma because of the increased generation of reactive oxygen species and defective antioxidant functions. Severe asthma patients have increased airway oxidant burden. Oxidative stress promotes airway remodeling, reduces the anti-inflammatory corticosteroid action and contributes to the relative insensitivity to corticosteroids. However, oxidative stress is not defined as a main pathophysiology mechanism of asthma.

#### *Allergen and oxidative stress*

Although indoor allergens, such as domestic animal hair, dust mite, cockroach and mouse, contribute to asthma morbidity. The high prevalence and attacks of asthma are linked to allergen exposures. The exposure to airborne allergens causes oxidative stress damage. However, it remains unclear whether indoor allergens exposures are linked to oxidative stress. These allergens may not only trigger a complex network of immune responses, but may also cause oxidative stress damage in the airways. House dust mites (HDMs) are the most common allergic sensitizers and risk factors for asthma. HDM-induced oxidative damage and DNA repair is regarded as a modulator of asthma-associated pathophysiology (2). Proteases from HDM may induce the innate immune response by binding protease-activation receptors (PARs), Toll-like receptors (TLRs), or C-type lectin receptors (CTRs), or they may function as molecular mimics (3). Protease-mediated HDM allergen (Der f1) induced reactive oxygen species production by neutrophils in asthmatic subjects (4).

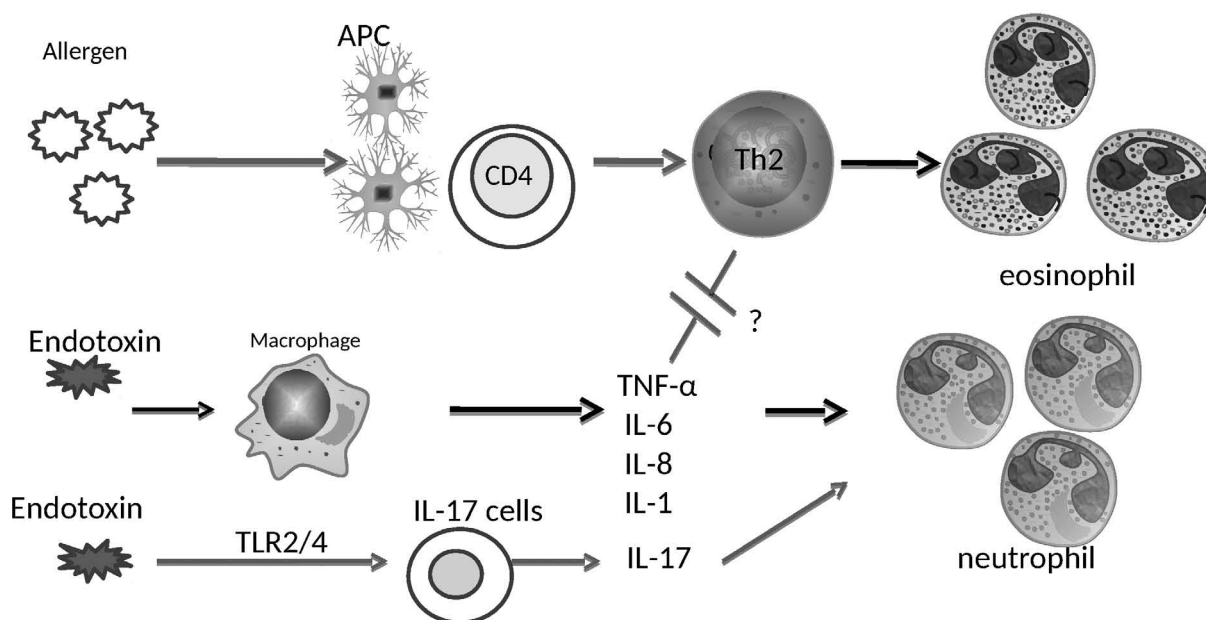
Nicotinamide adenine dinucleotide phosphate oxidase (NADPH-oxidase) is a complex enzyme that is responsible for increased reactive oxygen species (ROS) levels. The oxidative stress generated by the pollen NADPH oxidases (signal 1) augment the allergic airway inflammation that is induced by pollen antigens (signal 2). NADPH oxidase contributes to allergic airway inflammation in exposure to birch pollen extract. Birch pollen extract exhibited pollen-intrinsic NADPH oxidase activity. NADPH oxidase activity was shown to rapidly trigger ROS production within the airway epithelium upon topical exposure (5). Sub-pollen particles (SPPs) significantly

increase ROS levels. NADPH oxidase inhibitors attenuated SPP-induced ROS levels in airway epithelial cells and the subsequent airway inflammation. Apocynin (NADPH oxidase inhibitor) showed potential antioxidant activities and inhibitory effects on the activation of redox-sensitive transcription factors, such as NF $\kappa$ B and AP-1, in an allergen-induced mouse model (6).

#### *Environmental contaminants and oxidative stress in asthma*

Airborne particulate matter (PM) is an important environmental contaminant and is related to pulmonary diseases. PM is deposited at different levels of the respiratory tract depending on its size. PM<sub>2.5</sub> is regarded as particles less than 2.5 microns in diameter. PM<sub>2.5</sub> can penetrate deeply into the lung, irritate and corrode the alveolar wall. The diameter of PM<sub>10</sub> particles is less than 10 microns. PM<sub>10</sub> is accumulated in upper airways. PM components from fossil fuel combustion can induce the oxidative stress initiated by ROS. It has been shown that PM<sub>2.5</sub> particles, which were collected in a residential area of Seoul, might induce Th1 type inflammatory responses with oxidative stress and trigger adverse effects in human health (7). PM<sub>2.5</sub> particles tended to increase the respiratory burst of human blood neutrophils from the asthmatic group. Exposure to airborne black carbon has been associated with asthma development through increasing oxidative stress in the airways (8). PM<sub>10</sub> particles also increased the production and release of inflammatory cytokines and activation of the transcription factor NF $\kappa$ B in respiratory tract epithelium via oxidative stress (9).

Endotoxin causes neutrophilic inflammatory and airway macrophage immune-phenotypes (Fig. 1). Endotoxin was shown to enhance the response to inhaled allergens. Serum endotoxin concentrations were significantly correlated with sputum IL-6, IL-8, and chemokine motif ligand 2 concentrations. Airway endotoxin might contribute to corticosteroid insensitivity in asthmatic patients. Endotoxin exposure during sensitization to allergens shifts Th2 immunity towards Th17-mediated airway neutrophilic inflammation via TLR4 and TLR2 (10).



**Fig. 1.** Endotoxin induced a neutrophilic inflammatory phenotype in asthma. Endotoxin significantly increased IL-6, IL-8, and chemokine motif ligand 2 concentrations. Endotoxin shifted Th2 immunity towards Th17-mediated airway neutrophilic inflammation via TLR4 and TLR2

However, it remains unclear whether oxidative stress is essential for the endotoxin-induced neutrophil phenotype.

#### *Oxidative stress and asthma*

The imbalance of antioxidants and oxidants contributes to oxidative stress in asthma. The ameliorated glutathione redox balance suppressed chemokine production and eosinophil migration by altering the Th1/Th2 balance in a mouse asthma model (11). Mitochondria are critical sources of ROS for promoting the transforming growth factor-beta (TGF- $\beta$ ) activity that contributes to airway remodeling in allergic asthma. The ER stress pathway is important in asthma pathogenesis through the modulation of NF $\kappa$ B activation (12). Dust mites induce ER stress in airway epithelial cells. ER stress response inhibition may provide a potential therapeutic avenue in chronic asthma.

However, it remains unclear whether the oxidative stress status is associated with the degree of asthma. Children with severe asthma have increased oxidative stress associated with increased

glutathione disulfide (GSSG) formation and a shift in the glutathione (GSH) redox potential towards a more oxidized state. Severe asthma with airway obstruction showed the greatest oxidation level. Total antioxidant capacity (TAC) and reduced glutathione (GSH) levels in asthmatic patients were significantly decreased and inversely related to asthma severity. Severe asthma has increased airway oxidant burden. Oxidative stress promotes airway remodeling via TGF- $\beta$ 1 in children with severe asthma. However, some studies have found that the oxidative stress-related biomarker levels and plasma antioxidant status may not be related to asthma in the general population in the absence of more severe symptoms or exacerbations.

#### *Oxidative stress and corticosteroid resistance in asthma*

Corticosteroids are effective useful medicines for asthma therapy. However, some asthma types are refractory or insensitive to corticosteroids. Oxidative stress reduces the anti-inflammatory corticosteroid action and contributes to the relative corticosteroid

insensitivity in asthma. H<sub>2</sub>O<sub>2</sub> or cigarette smoke extract decreased the inhibition of budesonide and dexamethasone on LPS-induced IL-8 and TNF- $\alpha$  secretion levels from alveolar macrophages. Oxidative stress-induced PI3K activation led to glucocorticoid resistance, which was correlated with reduced histone deacetylase 2 tyrosine nitration (13). IL-17A-induced GC insensitivity is involved in IL-17A-activated the p38, extracellular signal-related kinase (ERK) and PI3K pathways. The oxidant-mediated reduction in HDAC-2 activity is, in part, a result of an elevation in the phosphoinositol 3-kinase (PI3K) delta/Akt signaling pathway (14). Passive smoking impairs HDAC2 function via PI3K signaling activation, which can contribute to corticosteroid-insensitive inflammation in children with severe asthma. DEX-dependent nuclear targeting of human GR is markedly inhibited after treatment with 1 mM H<sub>2</sub>O<sub>2</sub>, whereas pretreatment with *N*-acetyl-L-cysteine (NAC) effectively decreased this negative effect of H<sub>2</sub>O<sub>2</sub> (15).

The effect of glucocorticoids on oxidative stress remains unclear. Even when the asthma is well controlled, after 3 months of inhaled corticosteroids and long-acting  $\beta_2$ -agonist treatment, the lipid peroxide concentrations were still higher. Dexamethasone did not inhibit H<sub>2</sub>O<sub>2</sub>-induced activation of the NF $\kappa$ B pathway. Oxidative stress-induced corticosteroid resistance is prevented by phosphodiesterase (PDE)-3/PDE-4 inhibition in human alveolar macrophages. Corticosteroid insensitivity and reduced histone deacetylase-2 activity after oxidative stress induction were reversed by a non-selective PI3K inhibitor and low theophylline concentrations (16).

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## LETTER TO THE EDITOR

**CHANGES OF EXPRESSION OF ESTROGEN AND PROGESTERONE RECEPTORS, HUMAN EPITHELIAL GROWTH FACTOR RECEPTOR 2 AND KI-67 AFTER NEOADJUVANT CHEMOTHERAPY IN THE TREATMENT OF BREAST CANCER**M.L. LI<sup>1</sup>, Y. DONG<sup>2</sup>, S.L. LUAN<sup>1</sup>, Z.H. ZHAO<sup>1</sup> and F.L. NING<sup>1</sup><sup>1</sup>*Oncology Department, Binzhou Medical University Hospital, Binzhou, China;* <sup>2</sup>*Clinical Laboratory, Binzhou Medical University Hospital, Binzhou, China**Received February 25, 2016 – Accepted September 27, 2016*

Recent studies suggest that the development and prognosis of breast cancer is in close correlation to molecular subtype of breast cancer. Neoadjuvant chemotherapy has been extensively applied in the treatment of local breast cancer in advanced stage. In order to verify the correlation between expression changes of estrogen receptor, progesterone receptor, human epithelial growth factor receptor 2 and Ki-67 after neoadjuvant chemotherapy and neoadjuvant chemotherapy, we studied 120 patients with stage IIA-IIIc breast cancer who underwent neoadjuvant chemotherapy in Binzhou Medical University Hospital, Shandong, China from February 2011 to February 2015. Clinical characteristics were retrospectively analyzed. The expression of estrogen receptor, progesterone receptor, human epithelial growth factor receptor 2 and Ki-67 of patients were detected using the immunohistochemical method before and after neoadjuvant chemotherapy. The results suggest that the overall remission rate of neoadjuvant chemotherapy was 76.7% (92/120) of which 16.7% (20/120) of cases had complete remission, 60% (72/120) had partial remission and 23.3% (28/120) were stable. There were no cases of progressive disease. The property of estrogen receptor and the expression of Ki-67 of primary tumor were correlated to the remission rate of neoadjuvant chemotherapy ( $P < 0.05$ ). The expression of Ki-67 had a significant decline after neoadjuvant chemotherapy, and the difference had statistical significance ( $P < 0.05$ ). The difference in expression of estrogen receptor, progesterone receptor and human epithelial growth factor receptor 2 before and after neoadjuvant chemotherapy had statistical significance ( $P > 0.05$ ). Hence, it can be concluded that breast cancer patients with negative estrogen receptor expression and high Ki-67 expression before neoadjuvant chemotherapy can achieve better curative effects. Neoadjuvant chemotherapy cannot change the expression states of estrogen receptor, progesterone receptor and human epithelial growth factor receptor 2, but it can lower the expression level of Ki-67. Ki-67 can also be used for predicting the curative effect of neoadjuvant chemotherapy.

To the Editor,

Breast cancer, which has a yearly increase of 3-4%, detains a high mortality factor among females (1). Neoadjuvant chemotherapy is important in treating

breast cancer, especially middle and advanced stage (2). Neoadjuvant chemotherapy, which refers to the initial systemic treatment carried out before surgical removal or chemotherapy, can lower the pathological stage of

*Key word: breast cancer, neoadjuvant chemotherapy, Estrogen receptor, progesterone receptor, human epithelial growth factor receptor 2*

*Mailing address*

Dr Yan Dong,  
No.661, Huanghe 2nd Road,  
Binzhou, 256603, China  
Tel.: +86 0543 3323481  
Fax: +86 0543 3323481  
e-mail: dydongdy@163.com

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breast cancer patients, improve the success rate of breast conservation, inhibit the possible micro metastatic lesion and evaluate the sensitivity to chemotherapeutics.

Currently, manual palpation and imaging tests are the main methods for evaluating the effect of neoadjuvant chemotherapy, although they cannot be used for exploring the correlation between cancer cells and chemotherapy or to determine malignant biological behaviors of tumor at molecular level and genetic level (3, 4).

Some studies (5, 6) have suggested that the expressions of biological factors such as estrogen receptor (ER), progesterone receptor (PR), human epithelial growth factor receptor 2 (HER2) and Ki-67 antigen have different degrees of changes after neoadjuvant chemotherapy, which can influence the subsequent individualized treatment; however, the rules of the four indexes have not been defined. This study retrospectively analyzed the data of 120 patients with breast cancer who underwent neoadjuvant chemotherapy, aiming to compare the different expressions of ER, PR, HER2 and Ki-67 and calculating the factors which could be used for evaluating the curative effects of neoadjuvant chemotherapy. This work can provide a reference for the comprehensive treatment of breast cancer in the future.

## MATERIALS AND METHODS

### *Research subjects*

One hundred and twenty patients, aged from 32 to 78 years (average  $57 \pm 2.40$  years) with breast cancer who received treatment in the Affiliated Hospital of Binzhou Medical College, Shandong, China from February 2011 to February 2015 were enrolled in the study. Sixty-four patients were in menopause. The size of the lesion was evaluated by mammography, ultrasonic B, magnetic resonance imaging (MRI) or chest computed tomography CT. TNM stage was determined according to the criteria of Union for International Cancer Control (UICC); there were 8 cases of II A stage, 35 cases of IIB stage, 67 cases of IIIA stage and 10 cases of IIIB stage.

The patients enrolled in this study were required to meet the following criteria: pathologically proven invasive ductal carcinoma, undergoing neoadjuvant chemotherapy before surgery, with primary breast cancer in single breast, receiving treatment for the first time, with reliable clinical and pathological data. All patients

signed informed consent. Exclusion criteria included distant metastasis before neoadjuvant chemotherapy, having undergone surgery, radiotherapy, chemotherapy and molecular target treatment, with other malignant tumor, or with contraindication to chemotherapy due to cardiovascular and cerebrovascular diseases.

### *Major experimental materials*

Reagents used included mouse anti-human Ki-67 monoclonal antibody (Fuzhou Maixin Biotechnology Development Co., Ltd., Fujian, China), C-erb B-2 rabbit anti-human monoclonal antibody (Fuzhou Maixin Biotechnology Development Co., Ltd., Fujian, China) and others (Fuzhou Maixin Biotechnology Development Co., Ltd., Fujian, China). The main instruments used included Leica ASP 200S fully closed dehydrator (Leica Inc., Germany), Leica RM2245 fully automatic slicer (Leica Inc., Germany), Leica AUTO stainer XL (Leica Inc., Germany), OLYMPUSPM-20 microscope and OLYMPUSPM-C35DX imaging system (Olympus Inc., Japan) and OLYMPUSPM-C35DX (Olympus Inc., Japan).

### *Chemotherapy regimen*

All patients were confirmed having breast cancer by undergoing puncture with a bomb core needle (Bard Company, U.S.A.) under the guidance of ultrasound, or biopsy with the Mammotome System (Johnson & Johnson Pharmaceuticals Co., Ltd.) before neoadjuvant chemotherapy. Pathological examination and detection of the expression of ER, PR, HER2 and Ki-67 were carried out on patients who had biopsy-proven breast cancer. Taxane in combination with anthracycline was adopted as the scheme for neoadjuvant chemotherapy according to National Comprehensive Cancer Network Guide (7). Pirarubicin ( $60 \text{ mg/m}^2$ ) or epirubicin ( $90 \text{ mg/m}^2$ ) and docetaxel ( $75 \text{ mg/m}^2$ ) were intravenously injected on the first day; three weeks were taken as one course and the treatment lasted for six courses. The chemotherapy lasted for 2 to 6 courses. Preoperative evaluation was performed after one to two weeks after neoadjuvant chemotherapy. ER, PR, HER2 and Ki-67 were detected on biopsy specimens and large postoperative specimens using Streptavidin Peroxidase Conjugated Method (SP method).

### *Evaluation of curative effect*

Clinical effect was evaluated according to Response

Evaluation Criteria In Solid Tumors (RECIST) (8). Complete remission (CR) referred to disappearance of all lesions. CR included clinical complete remission (cCR) and pathological complete remission (pCR). Partial remission (PR) could be confirmed if the product of the maximum diameter and the maximum vertical diameter of lesion reduced at least 30%. Stable disease (SD) could be confirmed if the product of the maximum diameter and the maximum vertical diameter of lesion reduced less than 30% or increased less than 20%. Patients were determined having progressive disease (PD) if the product of the maximum diameter and the maximum vertical diameter increased for at least 20% or new lesions developed. CR and PR were determined as clinically effective, while PD and SD were determined as ineffective. The formula of the effective rate was: effective rate=(number of cases of CR+number of cases of PR)/total number of cases. pCR refers to no residual cancer cells in original tumor site.

#### *Detection of ER, PR, HER2 and Ki-67 and determination*

Tumor tissues were fixed with 10% formaldehyde and embedded with paraffin. They were then sliced into 3-micrometer sections. The expression of ER, PR, HER2 and Ki-67 were detected using the immunohistochemical Envision method according to the procedures specified by manufacturer's instructions. All the reagents used were purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd, Beijing, China and the detection was carried out following the instructions. The staining results were observed using an optical microscope. Positive cells were distinguished according to relevant criteria. The proportion of positive cells viewed in the tumor cells was registered. The expression of factors was considered having changes if the expression of ER, PR and Ki-67 transformed from (+ - +++) to (-) or from (-) to (+ - +++) and HER2 transformed from overexpression (3+) to non-overexpression (2+/-) or from non-expression (2+/-) to overexpression (3+) after neoadjuvant chemotherapy. Changes of positive expression degrees of ER, PR, HER2 and Ki-67 after neoadjuvant chemotherapy indicated quantitative changes of biological factors. The other patients were considered having unchanged expression of biological factors.

#### *Determination criteria of ER, PR, HER2 and Ki-67*

ER and PR were considered as positive staining if cell nucleus and cytoplasm were stained at the same time

and as negative staining if cytoplasm only was stained. ER and PR were considered as positive expression if the proportion of positive cells was equal to or higher than 1% and as negative if the proportion was lower than 1%. HER2 was considered as (+++) and overexpression if more than 30% of invasive cancer cells showed complete cytomembrane staining, and the result lower than (+/++) was not considered as overexpression. Ki-67 was determined as positive if brown-yellow particles appeared in cell nucleus. Under a high power field (10×40), ten fields were selected randomly. The percentage was calculated according to the positive proportion of tumor cells, and the average value was taken as the final result. A positive cell proportion equal to or higher than 14% was determined as high expression and a positive cell proportion lower than 14% was determined as low expression.

#### *Statistical analysis*

SPSS20.0 was used to process statistical data. The correlation between various parameters and effect of chemotherapy was tested by *Chi-squared* test.  $P < 0.05$  difference had statistical significance.

## RESULTS

#### *Observation of curative effect of neoadjuvant chemotherapy and clinical pathological characteristics*

One hundred and twenty patients with breast cancer completed 6 cycles of neoadjuvant chemotherapy. At the sixth cycle of neoadjuvant chemotherapy, the curative effect was evaluated. The overall remission rate (RR) of primary tumor was 76.7%; 16.7% of cases had CR, 60% of cases had PR and 23.3% of cases had SD. No patient had PD or pCR. Through correlation analysis, we found TNM stage of primary tumor, positive or negative property of ER and high or low expression of Ki-67 were correlated to RR ( $P < 0.05$ ); age and state of axillary lymph nodes and HER2 expression were in no obvious correlation to RR ( $P > 0.05$ ) (Table I).

#### *Changes of expression of ER, PR, HER2 and Ki-67 after neoadjuvant chemotherapy*

The expression of ER, PR and HER2 measured before and after neoadjuvant chemotherapy, had no significant differences. Eight cases (6.7%) had

**Table I.** Effects of different indexes on curative effect of neoadjuvant chemotherapy (N=120).

| Clinical pathological characteristics | RR        |           |           | $\chi^2$ | P     |
|---------------------------------------|-----------|-----------|-----------|----------|-------|
|                                       | SD or PD  | PR        | CR        |          |       |
| N                                     | 28(23.3%) | 72(60%)   | 20(16.7%) |          |       |
| Age                                   |           |           |           | 0.266    | 0.779 |
| <50                                   | 15(23.1%) | 38(58.5%) | 12(18.4%) |          |       |
| ≥50                                   | 13(23.6%) | 34(61.8%) | 8(12.6%)  |          |       |
| T stage                               |           |           |           | 7.941    | 0.014 |
| T <sup>2</sup>                        | 8(16.7%)  | 30(62.5%) | 10(20.8%) |          |       |
| T <sup>3</sup>                        | 15(24.6%) | 38(62.3%) | 8(13.1%)  |          |       |
| T <sup>4</sup>                        | 5(45.4%)  | 4(36.4%)  | 2(18.2%)  |          |       |
| N stage                               |           |           |           | 5.662    | 0.048 |
| N <sup>0</sup>                        | 4(44.4%)  | 3(33.3%)  | 3(33.3%)  |          |       |
| N <sup>1</sup>                        | 14(17.3%) | 54(66.7%) | 13(16%)   |          |       |
| N <sup>2</sup>                        | 10(34.5%) | 15(51.7%) | 4(13.8%)  |          |       |
| TNM stage                             |           |           |           | 7.668    | 0.034 |
| II A                                  | 3(37.5%)  | 3(37.5%)  | 2(25%)    |          |       |
| II B                                  | 5(14.3%)  | 21(60%)   | 9(25.7%)  |          |       |
| III A                                 | 15(22.4%) | 44(65.7%) | 8(11.9%)  |          |       |
| III B                                 | 5(50%)    | 4(40%)    | 1(10%)    |          |       |
| Axillary lymph nodes                  |           |           |           | 0.374    | 1.000 |
| Negative                              | 12(25%)   | 31(64.6%) | 5(10.4%)  |          |       |
| Positive                              | 16(22.2%) | 41(57%)   | 15(20.8%) |          |       |
| ER                                    |           |           |           | 7.154    | 0.012 |
| Negative                              | 8(12.9%)  | 46(74.2%) | 8(12.9%)  |          |       |
| Positive                              | 20(34.5%) | 26(44.8%) | 12(20.7%) |          |       |
| PR                                    |           |           |           | 0.259    | 0.611 |
| Negative                              | 13(20%)   | 40(61.5%) | 12(18.5%) |          |       |
| Positive                              | 15(27.3%) | 32(58.2%) | 8(14.5%)  |          |       |
| HER2                                  |           |           |           | 2.034    | 0.154 |
| Negative                              | 15(23.4%) | 40(62.5%) | 9(14.1%)  |          |       |
| Positive                              | 13(23.2%) | 32(57.1%) | 11(19.7%) |          |       |
| Ki-67                                 |           |           |           | 4.255    | 0.039 |
| Negative                              | 16(42.1%) | 18(47.4%) | 4(10.5%)  |          |       |
| Positive                              | 12(14.6%) | 54(65.9%) | 16(19.5%) |          |       |

changed ER expression while the expression of 2 cases (1.7%) transformed from positive to negative; the expression of 6 cases (5%) transformed from negative to positive (5%); 14 cases had quantitatively changed expression (11.7%) while the expression of 98 cases (81.7%) remained unchanged. As to HER2 expression, 2 cases (1.7%) transformed from negative

to positive. The expression of Ki-67 before and after neoadjuvant chemotherapy showed a statistical difference. Before chemotherapy, there were 66 cases of positive Ki-67 expression (55%) while after chemotherapy there were 44 cases of positive Ki-67 expression (36.7%). Before chemotherapy, there were 54 cases of negative Ki-67 expression

**Table II.** *Changes of expression of ER, PR, HER2 and Ki-67 after neoadjuvant chemotherapy (N=120).*

| Index | Before neoadjuvant chemotherapy |           | After neoadjuvant chemotherapy |           | $\chi^2$ | P     |
|-------|---------------------------------|-----------|--------------------------------|-----------|----------|-------|
|       | Positive                        | Negative  | Positive                       | Negative  |          |       |
| ER    | 58(48.3%)                       | 62(51.7%) | 62(51.7%)                      | 58(48.3%) | 0.926    | 0.336 |
| PR    | 55(45.8%)                       | 65(54.2%) | 59(49.2%)                      | 61(50.8%) | 0.134    | 0.714 |
| HER2  | 56(46.7%)                       | 64(53.3%) | 58(48.3%)                      | 62(51.7%) | 0.076    | 0.783 |
| Ki-67 | 82(68.3%)                       | 38(31.7%) | 60(50%)                        | 60(50%)   | 4.062    | 0.044 |

**Table III.** *Changes of Ki-67 in groups with different curative effects.*

| Curative effect(N)    | Median (%) | P25 (%) | P75 (%) | Z      | P     |
|-----------------------|------------|---------|---------|--------|-------|
| Effective group(92)   |            |         |         | -5.036 | 0.000 |
| Before chemotherapy   | 30.00      | 15.00   | 65.00   |        |       |
| After chemotherapy    | 10.00      | 42.50   | 36.30   |        |       |
| Ineffective group(28) |            |         |         | -0.484 | 0.629 |
| Before chemotherapy   | 41.20      | 18.80   | 61.30   |        |       |
| After chemotherapy    | 36.60      | 10.00   | 63.80   |        |       |
| Overall               |            |         |         | -4.985 | 0.000 |
| Before chemotherapy   | 32.50      | 15.00   | 5.00    |        |       |
| After chemotherapy    | 10.00      | 65.00   | 40.00   |        |       |

*In the comparison of Ki-67 changes of the effective group and ineffective group after neoadjuvant chemotherapy, Z=-2.151, P=0.031.*

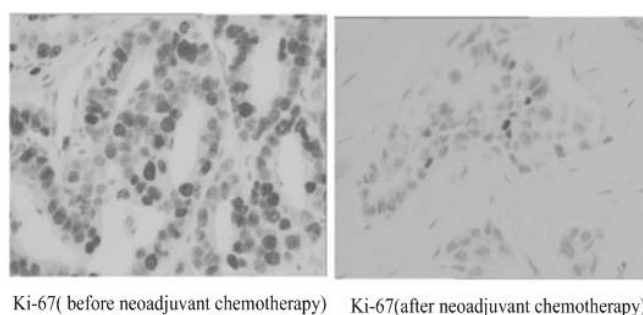
(45%) while after chemotherapy, there were 76 cases of negative Ki-67 expression (63.3%). Thirty-four cases had changed Ki-67 expression state (28.3%) of which, 28 cases (23.3%) transformed from positive to negative and 6 cases (5%) transformed from negative to positive. Twenty cases (16.7%) had quantitatively changed Ki-67 expression. Sixty cases

had unchanged expression state. Details are shown in Table II.

#### *Changes of Ki-67 after chemotherapy*

Ki-67 of 120 patients with breast cancer showed a remarkable decline after neoadjuvant chemotherapy (Fig. 1). The median expression rate of Ki-67





Ki-67( before neoadjuvant chemotherapy) Ki-67(after neoadjuvant chemotherapy)

**Fig. 1.** Immunohistochemical staining results of Ki-67 before and after neoadjuvant chemotherapy.

decreased from 32.5% before chemotherapy to 10% after chemotherapy, and the difference had statistical significance ( $P=0.000$ ). The median expression rate of Ki-67 of the effective neoadjuvant chemotherapy group (PR+CR) showed a significant decline after chemotherapy, from 30% to 20% ( $P=0.000$ ), while the median expression rate of the ineffective neoadjuvant chemotherapy decreased from 41.2% before chemotherapy to 36.6% after chemotherapy, indicating no significant difference ( $P=0.629$ ).

## DISCUSSION

The value of expression of biological factors in breast cancer in predicting the curative effect and prognosis of neoadjuvant chemotherapy has become more and more remarkable. ER and PR can regulate the growth, differentiation and proliferation of hormone dependent breast cancer (9). Effects of neoadjuvant chemotherapy on the expression of ER and PR are controversial. Schneider et al. (10) considered that neoadjuvant chemotherapy had no influence on the expression of ER and PR. However, some Authors had a different opinion. Neubauer H et al. (11) found the expression of ER and PR had obvious changes after neoadjuvant chemotherapy. Patients with breast cancer were observed with overexpression of HER2 (15%-25%) (12). Ki-67 is currently a hot biological index in research concerning multiple malignant tumors, especially breast cancer (13). The expression level of Ki-67 can reflect the proliferation rate of malignant tumors.

The results of the present study suggest that

the expression states of ER and Ki-67 before neoadjuvant chemotherapy might reflect the sensitivity to neoadjuvant chemotherapy, and patients with negative ER expression and high Ki-67 expression could achieve better curative effects. Neoadjuvant chemotherapy can effectively lower the expression levels of Ki-67, therefore, Ki-67 can be regarded as one of the indexes used for predicting its effectiveness. Moreover, neoadjuvant chemotherapy had no influence on the expression of ER, PR and HER2 which can therefore be considered a basis for the formulation of individualized treatment schemes for patients with breast cancer.

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## LETTER TO THE EDITOR

THE IMPACT OF ELECTROMAGNETIC FIELD AT A FREQUENCY OF 50 HZ AND A MAGNETIC INDUCTION OF 2.5 mT ON VIABILITY OF PINEAL CELLS *IN VITRO*A. KOZIOROWSKA<sup>1</sup>, E. PASIUD<sup>2</sup>, M. FILA<sup>2</sup> and M. ROMEROWICZ-MISIELAK<sup>2</sup><sup>1</sup>Faculty of Mathematics and Natural Sciences, University of Rzeszow, Poland;<sup>2</sup>Institute of Applied Biotechnology and Basic Sciences, University of Rzeszow, Poland

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The impact of electromagnetic fields (EMF) on the pineal gland has been described in numerous studies, but many questions still remain unanswered. The aim of the experiment described in this study was to evaluate the effect of EMF on the viability of the pineal gland cells of pig *in vitro*. Primary culture of the pineal gland cells has been exposed to the influence of an EMF at a frequency of 50 Hz with 1, 2 or 3 hours and for 3 hours every 2 or 3 days. After the experiment, viability of cells was assessed by MTT assay and compared to a control culture not exposed to electromagnetic fields. We noticed that in respect to the control, exposure of the cells to the EMF induced a significant increase in viability of cells at 2 and 3 hours of exposure. After three days of 3-hour exposure to EMF, we observed a significant decrease in cell viability in relation to the control. The results of these studies suggest that EMF can have a significant biological effect on the cells of the pineal gland in a time-dependent exposure to its action.

To the Editor,

In recent decades the electromagnetic environment in which humans live has changed greatly. In addition to the Earth's natural electromagnetic field (30-70  $\mu$ T), which always accompanied people, many artificial sources of EMF with unnatural parameters have been created. The human body does not have receptors that allow it to receive information about the existence of fields in close proximity. The effects of the biological effects of EMF on the human body depend on the frequency, intensity, type of fields source and the exposure time. The effects of EMF have long been studied, and are still not fully explained.

EMF can be divided according to frequency. Fields with a frequency of 50 Hz, which are generated by transmission lines, transformer stations, as well as all the equipment supplied by the network, are referred

to as a quasi-stationary slow variable extremely low frequency field. EMF can be considered as two independent components - electric and magnetic. In the case of high frequencies fields we can talk about the properties of wave and about electromagnetic radiation.

The properties of electric and magnetic field and the relationship between them are described in the fundamental equations of Maxwell:

$$(i) \quad \oint_s \vec{D} d\vec{s} = \int_v \rho v \cdot dv,$$

This is the Gauss law for electricity. It means that the source of electric field is charges.

$$(ii) \quad \oint_L \vec{E} \cdot d\vec{l} = - \frac{d\Phi_B}{dt}$$

This is the Faraday law of induction. It means

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Mailing address:

Anna Koziorowska,  
Al. Rejtana 16c,  
35-959 Rzeszów, Poland  
Tel.: +48 17 851 86 09  
Fax: +48 17 851 85 16  
e-mail: akozioro@ur.edu.pl

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that time-varying magnetic field generates a vortex electric field.

$$(iii) \quad \oint \vec{B} \cdot d\vec{A} = 0$$

This is the Gauss law for magnetism. It means that the net magnetic flux out of any closed surface is zero.

$$(iv) \quad \oint_L \vec{H} \cdot d\vec{l} = I + \frac{d\Phi_D}{dt}$$

This is the Amper law. It means that flowing current and changing electric field produces a vortex magnetic field. where: E - electric field [V/m], H - magnetic field, [A/m], B - magnetic induction [T], D - electric induction [C/m<sup>2</sup>], ρ - charge density, I - current density [A/m<sup>2</sup>], Φ<sub>D</sub> - electric flux, Φ<sub>B</sub> - magnetic flux.

Living organisms can be regarded as a system of solid bodies and liquids. Biochemical processes within them, and all tissues have magnetic and electric properties. High frequency EMF impact on the body causes an increase of body temperature. EMF of low frequencies cause A current which flows through the body. In tissues treated with EMF, electromotive force can be induced.

In biological objects exposed to EMF physical phenomena associated with the formation of the Lorenz force occur. The force causes the creation of electromotive force in a moving conductor, creating a force acting on the moving particles, the torsional action on the permanent magnetic dipoles, and the paramagnetic and diamagnetic particles. The impact of EMF is also associated with the impact on the spin states of electrons (1).

The pineal gland is a small unpaired gland just outside the central part of the brain, responsible for producing melatonin, a hormone involved in the regulation of biological rhythms. Melatonin carries a neurohormonal time signal to the body, with information about the changes in environmental light intensity. In the mammalian pineal gland the level of melatonin synthesis increases at the start of night, and is directly secreted into the blood and cerebrospinal fluid (2). Information about changing lighting conditions (day-night cycle) is encoded

in a cyclic change of the amplitude of melatonin secretion. The circadian rhythm of melatonin production in the pineal gland of mammals is modified by light (electromagnetic visible waves). Chronobiological light information flows from the retina to the pineal gland by the neural and humoral pathway (3). Numerous studies have also shown the impact of EMF of extremely low frequency (ELF) as well as exposure to constant magnetic field on the synthesis of melatonin. Both the exposure to light and invisible electromagnetic field at night decreased production of melatonin in the pineal gland. Also, exposure to sinusoidal EMF showed the impact on nocturnal melatonin levels (4).

Melatonin is the most effective natural protector of cells and anti-tumor agents having the ability to eliminate free radicals (5). Free radicals stimulate cells to divide, carrying the risk of inducing tumors (6). Melatonin also reduces the release of estrogen, of which high levels can theoretically raise the risk of breast cancer (7).

It is considered that each factor that inhibits the secretion of melatonin or leads to pineal gland dysfunction may contribute to increase risk of cancer.

The aim of this study was to evaluate the viability of cells of the pineal gland of pigs exposed to EMF-ELF at different exposure times.

## MATERIALS AND METHODS

### *Isolated pinealocyte culture*

Pineal glands were obtained from male pigs (n=6) from a slaughterhouse. The pineal glands were removed no later than 3 min after the heart stopped beating.

Pinealocytes were obtained by trypsin digestion. The glands were removed and immediately placed in ice-cold Dulbecco's Modified Eagle Medium (DMEM). The tissue was incubated at 37°C for 15 min in trypsin (0.25%) solution. After removal of trypsin and its blockage, the cells were mechanically dispersed and resuspended in DMEM supplemented with 7.5% fetal calf serum, 7.5% horse serum, 100U/mL penicillin and 100μg/mL streptomycin to a final concentration of 0.5 x 10<sup>5</sup> cells/mL. The total number of cells and survival was estimated by trypan blue exclusion. The survival rate was 90% or higher. Cells were cultivated in 25 cm<sup>2</sup> culture flasks at



37°C in 5% CO<sub>2</sub>/95% air for 24 h. After 24 h, the cells in suspension were predominantly pinealocytes, which were separated after the removal of all the culture medium content. The cells were centrifuged and resuspended in DMEM at a concentration of 50,000 cells/mL, transferred to a 96-well plate (100 µl per well), and kept at 37°C in 5% CO<sub>2</sub>/95% air for 18 h prior to the electromagnetic field exposure. Recovery of pineal cells after enzymatic digestion was approximately 5 x 10<sup>6</sup> cells per gland, with greater than 95% viability based on trypan blue exclusion.

#### *Electromagnetic field exposure*

The covered plates were placed in an EMF exposure system for 1, 2 or 3 h or for 3 h for two or three days. The cell cultures were exposed to sinusoidal electromagnetic fields at 50 Hz frequency and magnetic induction 2,5mT. The EMF was generated by Astar generator (Magneris, Astar).

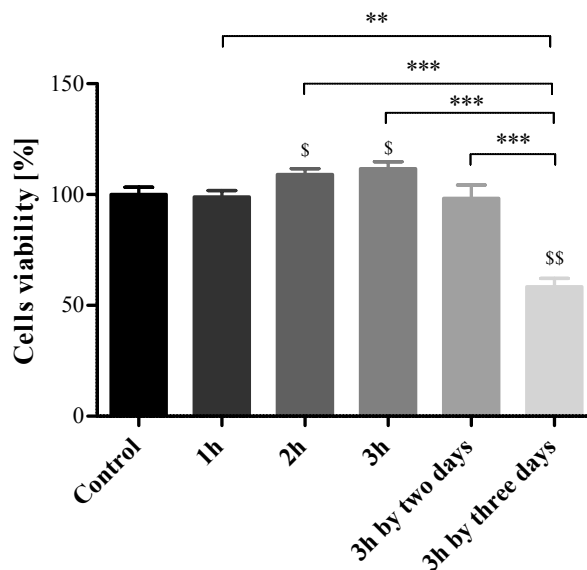
#### *Viability test*

Viability was determined by quantitative colorimetric MTT assay (Sigma-Aldrich). Pineal cells were seeded at a density of 0.5 x 10<sup>5</sup> cells/well in 96-well plates (100µL/well). Immediately following EMF-ELF exposure and control (no exposure) to every well 50 µl were added of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) at a final concentration of 0.5mg/mL. Following a 4 h incubation period with MTT,

media was removed and the formazan crystals trapped in cells were dissolved in MTT solvent (4 mM HCl, 0.1% Nanodet P-40 all in isopropanol). The absorbance at 540 nm was measured with a plate reader (Victor, Perkin Elmer; USA). Cell viability was defined based on the percentage of optic density (OD) in each group as compared to the control group.

## RESULTS

A series of five experiments (for different times of exposure) were carried out in which cells derived from pig pineal glands were treated with an EMF at a frequency of 50 Hz and a magnetic induction of 2.5 mT. The results of pineal cell viability after EMF exposure are presented in Fig. 1. Pineal gland cell viability after exposure to EMF differ in a time-dependent manner on field exposure in relation to the controls. 2- and 3-hours of exposure resulted in a significant ( $P \leq 0.05$ ) increase of pineal gland cell viability relative to the control (cells not exposed to EMF). After three days, a three-hour exposure to EMF, showed significant ( $P \leq 0.01$ ) decrease in cell viability in relation to the control. Cell viability on this day was also significantly lower than in all analyzed exposure times.



**Fig. 1.** Effect of electromagnetic field exposition on cells viability of pig pineal gland cells in vitro. MTT assay of pineal gland cells treated with 50Hz electromagnetic field. Proliferation rate is presented as the mean  $\pm$  SEM of the percentage of viable cells \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ ; \$\$\$ $P \leq 0.01$  (in relation to the control).

## DISCUSSION

Melatonin production in animals and humans is directly related to the presence of electromagnetic radiation in the range registered by receptors in the retina of the eye (visible light). At night, the level of the synthesis of melatonin in the pineal gland significantly increases. During the day, the light falling on the retina is a signal to reduce the production of this hormone (6). In 1981, Wilson and colleagues were the first to show that under *in vitro* conditions ELF-EMF significantly reduces the synthesis of melatonin (8). The inhibitory impact of ELF-EMF on the synthesis of melatonin has been confirmed in further studies on both humans and animals (9). Selmaoui and Touitou (1995), in studies on rats, showed that decrease in the level of melatonin in the blood serum after exposure to EMF at a frequency of 60Hz is associated with lower levels of serotonin N-acetyltransferase, one of the key enzymes in the pathway of synthesis of melatonin (10).

Melatonin is one of the most effective factors with an antitumor effect (5). Any factor that disrupts the rhythm of melatonin synthesis or affects the functioning and structure of the pineal gland can potentially contribute to increase cancer risk. The aim of this study was to evaluate the viability of pineal cells exposed to electromagnetic fields of extremely low frequency under *in vitro* conditions.

A series of five experiments (for different times of exposure) were carried out in which the cells of the pineal gland were exposed to an electromagnetic field at a frequency of 50 Hz and a magnetic induction 2,5mT. The exposure to EMF was carried out for 1, 2 or 3 hours. In the following two experiments, cells were exposed to EMF for 3 hours for two or three consecutive days. The results indicate a significant increase in viability of pineal cells at 2 and 3 hours of exposure to EMF. After 2 or 3 hours of the pineal cell exposure to ELF-EMF, we observed a significant increase in their viability, expressed in the ability of mitochondrial succinate dehydrogenase to convert MTT into formazan. When repeated for three consecutive days, three-hour exposure of the pineal cells to EMF led to a significant decrease in their viability (Fig. 1).

In studies on the effect of EMF-ELF on living organisms, analyses have been carried out of different types and parameters of the fields in a wide range of biological models including animal cell lines. Despite the large amount published research, it is difficult to get a clear answer whether the influence of EMF ELF gives a significant, positive or negative biological effect or whether it is an inert medium. Our results for the different cell lines suggest that the reactivity of ELF-EMF is related to the nature and parameters of field, exposure time, and its action is different depending on the cell line. Afinogenov and colleagues (2009) examined the impact of constant, alternating and cyclotron EMF-ELF (50Hz, 80 $\mu$ T) on the viability of human fibroblasts. Only in the case of cyclotron EMF was there a significant increase in cell viability at all analyzed exposure times (1, 2, 4 and 6 hours). Exposition of fibroblasts to constant EMF resulted after 4 hours of incubation in a significant increase in cells viability, but the prolongation of exposition time to 6 hours resulted in a decrease in cells viability compared to control.

In turn, Ansari and Hei (2001), in the studies on human-hamster hybrid A(L) cells, showed that EMF (60Hz, 100 $\mu$ T) in both short (24 hours) and long-term (7 days) exposure did not affect the survival of these cells (11). Kawczyk-Krupka and colleagues (2002) observed a significant decrease in viability of the murine macrophage cells (murine macrophages J7774.2) after exposure to EMF at a frequency of 25 Hz and magnetic induction 2.4mT (12).

Exposure to EMF-ELF can lead to a metabolic response in the body (13). There are many potential mechanisms by which EMF might induce its biological effect. These include the polarization of cell membrane or free radical mechanism (14). The increase in exposure to EMF may change the balance of the cells by the generation of reactive oxygen species (ROS) (25). The model of the EMF action mechanism on biological systems by Scaiano (1994) assumes that exposure to EMF-ELF may result in higher free radical concentrations by increasing their stability and lengthening their lifetime, thereby enhancing the probability of radical reactions with cellular components (15). Numerous studies have demonstrated that overproduction of ROS in the

cells can increase the incidence of cellular component damage, primarily the lipid membrane, and nucleic acids (16). Moreover, chronic exposure to ELF-EMF can lead to a decrease in melatonin plasma levels, one of the most effective scavengers of free radicals, which is another mechanism for induction of oxidant stress by ELF-EMF radiation (17). Katsir and Parola (1998), in their study on the effect of EMF on the proliferation of chick embryo fibroblasts, showed that increased cell division as a result of ELF-EMF frequency decreases when substances with free radical-scavenging properties are present in the culture medium (18). The brain is particularly sensitive to oxidative damage because of the high metabolic rate and the composition rich in polyunsaturated fatty acids (19). The results of this study showed that EMF-ELF can influence changes in the viability of pineal cells in a time-dependent manner to its exposure. Despite the large amount of research on the biological effects that may be caused by EMF-ELF, knowledge of the subject is still ambiguous. In order to clarify the effects of EMF on cells more detailed studies are necessary.

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## LETTER TO THE EDITOR

**DECREASED IMMUNOREACTIVITY OF VISFATIN IN THE PANCREAS  
AND LIVER OF RATS WITH RENOVASCULAR HYPERTENSION**Ż. PIOTROWSKA<sup>1</sup>, I. JANIUK<sup>2</sup>, A. LEWANDOWSKA<sup>1</sup> and I. KASACKA<sup>1</sup><sup>1</sup>*Department of Histology and Cytophysiology, Medical University of Białystok, Białystok, Poland;*<sup>2</sup>*Department of Dietetics and Food Assessment, Institute of Health Sciences, Siedlce University of Natural Sciences and Humanities, Siedlce, Poland**Received May 23, 2016 – Accepted August 8, 2016*

Hypertension is one of the major endocrine and metabolic disorders, in which visfatin plays a significant role. The objective of this study was to evaluate the immunoreactivity of visfatin in pancreas and liver of “two kidney, one clip” (2K1C) renovascular hypertension model in rats. The studies were carried out on the pancreas and liver of rats. After a 6-week period of the renal artery clipping procedure, 2K1C rats developed a stable hypertension. Paraffin sections were stained with hematoxylin and eosin (for general histological examination) and processed for immunolocalization of visfatin. The intensity of immunohistochemical reaction was measured using Nikon NIS-Elements Advanced Research software. The hypertension significantly weakened the immunohistochemical reaction exhibiting visfatin in the pancreas and liver of hypertensive rats, compared to control animals. The changes induced by hypertension in the visfatin-containing cells in the pancreas and liver of the rats are discussed and needs further study.

To the Editor,

The list of biological substances secreted by adipose tissue is constantly being extended. One of the recently discovered adipokines is visfatin, a 52-kDa protein. The peptide is distributed predominantly in visceral fat but it is produced also in central nervous system and peripheral organs such as heart, lungs, gastrointestinal track, liver and pancreas (1, 2).

Visfatin is involved in the regulation of feeding behaviour and energy balance (3). Peptide also plays an important role in maintaining glucose homeostasis. Visfatin enhances insulin secretion and improves survivability of islet  $\beta$ -cells (4). It was demonstrated that visfatin has affinity to the insulin receptor, whereby it causes glucose uptake into muscle cells, adipocytes and hepatocytes (1).

The latest reports demonstrated that visfatin

influence the blood pressure by causing vessel relaxation (5). On the other hand, it was shown that peptide contributes to endothelium dysfunction, which might lead to insufficient amount of endothelium-generated vasodilators, enhanced vascular resistance and abnormal blood pressure (5).

Several studies indicated an increased level of circulating visfatin in hypertension stage (6, 7). As apparent from the literature review, changes in visfatin content were measured only in blood samples, and it was not established whether hypertension affect the visfatin synthesis in metabolically important organs such as liver and pancreas.

Close associations between hypertension and impaired carbohydrate homeostasis and importance of visfatin in regulation of pancreatic endocrine function, glucose metabolism and glycogenesis in

*Key words: visfatin, pancreas, liver, rat, renovascular hypertension, immunohistochemistry*

*Mailing address:*

Prof. Irena Kasacka,  
Department of Histology and Cytophysiology,  
Medical University of Białystok, Mickiewicza 2C Street,  
15-222 Białystok, Poland  
Tel.: +48 85 748 54 58 - Fax: +48 85 748 55 16  
e-mail: kasacka@umb.edu.pl

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hepatocytes, prompted us to undertake the present research, which aims at evaluating the visfatin-producing cells in pancreas and liver of renovascular hypertensive rats.

## MATERIALS AND METHODS

### *Experimental animals*

The assumptions, the aim and the plan of the study, as well as the approach to animals were approved by the Senate Commission for the Supervision of Studies on Human and Animal Subjects, Medical University in Białystok (Resolution no. 39/2013)

The study was performed on sixteen ( $n=16$ ) young male Wistar rats, their body weight at the beginning of the experiment was within 160-180 g (mean body weight:  $170\pm 10$  g). The rats were housed in polypropylene cages in groups of two rats per cage and received laboratory chow *ad libitum*. After a 1-week period of acclimatization, the systolic blood pressure of each rat was measured, after which the surgical procedure for induction of renovascular hypertension was performed.

The experimental animals were divided into two groups: a control group of 6 rats underwent sham operation, and a study group of 10 rats with induced hypertension.

### *2K1C renovascular hypertension*

Induction of experimental hypertension was performed according to the procedure described by Goldblatt et al. (8). After the rats were anesthetized by exposure to pentobarbital (40 mg/kg, i.p.), a 3-cm retroperitoneal flank incision was performed under sterile conditions. The left kidney was exposed and the renal artery was carefully dissected free of the renal vein. The renal artery was then partially occluded by placing a silver clip with an internal diameter of 0.20 mm on the vessel. The wound was closed with a running 3-0 silk suture ( $n = 10$ ). Sham operated rats ( $n = 6$ ) underwent identical surgical procedures, except that a clip was not applied to the renal artery. After the surgery, the rats were kept in single cages.

Six weeks after the renal artery clipping procedure, all animals were weighed and the systolic arterial pressure was measured by the tail-cuff method (9). Arterial pressure was evaluated by using a Student Oscillograph Rat Tail Blood Pressure Monitor, Harvard, England.

The BP measurements were considered valid only when three consecutive readings did not differ by more than 5 mmHg. The average of the three measured values was then recorded. After this time, eight 2K1C rats ( $n = 8$ ) developed stable hypertension (mean blood pressure values  $162.6\pm 3.2$  mmHg).

### *Experimental material collection and fixation*

Six weeks after surgery, fragments of pancreas and liver were collected under deep pentobarbital anesthesia (50 mg/kg b.w.) from the hypertensive and control animals. The specimens were fixed in Bouin's fluid and embedded in paraffin in a routine manner. 4- $\mu$ m thick sections were cut and stained by hematoxylin-eosin (H+E) for general histological examination and processed by immunohistochemistry for visfatin detection. Weight data of the left and right kidneys were additionally collected.

### *Detection of visfatin by immunohistochemistry*

In the immunohistochemical study, the EnVision method was used according to Herman and Elfont (10). Immunostaining was performed by the following protocol: paraffin-embedded sections were deparaffined and hydrated in pure alcohols. For antigen retrieval, the sections were subjected to pretreatment in a pressure chamber and heated for 1 min at 120 kPa and 125°C, using Target Retrieval Solution (S 1699, Dako Denmark A/S, Produktionsvej 42, DK-2600, Glostrup, Denmark). After cooling to room temperature, the sections were incubated with Peroxidase Blocking Reagent (S Dako Denmark A/S, Produktionsvej 42, DK-2600, Glostrup, Denmark) for 10 min to block endogenous peroxidase activity. Subsequently sections were incubated with primary antibody for Visfatin (No H-003-93, purchased from Phoenix Pharmaceuticals, Inc., Mountain View, CA, USA. The antibody was previously diluted in Antibody Diluent (S 0809 Dako Denmark A/S, Produktionsvej 42, DK-2600, Glostrup, Denmark) in relation 1:10,000. Sections were incubated overnight at 4°C in a humidified chamber. This procedure was followed by incubation with secondary antibody (conjugated to horseradish peroxidase-labelled polymer) (EnVision+ Kit HRP Rabbit K4011 Dako Denmark A/S, Produktionsvej 42, DK-2600, Glostrup, Denmark). The bound antibodies were visualized by 1-min incubation with liquid 3,3'-diaminobenzidine substrate chromogen. The sections were finally counterstained in hematoxylin

QS (H – 3404, Vector Laboratories; Burlingame, CA, USA), mounted and evaluated under light microscope. Appropriate washing with Wash Buffer (S 3006 Dako Denmark A/S, Produktionsvej 42, DK-2600, Glostrup, Denmark) was performed between each step.

Specificity tests, performed for the Visfatin antibody included: negative control, where the antibodies were replaced by normal rabbit serum (Vector Laboratories; Burlingame, CA, USA) at respective dilution, and a positive control was prepared with specific tissue (as recommended by the manufacturer, we used mouse liver for our research concerning visfatin).

#### *Microscopic and quantitative analysis*

The results of routine and immunohistochemical staining were submitted for evaluation in an Olympus BX41 microscope with Olympus DP12 camera. From each animal, five sections of pancreas and liver were studied. Visfatin-positive structures were searched for and their localization was observed. From all sections of pancreas and liver, five randomly selected microscopic fields, each field of 0.785 mm<sup>2</sup>, at magnification of 200x (20x the lens and 10x the eyepiece), were documented. Subsequently, images were submitted to morphometric evaluation by using NIS-Elements Advanced Research software of Nikon.

In each analyzed image from pancreas and liver the intensity was measured of the immunohistochemical reaction. The intensity of immunoreaction was measured by using 0 to 256 grey scale level, where a completely black pixel was given a value of 0, whereas one with a value of 256 is completely white or bright.

All data were statistically analyzed by means of software computer package Statistica Version 10.0. The corresponding mean values were computed automatically; significant differences were determined by Student's *t*-test; *p*<0.05 was taken as the level of significance.

## RESULTS

No significant differences were found between the two control groups of rats; therefore, only the results from the sham-operated animals were used for comparison with experimental animals.

After six weeks of experiment, the rats which had undergone the procedure of renal artery occlusion

had elevated levels of blood pressure in comparison with control animals. Long-term restriction of blood supply resulted in the substantial atrophy of the left kidney, while the mass of right unclipped kidney was slightly increased, when compared to kidneys of normotensive rats (Table I).

Routine histopathological examination showed no identifiable pathological changes in all studied pancreas and liver specimens. Immunohistochemical staining using anti-visfatin revealed the presence of the peptide in the pancreas and liver of each studied animal; however the intensity of immunoreaction varied between experimental and normotensive rats.

In rat pancreas, visfatin- immunosignal was observed in endocrine cells forming pancreatic islets, as well as in secretory acini and ducts of exocrine part of the organ (Fig. 1 A-D). Visfatin was also detected in blood vessels supplying pancreatic parenchyma (Fig. 1 D).

The antisera against visfatin stained the majority of islet cells in the pancreas of control rats. Visfatin-containing cells, with strong immunoreaction occupied nearly the entire section of pancreatic islets (Fig. 1 A, B). The intense immunostaining was also observed in apical domain of acini cells and in the epithelium lining of the intralobular and interlobular ducts (Fig. 1 B). Likewise, high immunoreactivity was observed in endothelium and myocytes of vasculature.

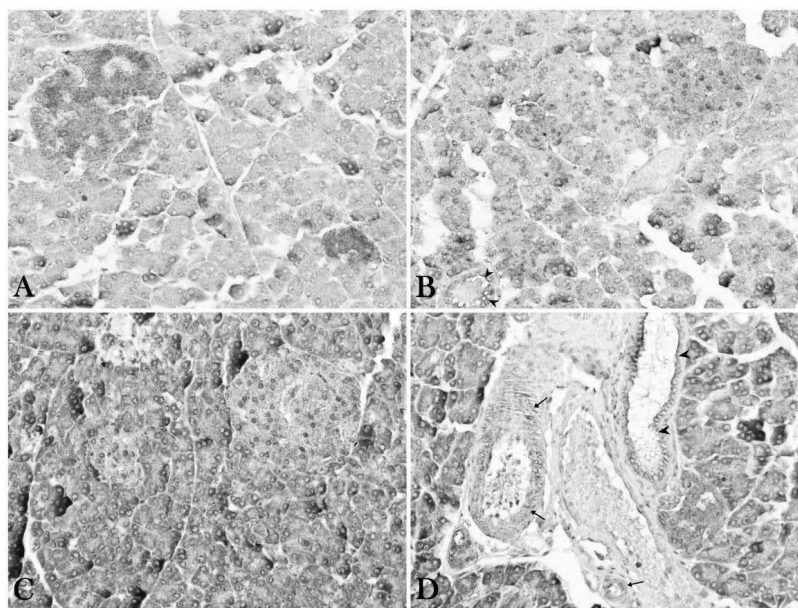
In 2K1C rats, similarly to sham-operated animals, visfatin-containing cells were observed in the vast area of the islets. However, the intensity of reaction in visfatin-positive endocrine cells was significantly lower as compared with the control group (Fig. 1 C, D). The weaker immunoreactivity was noticed also in the exocrine part and blood vessels of pancreas of hypertensive rats (Fig. 1 D).

Immunodetection of visfatin in rat liver revealed the presence of the peptide in hepatocytes (Fig. 2 A, B). In the liver of normotensive animals, positive reaction occurred in numerous hepatic cells. The visfatin-IR cells found in liver of control rats were stained an intense brown colour (Fig. 2 A). Less abundant visfatin-containing hepatocytes and weaker visfatin-immunoreactivity were observed in the liver of hypertensive rats, in comparison with the

**Table I.** Body weight, mass of kidney, values of BP of normotensive and renovascular hypertensive rats.

| Group of rats | Bodyweight (g) | Mass of kidney (g) |            | Values of BP<br>(mmHg) |
|---------------|----------------|--------------------|------------|------------------------|
|               |                | right              | left       |                        |
| Control       | 443±44.6       | 1.37±0.19          | 1.35±0.13  | 120.2±5.89             |
| 2K1C rats     | 437±56.8       | 1.85±0.2 *         | 0.31±0.1 * | 162.6±2.19 *           |

(mean ± SD) \* $p < 0.05$



**Fig. 1.** Immunodetection of visfatin in rat pancreas. **A)** control rat; strong immunosignal in pancreatic islets; **B)** normotensive rat; intense immunostaining in the endocrine cells of Langerhans islets, as well as in secretory acini and epithelium lining the ducts of exocrine pancreas (arrowheads); **C)** 2K1C rat; weaker immunoreactivity in islet cells; **D)** rat with hypertension; weak to moderate immunohistochemical reaction in pancreatic islets, acini cells, epithelium of exocrine ducts (arrowheads) and myocytes of blood vessels (arrows).

sham-operated animals (Fig. 2 B).

The microscopic evaluation was confirmed by the morphometric analysis, which showed significant reduction in intensity of immunohistochemical reaction in pancreas and liver of rats with experimental hypertension (Table II).

## DISCUSSION

In the present research, we have accurately exhibited immunoreactivity of visfatin in pancreatic islet cells and exocrine part of the pancreas, as well

as in the hepatocytes of tested rats and furtherly demonstrated the differences in its expression among normotensive and hypertensive animals.

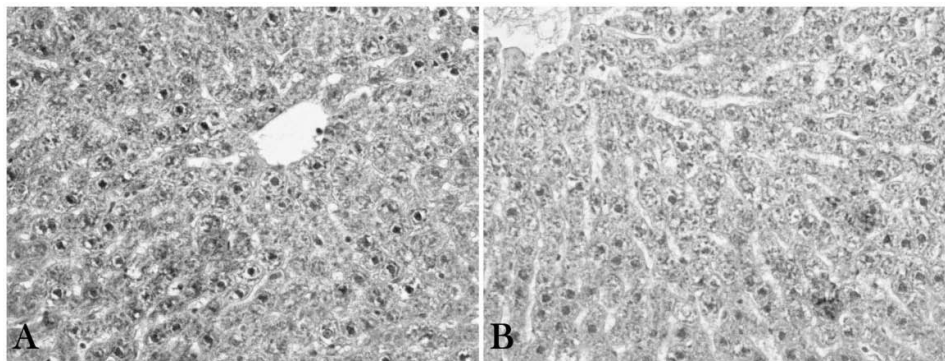
No reports related to the evaluation of immunoreactivity of visfatin in the pancreas and liver in the conditions of hypertension were found in the available literature.

Pathogenesis of renovascular hypertension involves numerous interrelated processes, among which the greatest importance is assigned to hyperactivity of renin-angiotensin-aldosterone (RAA) system (11). *In vitro* and clinical studies

**Table II.** *Intensity of immunohistochemical reaction in pancreas and liver of control and 2K1C rats.*

| Group of rats | Intensity of immunohistochemical reaction<br>scale from 0 (black pixel) to 256 (white pixel) |               |
|---------------|----------------------------------------------------------------------------------------------|---------------|
|               | Pancreas                                                                                     | Liver         |
| Control       | 141.8±16.4                                                                                   | 124.7±25.77   |
| 2K1C rats     | 150.0±13.73 *                                                                                | 176.9±17.56 * |

\* $p < 0.05$  (mean  $\pm$  SD)

**Fig. 2.** *Immunohistochemical reaction determining visfatin in rat liver. A) control animal; intensive visfatin-staining in numerous hepatocytes; B) hypertensive rat; visfatin-immunoreactivity in fewer hepatic cells.*

indicated that angiotensin II has a negative effect on visfatin synthesis (12, 13). Therefore, weakening of immunohistochemical reaction in pancreas and liver of 2K1C rats might result from down-regulation of visfatin production by components of RAA system.

Hypertension leads to excessive fibrogenesis in the majority of organs, especially in the heart, kidneys and liver. Recent reports demonstrated that visfatin increases the release of profibrotic mediators (14). It was also indicated that visfatin level in patient's blood increase with severity of liver fibrosis (15). The reduced visfatin biosynthesis observed in liver of 2K1C rats might be one of the mechanisms aimed at restricting the disease-associated hepatic fibrosis.

It is known that visfatin significantly affects insulin release and promotes  $\beta$ -cell longevity (4). Consequently, impaired expression of visfatin in pancreas might entail loss of insulin-producing

cells, and presumably lead to the development of diabetes. Diminished visfatin production in pancreas of rats with hypertension, reported in our research, might constitute a possible mechanism explaining the strong interdependence between elevated blood pressure and hyperglycemia.

Further studies are needed to clarify the mechanism of reducing the expression of visfatin in the pancreas and liver in hypertension.

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## LETTER TO THE EDITOR

## EFFECTS OF SEVOFLURANE ON CARDIOPULMONARY FUNCTION IN PATIENTS UNDERGOING CORONARY ARTERY BYPASS

J. ZHANG<sup>1</sup> and S. WANG<sup>2</sup><sup>1</sup>Department of Anesthesia, The East Area of the Central Hospital of Xinxiang, Xinxiang, PR China;<sup>2</sup>Quality and Technical Supervision and Inspection Center of Xinxiang, Xinxiang, PR China

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The objective of the current study was to investigate effects of sevoflurane on cardiopulmonary function in patients undergoing coronary artery bypass grafting (CABG). In this study, 60 cases of patients with coronary heart disease (CHD) were selected and randomly divided into the sevoflurane group (group S) and the control group C (group C) with 30 cases in each group. The two groups received intravenous anesthesia. The patients of group C were only given oxygen mask and physiological saline to keep vein open; while the patients of group S were administered with 1% sevoflurane immediately after the beginning of cardiopulmonary bypass (CPB) until the end of the treatment. The cardiopulmonary functions at 30 min before operation ( $T_0$ ), postoperative 2 h ( $T_1$ ), 6h ( $T_2$ ), 24h ( $T_3$ ) and 48 ( $T_4$ ) were observed. The mean arterial pressure (MAP) of the group S at  $T_1$ ,  $T_2$ ,  $T_3$  was lower than that of the group C, as were the heart rate (HR) and left ventricular ejection fraction (LVEF). The creatine kinase isoenzyme (CK-MB) during  $T_1$  to  $T_4$  in the group S was less than that of the group C, and there were significant differences between the two groups ( $P < 0.05$ ). The tidal volume (Vt), vital capacity (Vc) and oxygenation index ( $\text{PaO}_2/\text{FiO}_2$ ) of the two groups during  $T_1$  and  $T_2$  were decreased, while respiratory frequency (RR) and alveolar-arterial blood oxygen partial pressure ( $\text{PA-aO}_2$ ) were increased and they began to decrease during  $T_3$  and  $T_4$ . Vt and Vc of the group S were higher during  $T_1$  and  $T_2$  periods than those of the group C, while RR was lower than that of the group C;  $\text{PaO}_2/\text{FiO}_2$  during  $T_1$  to  $T_4$  period of group S was higher than that of group C, while  $\text{PA-aO}_2$  was significantly lower than that of the control group ( $P < 0.05$ ). In conclusion, although LVEF was not improved in the sevoflurane group, sevoflurane may contribute to stabilizing the cardiopulmonary function and preventing from myocardial injury.

To the Editor,

Coronary artery bypass grafting (CABG) is a common surgical treatment of the coronary artery diseases in the world. However, some clinical studies have shown that this operation could affect cardiopulmonary functions, especially ischemia-reperfusion injury in heart and weak pulmonary ventilation and diffusion capacity caused by ascending aorta blocking (1). Therefore, more

effective treatment methods are urgently needed.

In recent years, some foreign anesthesiology studies have found that sevoflurane, a common inhalation anesthesia drug, had a favorable anaesthetic effect, keeping the patients with coronary artery diseases from cardiopulmonary injury caused by ischemia-reperfusion (2). However, there are few clinical studies on CABG in the cardiopulmonary bypass (CPB) state.

*Key words:* sevoflurane, cardiopulmonary bypass, coronary artery bypass, cardiac function, respiratory function

Mailing address:

Dr. Jichen Zhang,  
The East Area of the Central Hospital of Xinxiang,  
No. 56 Jinsui Road, Xinxiang City, 453000,  
People's Republic of China  
e-mail: jichenzhang2015@sina.com

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INTEREST RELEVANT TO THIS ARTICLE.

The objective of the current study was to investigate effects of sevoflurane on cardiopulmonary function in patients undergoing CABG. The results demonstrated that, although LVEF was not improved in the sevoflurane group, sevoflurane may contribute to stabilizing the cardiopulmonary function and preventing from myocardial injury.

## MATERIALS AND METHODS

### *Patient information*

Sixty cases of CHD conforming to CABG surgical indications were selected from January 2013 to December 2015 in the Cardiac Surgery Department of The East Area of the Central Hospital of Xinxiang, and were randomly divided into a sevoflurane group (group S) (30 cases) and a control group (group C) (30 cases). The local ethics committee approved this study, and all the subjects gave written informed consent.

There were 22 males and 8 females in group C, aged 54-69 years (average  $61.75 \pm 6.32$  years), with weight of 52-76 kg and average weight of  $(62.86 \pm 8.45)$  kg. According to the American Society of Anesthesiologists (ASA) criteria, there were 20 cases with ASA II and 10 cases with ASA III. According to the New York Heart Association (NYHA) criteria, there were 6 cases with NYHA II and 24 cases with NYHA III. In group S, there were 22 males and 8 females, aged 53-70 years (average  $61.83 \pm 6.35$  years), and with weight of 51-77 kg, and average weight of  $62.75 \pm 8.86$  kg. There were 21 cases with ASA II, 9 cases with ASA III, 7 cases with NYHA II and 23 cases with NYHA III. In addition, there were no significant differences in gender, age, weight, ASA classification, NYHA classification or other preoperative clinical data between the two groups ( $P > 0.05$ ).

### *Inclusion criteria*

More than 50% of the patients in this study had problems in the left main coronary artery, and more than 70% of the patients had lesion in the proximal anterior descending coronary artery and stenosis in another vascular diameter. The patients were those with ASA II and ASA III, and with NYHA II or ASA III according to the criteria of cardiac functions. The surgery carried out conformed to the Guideline of CABG Indication in the United States in 2011.

### *Exclusion criteria*

The exclusion criteria were as follows: allergic to narcotic drugs, having been given sevoflurane, history of myocardial infarction, or acute or chronic heart failure, disorders in liver or kidney, chronic obstructive pulmonary disease (COPD), infectious disease, malignant tumor or other diseases.

### *Anesthesia method*

Anesthesia was carried out on all the patients in the two groups according to Jang et al. (1), and oxygen was given immediately after their entering the operating theatre. The vital signs including respiration, HR, pulse and blood oxygen saturation were measured by cardiogram monitor. Firstly, local anesthesia was carried out on the patients by using 2% lidocaine. The invasive arterial blood pressure of the patients was monitored by using sphygmomanometer during surgery. Secondly, they were intravenously injected with 0.05 mg/kg Midazolam, 3 mg/ml propofol, 1  $\mu$ g/kg sufentanil and 0.6 mg/kg rocuronium bromide. Mechanical ventilation was carried out at 8-10 ml/kg tidal volume, 12 times/min respiratory rate (RR), 1:2 of inspiration/expiration and 35-45 mmHg PetCO<sub>2</sub>. Intravenous infusion of 3-5 mg\*kg<sup>-1</sup>\*h<sup>-1</sup> Dimethylcarbinol and 0.5-1 mg\*kg<sup>-1</sup>\*h<sup>-1</sup> sufentanil were used to maintain the anesthesia. 1.0% sevoflurane was given to the patients of group S immediately when carrying out CPB. Sevoflurane was not used after finishing the CPB. The patients in group C were only given oxygen and maintained venous channel unobstructed. The preoperative BISxp was kept between 40-60, 50-100  $\mu$ g phenylephrine was intravenously and intermittently injected to keep MAP at more than 70 mmHg.

### *Observation indexes*

The observation indexes of the two groups included the changes of cardiac function indexes (MAP, HR, LVEF and CK-MB) and the lung function indexes (Vt, Vc, RR, PaO<sub>2</sub>/FiO<sub>2</sub> and PA-aO<sub>2</sub>) at T<sub>0</sub>, T<sub>1</sub>, T<sub>2</sub>, T<sub>3</sub> and T<sub>4</sub>.

### *Statistical analysis*

SPSS17.0 software was used for statistical analysis. Measurement data was expressed as standardized mean difference (SMD) ( $\bar{x} \pm s$ ). Student's t-test was used for analysis. One-way ANOVA was used within groups, and  $P < 0.05$  was considered statistically significant.

## RESULTS

Surgery on the patients of the two groups was conducted smoothly with a good anesthetic effect and without adverse events.

*Comparison of the cardiac function indexes between the two groups during different time points*

The differences of MAP, HR, LVEF and CK-MB between the two groups during  $T_0$  period were not statistically significant ( $P > 0.05$ ). As shown in the data, MAP and HR of the two groups presented a rising trend during  $T_1$  and  $T_2$ , while LVEF presented a declining trend; and CK-MB had an increasing trend during  $T_1$  to  $T_3$  in both groups. The differences of MAP and LVEF of group S and the repeated ANOVA of MAP, HR, LVEF and CK-MB of group C during different periods were statistically significant ( $P < 0.05$ ); while the repeated ANOVA of HR of group S at different time points was not statistically significant ( $P > 0.05$ ). MAP, HR and LVEF of group S during  $T_1$ ,  $T_2$  and  $T_3$  were lower than those of group C, and CK-MB of group S during  $T_1$  to  $T_4$  was lower than that of group C, and the difference between the two groups was statistically significant ( $P < 0.05$ ), as shown in Table I.

*Comparison of the respiratory function indexes between two groups at different time points*

The differences of Vt, Vc, RR,  $\text{PaO}_2/\text{FiO}_2$  and PA-a $\text{O}_2$  of the two groups during  $T_0$  were not statistically significant ( $P > 0.05$ ). The repeated ANOVA of Vt, Vc, RR,  $\text{PaO}_2/\text{FiO}_2$  and PA-a $\text{O}_2$  of the two groups during  $T_1$ ,  $T_2$ ,  $T_3$  and  $T_4$  were statistically significant ( $P < 0.05$ ). Vt, Vc and  $\text{PaO}_2/\text{FiO}_2$  of the two groups declined during  $T_1$  and  $T_2$ , while RR and PA-a $\text{O}_2$  were on a rising trend but declined during  $T_3$  and  $T_4$  period. Vt and Vc of group S during  $T_1$  and  $T_2$  were higher than those of group C, while RR was lower than that of the group C.  $\text{PaO}_2/\text{FiO}_2$  during  $T_1$  to  $T_4$  was higher than that of group C, while PA-a $\text{O}_2$  of group S was lower than that of group C, and the difference between the two groups was statistically significant ( $P < 0.05$ ), as shown in Table II.

## DISCUSSION

Although CABG is a common surgical treatment of coronary artery diseases, the method affects cardiopulmonary functions (3, 4). Therefore, decreasing the harm of CABG in CPB state to cardiopulmonary function would contribute to good prognosis and reducing complications (5,

**Table I.** Comparison of the cardiac function indexes between the two groups at different periods.

| Indexes       | Groups  | $T_0$       | $T_1$                    | $T_2$                    | $T_3$                    | $T_4$                   | F     | P      |
|---------------|---------|-------------|--------------------------|--------------------------|--------------------------|-------------------------|-------|--------|
| MAP (mmHg)    | Group S | 103.95±9.17 | 104.78±8.15 <sup>▲</sup> | 108.47±7.48 <sup>▲</sup> | 100.19±7.64 <sup>▲</sup> | 103.32±7.55             | 39.15 | < 0.05 |
|               | Group C | 104.69±8.15 | 106.54±10.64             | 112.68±11.55             | 107.14±12.31             | 104.75±11.47            | 58.42 | < 0.05 |
| HR (time/min) | Group S | 76.45±7.86  | 77.64±8.09 <sup>▲</sup>  | 77.75±8.21 <sup>▲</sup>  | 76.55±8.69 <sup>▲</sup>  | 76.26±8.37              | 2.73  | > 0.05 |
|               | Group C | 77.15±8.39  | 87.62±10.28              | 90.45±10.97              | 86.15±10.38              | 78.77±9.87              | 51.26 | < 0.05 |
| LVEF (%)      | Group S | 57.18±3.64  | 51.39±4.85               | 50.69±3.76               | 51.39±4.85               | 57.38±4.52              | 2.78  | > 0.05 |
|               | Group C | 56.69±3.76  | 54.65±4.56 <sup>▲</sup>  | 53.79±3.64 <sup>▲</sup>  | 54.25±4.09 <sup>▲</sup>  | 55.39±4.85              | 35.14 | < 0.05 |
| CK-MB (u/L)   | Group S | 17.22±4.23  | 28.61±5.89 <sup>▲</sup>  | 49.52±9.25 <sup>▲</sup>  | 69.28±10.25 <sup>▲</sup> | 58.36±9.85 <sup>▲</sup> | 20.36 | < 0.05 |
|               | Group C | 16.79±4.65  | 42.36±9.15               | 68.25±11.26              | 109.75±13.25             | 88.45±11.02             | 55.42 | < 0.05 |

Note: compared with group C, <sup>▲</sup> $P < 0.05$

**Table I.** Comparison of the cardiac function indexes between the two groups at different periods.

| Indexes                                   | Groups  | T <sub>0</sub> | T <sub>1</sub>            | T <sub>2</sub>            | T <sub>3</sub>            | T <sub>4</sub>            | F      | P      |
|-------------------------------------------|---------|----------------|---------------------------|---------------------------|---------------------------|---------------------------|--------|--------|
| Vt (L)                                    | Group S | 0.69±0.24      | 0.60±0.21 <sup>▲</sup>    | 0.60±0.19 <sup>▲</sup>    | 0.69±0.20                 | 0.69±0.25                 | 12.38  | < 0.05 |
|                                           | Group C | 0.68±0.26      | 0.58±0.30                 | 0.57±0.27                 | 0.68±0.31                 | 0.69±0.32                 | 9.45   | < 0.05 |
| Vc (L)                                    | Group S | 3.34±0.39      | 3.01±0.35 <sup>▲</sup>    | 3.03±0.34 <sup>▲</sup>    | 3.32±0.41                 | 3.29±0.40                 | 8.16   | < 0.05 |
|                                           | Group C | 3.25±0.37      | 2.78±0.41                 | 2.75±0.39                 | 3.18±0.44                 | 3.20±0.38                 | 12.33  | < 0.05 |
| RR (time/min)                             | Group S | 18.25±3.65     | 19.97±4.5 <sup>▲</sup>    | 20.57±4.67 <sup>▲</sup>   | 18.55±4.57                | 18.15±4.36                | 15.16  | < 0.05 |
|                                           | Group C | 18.35±7.69     | 21.67±3.67                | 21.55±4.25                | 18.66±4.58                | 18.74±4.39                | 22.37  | < 0.05 |
| PaO <sub>2</sub> /FiO <sub>2</sub> (mmHg) | Group S | 478.41±30.68   | 436.45±36.45 <sup>▲</sup> | 385.42±38.79 <sup>▲</sup> | 453.81±41.77 <sup>▲</sup> | 473.81±35.72 <sup>▲</sup> | 56.35  | < 0.05 |
|                                           | Group C | 482.46±31.07   | 404.24±32.57              | 357.75±38.39              | 426.87±42.73              | 466.35±38.45              | 49.78  | < 0.05 |
| PA-aO <sub>2</sub> (mmHg)                 | Group S | 53.27±5.63     | 98.46±12.37 <sup>▲</sup>  | 118.29±21.32 <sup>▲</sup> | 78.36±6.38 <sup>▲</sup>   | 56.15±5.98 <sup>▲</sup>   | 32.12  | < 0.05 |
|                                           | Group C | 52.89±5.15     | 121.38±17.22              | 126.52±24.36              | 88.42±7.25                | 63.74±6.36                | 101.17 | < 0.05 |

Note: compared with group C, <sup>▲</sup>P<0.05

6). The data in this investigation illustrate that, although LVEF was not improved in the sevoflurane group, the sevoflurane may contribute to stabilizing the cardiopulmonary function and preventing myocardial injury.

LVEF could be used to assess the left ventricular pump function, and CK-MB can reflect the myocardial function injury (7). In this study, MAP and HR indexes at postoperative 2 h, 6 h and 24 h did not change significantly, while the CK-MB index of postoperative 2 h, 6 h, 24 h and 48 h increased significantly (8), indicating that sevoflurane might stabilize HR and protect myocardium (9). Vt, Vc and RR can reflect pulmonary ventilation function (10). PaO<sub>2</sub>/FiO<sub>2</sub> and PA-aO<sub>2</sub> can reflect the pulmonary ventilation function (11). In this study, Vt, Vc and PaO<sub>2</sub>/FiO<sub>2</sub> of the two groups during T<sub>1</sub> and T<sub>2</sub> were decreased, while RR and PA-aO<sub>2</sub> were increased and they began to decrease during T<sub>3</sub> and T<sub>4</sub>. Vt and Vc of group S were higher during T<sub>1</sub> and T<sub>2</sub> periods than those of group C, while RR was lower than that of the group C. PaO<sub>2</sub> / FiO<sub>2</sub> during T<sub>1</sub> to T<sub>4</sub> period of group S was higher than that of group C, while PA-aO<sub>2</sub> was significantly lower than that of groupC, which were

consistent with previous studies (12, 13).

CABG would affect the cardiopulmonary function in patients with CHD under CPB state, and although LVEF was not improved in the sevoflurane group, sevoflurane may contribute to stabilizing the cardiopulmonary function and preventing from myocardial injury.

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## LETTER TO THE EDITOR

## USE OF LACTATED RINGER'S SOLUTION DOES NOT ELIMINATE THE RISK OF STRONG ION DIFFERENCE-RELATED METABOLIC ACIDOSIS FOLLOWING ON-PUMP CARDIAC SURGERY

T. JOVAISA<sup>1</sup>, V. VICKA<sup>2</sup>, G. LINKAITĖ<sup>3</sup>, J. GUSEINOVAITĖ<sup>3</sup>, D. RINGAITIENĖ<sup>2</sup>  
and I. NORKIENĖ<sup>2</sup>

<sup>1</sup>*Clinic of Anaesthesiology, Lithuanian University of Health Sciences, Kaunas, Lithuania;* <sup>2</sup>*Clinic of Anaesthesiology and Intensive Care, Vilnius University, Vilnius, Lithuania;* <sup>3</sup>*Faculty of Medicine, Vilnius University, Vilnius, Lithuania*

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There is a growing interest in the effects of plasma sodium levels on postoperative outcomes. A trend of using balanced crystalloid solutions is based on the extensive data on chloride and the strong ion difference-related acidosis. However, effects of sodium are often overlooked in this context. The aim of the study was to establish the effects of the routine use of Lactated Ringer's Solution (RL) on postoperative changes in sodium concentrations and whether these changes result in metabolic acidosis. We performed database analysis of 358 consecutive elective on-pump cardiac surgery cases in a tertiary referral university hospital. Approval from the institutional ethics committee was obtained for this study. Intraoperative fluid balance was  $2726 \pm 1073$  ml and the total volume of intravenous infusions in the first 24 hours was  $5865 (\pm 1073)$  ml, 95% of which was RL; 58% of the patients had metabolic acidosis with a base excess below  $(-2)$  mmol L<sup>-1</sup> on arrival at the intensive care unit. There was a significant correlation between a strong ion difference and base excess ( $p < 0.01$ ). A significant improvement in metabolic acidosis was noted within the first 24 hours, from a base excess of  $(-2.49 \pm 2.8)$  to  $0.32 \pm 2.6$  mmol L<sup>-1</sup> ( $p < 0.001$ ). All of the improvement in the base excess is explained by a change in the strong ion difference from the mean value of  $31 \pm 4.3$  to  $34.2 \pm 3.6$  mmol L<sup>-1</sup> ( $p < 0.001$ ). Changes in the strong ion difference were primarily driven by changes in the serum sodium concentration, which were three-fold higher compared to those of chloride  $[-2.36 (\pm 2.6)$  mmol L<sup>-1</sup> ( $p < 0.001$ ) and  $0.84 (\pm 3.2)$  mmol L<sup>-1</sup>, respectively ( $p = 0.01$ )]. In conclusion, our data confirm that there is a direct correlation between a strong ion difference and base excess following on-pump cardiac surgery. The use of RL prevented significant hyperchloraemia, but did not eliminate the risk of strong ion difference-related metabolic acidosis. The change in the strong ion difference was primarily linked to perioperative changes in the serum sodium concentration.

To the Editor,

The perioperative use of normal saline is linked to hyperchloraemic or strong ion difference (SID)-related acidosis (1), with some evidence of detri-

mental effects, including prolonged hospital stay, increased incidence of wound infection, renal failure, increased number of ventilator days and prolonged requirement for intensive care (2-4). It has been

*Key words: cardiac surgery, metabolic acidosis, strong ion difference, lactated Ringer's solution, hyponatraemia*

## Mailing address:

Dr T. Jovaisa, Clinic of Anaesthesiology,  
Lithuanian University of Health Sciences,  
1 Eiveniu Street, Kaunas 50009, Lithuania  
Tel.: +370 373 26221 - Fax: +370 373 26371  
e-mail: tomas.jovaisa@nhs.net

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suggested that these electrolyte disturbances can be avoided by the use of balanced crystalloid solutions, including Lactated Ringer's Solution (RL) (5). However, this topic remains controversial, as, even though a number of publications have noted significant changes in SID following cardiac surgery, one recent publication questioned the link between SID and hydrogen ion concentration when using a balanced solution for infusions perioperatively (6).

Recent large cohort studies on major-surgery patients demonstrated that significant changes in perioperative plasma sodium concentrations are linked to increased morbidity and mortality (7–9). This is important because the large-volume infusions of RL during cardiac surgery are likely to affect plasma sodium levels, as this crystalloid is relatively hyponatraemic. Large volumes of RL infused perioperatively may dilute the sodium in blood plasma and acidify it, resulting in an adverse outcome after cardiac surgery.

In this study, our aim was to establish the effects of the routine use of RL on postoperative sodium and chloride levels, changes in SID and whether these changes result in metabolic acidosis. As pH is affected by both respiratory and metabolic components, we attempted to establish a link between an SID and base excess (BE), thus eliminating the potential compensatory effect of respiratory function (10).

## MATERIALS AND METHODS

An institutional database analysis of elective cardiac surgery cases in a tertiary referral university hospital was performed. Operations were defined according to the Society of Thoracic Surgeons (STS) type of surgery (coronary bypass surgery, replacement of the aortic and mitral valves, reconstruction of the mitral valve and a combination of these operations). Inclusion criteria: operations using cardiopulmonary bypass (CPB) where RL was the sole crystalloid solution used (including the CPB circuit, intra- and postoperative IV infusions). In order to minimise co-founding factors, cases in which postoperative lactate levels exceeded  $2 \text{ mmol L}^{-1}$  were excluded from the final analysis. Approval of the institutional ethics committee was obtained and all patients gave informed, signed consent to participate in the study.

Routine, standard surgical and anaesthetic techniques were used in all cases. CPB was performed according to the institutional protocol. The CPB circuit was primed with RL and Manitol solution was used as a priming additive. During the CPB, all patients were treated under moderate hypothermia ( $32\text{--}34^{\circ}\text{C}$ ), mean arterial pressure was kept above 60–70 mmHg and the blood flow was maintained at  $2.4 \text{ L min}^{-1} \text{ m}^{-2}$  of body surface area. Myocardial protection during the aortic cross-clamping period was achieved using minimally diluted tepid blood cardioplegia. Anticoagulation was achieved using an unfractionated heparin loading dose of  $300 \text{ IU kg}^{-1}$  and additional doses if needed to maintain an activated clotting time of 450–480 seconds. Heparin reversal was achieved using protamine sulphate. After surgery, patients were admitted directly to the intensive care unit (ICU) and weaned from mechanical ventilation according to the standard institutional protocol.

RL was the sole crystalloid solution used in this cohort of patients, including the bypass machine and intra- and postoperative IV infusions. This crystalloid is composed of 130 mEq of sodium ion, 109 mEq of chloride ion, 28 mEq of lactate, 4 mEq of potassium ion and 3 mEq of calcium ion in 1 L, amounting to an osmolality of  $273 \text{ mOsm L}^{-1}$ , and its composition is referred to as a balanced solution.

Arterial blood gases were analysed at the point of the patient's arrival to the ICU and 24 hours after surgery. The analysis was performed using the on-site arterial blood-gas analysis machine, RAPIDPoint 500 (Siemens, Germany). Sodium-chloride difference was used as a marker of SID. As pH is affected by both respiratory and metabolic components, correlations were made between an SID and BE, thus eliminating the potential compensatory effect of the respiratory function and evaluating only the metabolic component of the acidosis.

### *Statistical analysis*

Statistical analysis was performed using the SPSS statistical package. All values are presented as means with standard deviation. Pearson correlation and multivariate analysis were used to establish a link between a SID and BE. *P*-values of less than 0.05 were considered statistically significant and adjusted *R*-squared was assessed for the overall validity of the regression model. A paired samples *t*-test was used to compare the means between the two time points.

## RESULTS

Three hundred and fifty-eight consecutive surgeries were performed during the study period. Following exclusions for the type of surgery, crystalloid use, data completeness and lactate levels, 102 cases were included in the final analysis. All patients had positive intraoperative fluid balance ( $2726 \pm 1073$  ml), resulting in significant haemodilution as measured by significant reduction of haematocrit values from  $39.6 \pm 4$  to  $32.2 \pm 5$  ( $p < 0.001$ ). 95% of the transfused volume in the first 24 hours was RL. The proportion of non-crystalloid volume was not significant (Table I).

Low SID values and a high incidence of metabolic acidosis were recorded postoperatively in spite of the intraoperative use of RL. Fifty-eight per cent of patients had metabolic acidosis with a BE below  $(-2)$  mmol L<sup>-1</sup> on arrival in the ICU. Significant spontaneous improvement occurred within the first 24 hours. A correlation between changes in SID and BE was

significant ( $p < 0.01$ ); the adjusted *R*-squared value for multivariate analysis was 0.58, suggesting satisfactory validity of the model. All of the improvements in the BE are explained by the change in the SID, as the mean difference between the two time points was 2.8 mmol L<sup>-1</sup> for BE and 3.2 mmol L<sup>-1</sup> for the SID (Fig. 1).

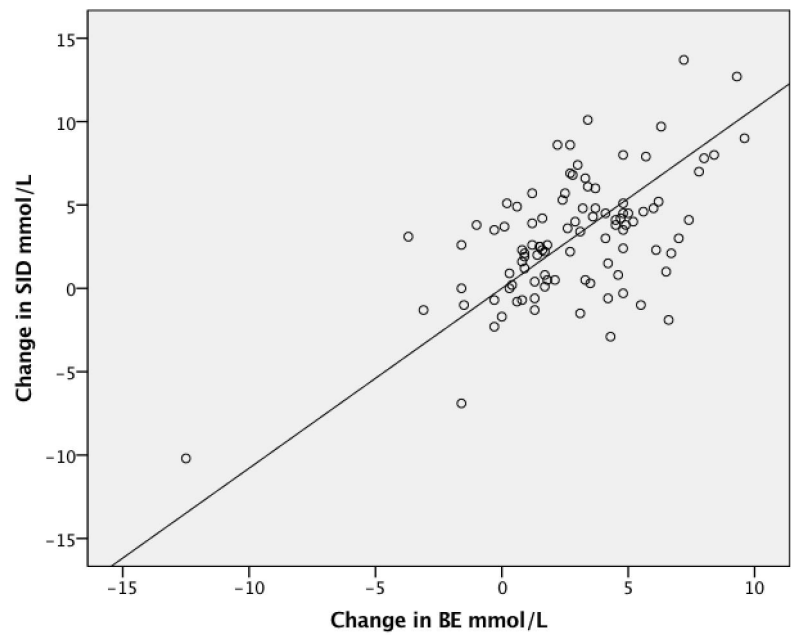
Changes in the SID were primarily driven by changes in the serum sodium concentration, which were three-fold higher compared to those of chloride [ $2.36 (\pm 2.6)$  mmol ( $p < 0.001$ ) and  $0.84 (\pm 3.2)$  mmol ( $p = 0.01$ ), respectively]. In cases where postoperative SID increased, reduction in plasma chloride concentration was less pronounced than increase in sodium (Fig. 2).

There was no difference in the pH between the two time points, but this could be explained by significantly lower postoperative mean pCO<sub>2</sub> level ( $41.3 \pm 6.7$  mmHg) compared with  $43.6 \pm 5.8$  mmHg at 24 hours ( $p = 0.003$ ).

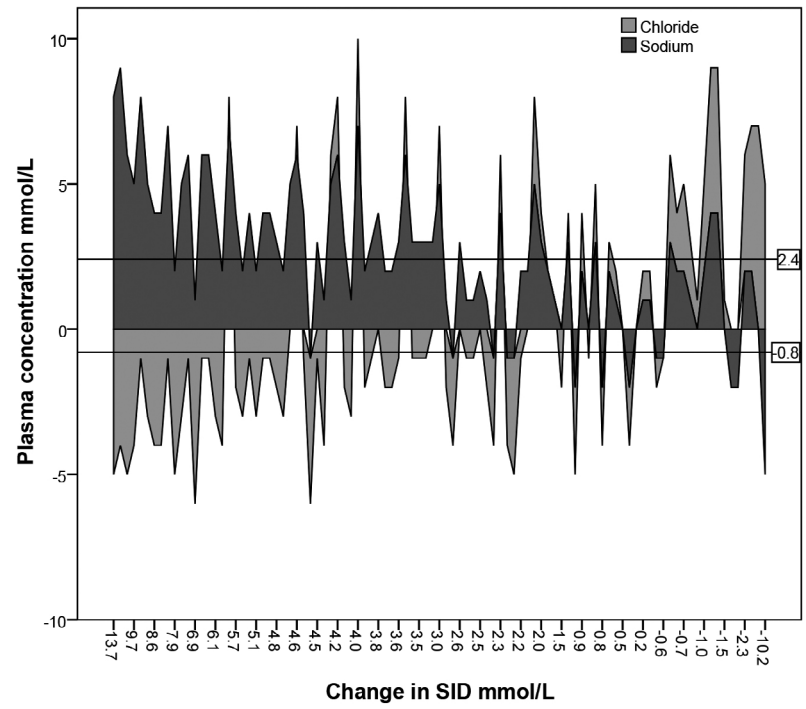
**Table I.** Summary of results.

| Measurement                                         | Postoperatively | After 24 hours | <i>P</i> -value |
|-----------------------------------------------------|-----------------|----------------|-----------------|
| Cumulative infusion volume (ml)                     | 2726±1074       | 5865±1073      |                 |
| Intraoperative transfusion of packed red cells (ml) | 209±401         |                |                 |
| Strong ion difference (mmol L <sup>-1</sup> )       | 31.0±4.3        | 34.2±3.6       | <0.001          |
| Base excess (mmol L <sup>-1</sup> )                 | (- )2.49 ± 2.8  | 0.32±2.6       | <0.001          |
| Sodium (mmol L <sup>-1</sup> )                      | 136.5±3.2       | 138.9±2.8      | <0.001          |
| Chloride (mmol L <sup>-1</sup> )                    | 105.5±4.3       | 104.7±3.4      | <0.01           |

*Values presented as means ± standard deviation*



**Fig. 1.** Correlation between the mean difference between the strong ion difference (SID) and the base excess (BE) at two time points.



**Fig. 2.** Sodium and chloride effect on a change in Strong Ion Difference (SID). Reference lines show mean changes in postoperative sodium and chloride concentrations.



## DISCUSSION

Metabolic acidosis following cardiopulmonary bypass is a well-known phenomenon. The postoperative base deficit in our study is comparable to that published previously (11). Many attempts have been made to identify the cause of this acidosis and introduce therapies that would allow modification of these effects. Significant changes in SID have been published previously and there has been some success in reducing acidosis by using modified pump priming solutions (11, 12). However, the total volume of prime fluids is significantly lower than the overall IV infusion volume and intraoperative fluid balance. In our study, the mean intraoperative fluid balance was more than 2.5 litres; even higher intraoperative IV infusion volumes have been reported in the literature (13). Therefore, the choice of intravenous fluid is likely to have a significant effect on the postoperative SID and acid–base balance. This statement correlates well with the results of previous studies evaluating the effect of intravenous fluids on perioperative acidosis that noted an increased risk associated with the administration of non-buffered fluids.

Hyperchloraemic acidosis is often used as a generic term to describe SID-related acid–base balance disturbances. We hypothesised that the perioperative use of a more physiologically balanced solution should result in lower postoperative chloride levels, less pronounced changes in the SID and subsequently less metabolic acidosis after on-pump cardiac surgery. Contrary to this, we have demonstrated that sodium changes may lead to acidosis and be more important than those of chloride. Lower sodium levels in the immediate postoperative period could be explained by dilution with RL, which contains 130 mmol L<sup>-1</sup> of sodium and results in relative hyponatraemia due to rapid RL infusion rates during surgery that frequently exceeds 500 mL/h. Interestingly, this trend has reversed and plasma sodium levels increased in the postoperative period in spite of ongoing infusion of RL. It is likely that compensatory sodium-handling mechanisms become relatively more efficient when a lower rate of postoperative RL infusions was used (14).

The acid–base effects of hyponatraemia in the immediate postoperative period may be easily overlooked due to haemodilution and respiratory compensation. Large volumes of RL are likely to dilute albumin and other weak acids, masking the true impact of electrolyte disturbance. Lower PaCO<sub>2</sub> levels in the immediate postoperative period are related to mechanical ventilation and increased CO<sub>2</sub> clearance resulting in additional respiratory compensation of metabolic acidosis. This is another important factor explaining why there was no difference in pH, whereas correlation with base excess was statistically significant. Persistent respiratory compensation after cessation of mechanical ventilation would indicate increased work of breathing, which may be detrimental in some patient groups, but significantly larger cohorts would be necessary to prove this.

The main limitation of this study is the retrospective design. However, due to the inclusion and exclusion criteria we were able to achieve a relative homogeneity in this group of patients. Furthermore, we excluded patients with abnormal postoperative lactate levels, therefore significantly reducing the risk that acid base changes were significantly affected by other cofounders, for example hypoperfusion.

The clinical effects of perioperative sodium changes had not been extensively investigated until very recently. Two large cohort retrospective studies demonstrated that both preoperative hyponatraemia and perioperative changes in plasma sodium concentration are independent predictors of increased mortality (7, 8). In spite of its limitations, our study adds to the increasing pool of evidence that perioperative changes in plasma sodium concentration are an important variable and its control may affect patient outcomes. Our data confirm that there is a direct correlation between an SID and BE following on-pump cardiac surgery. The use of RL prevented significant hyperchloraemia, but did not eliminate SID-related metabolic acidosis. A change in SID was primarily linked to perioperative changes in the serum sodium concentration.

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## LETTER TO THE EDITOR

## ROLE OF VITACAMPHORE IN IMPROVING CENTRAL PRO-INFLAMMATORY CYTOKINES FOLLOWING TRANSIENT GLOBAL ISCHEMIA

J. LIN, L. PANG, X-L. LIU and J. XING

*Department of Emergency Medicine, The First Hospital of Jilin University, Changchun, Jilin, China**Received August 31, 2016 – Accepted September 26, 2016*

The first two authors contributed equally to this work

**Pro-inflammatory cytokines (PICs) including interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) are responsive to ischemic stress. This process thereby modulates the responsiveness of many cell types under diseased conditions. The current study was to examine the role played by vitacamphore (VCP) in regulating the levels of PICs and protein expression of PIC receptors in the cerebral cortex and hippocampus of rats after cardiac arrest (CA)-induced transient global ischemia. CA was induced by asphyxia followed by cardiopulmonary resuscitation (CPR) in rats. ELISA and Western blot analyses were employed to determine PICs and their receptors in the cortex and hippocampus. Our results show that IL-1 $\beta$ , IL-6 and TNF- $\alpha$  were significantly elevated in the cortex and hippocampus after CA. This was accompanied with increasing of PIC receptors, namely IL-1R, IL-6R and TNFR1. Systemic injection of VCP attenuated amplification of PIC signal pathway in these brain regions. VCP also improved Neurological Severity Score and brain tissue edema in CA rats. Notably, VCP resulted in a significant increase in survival of CA rats as compared with controls. In conclusion, VCP is likely to play a beneficial role in modulating transient global ischemia induced by CA via PIC signal mechanisms.**

To the Editor,

Pro-inflammatory cytokines (PICs) i.e., interleukins, lymphokines and cell signaling molecules are released by numerous cells including leukocytes, myocytes, microglia and astrocyte cells (1). In particular, as mediators of immune and inflammatory reactions, interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) play an important role in responding to cerebral ischemic insults (2, 3). These PICs modulate the responsiveness of many cell types in a number of diseases. During diseased states, PICs can recruit cells to inflammatory sites and alter cell survival, division, proliferation and differentiation (4).

A rat model of asphyxial cardiac arrest (CA)

followed by cardiopulmonary resuscitation (CPR) has been widely used to study transient global ischemia. Prior studies have demonstrated that hypoxia inducible factor-1 (HIF-1) subtype HIF-1 $\alpha$  is expressed in the brain tissues, including the cerebral cortex and hippocampus, and is engaged in neuronal apoptosis after induction of global ischemia (5, 6). In particular, our recent study using this model further demonstrated that CA increases IL-1 $\beta$ , IL-6 and TNF- $\alpha$  and upregulates their receptors IL-1R, IL-6R and TNFR1 in the hippocampus (7). Systemic administration of ML228, an activator of HIF-1 $\alpha$  (8), attenuates these exaggerated PIC signal pathways (7) and, as a result, HIF-1 $\alpha$  improves neuronal apoptosis and neurological deficits induced by CA (9).

*Key words: vitacamphore, cytokines, cardiac arrest, cardiopulmonary resuscitation, cortex, hippocampus*

Mailing address:

Dr. Jihong Xing,  
Department of Emergency Medicine,  
The First Hospital of Jilin University,  
71 Xinmin Street, Changchun, Jilin 130021, China  
e-mail: jhxing79@gmail.com

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Vitacamphore (VCP) is an aqueous solution of *trans*- $\pi$ -oxocamphor (10), and intravenous injection of VCP has been reported to improve hypoxic and ischemic injuries due to cardiac and circulatory failure in patients (11, 12). Nevertheless, the underlying mechanisms leading to the effects of VCP are lacking. In light of the effective signal pathways responsible for VCP in response to ischemic conditions following CA, in the present study, we examined the role played by VCP in regulating PIC mechanisms in rat cerebral cortex and hippocampus after CA-induced global ischemia. We hypothesized that VCP plays a beneficial role in modulating the levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  and protein expression of their receptors IL-1R, IL-6R and TNFR1 in these brain regions, thereby leading to improvements of neurological deficits and survival.

## MATERIALS AND METHODS

### *Animals*

All the animal procedures were approved by the Institutional Animal Care & Use Committee of Jilin University, which were in compliance with the Guideline for the Care and Use of laboratory Animals of the U.S. National Health Institute. Male Sprague–Dawley rats (200–300g) were used in our experiments.

### *Transient global ischemia*

The ischemia was produced in the CA model induced by asphyxia as described in our previous publications (7, 9). Briefly, rats were anesthetized with an isoflurane-oxygen mixture (2-5% isoflurane in 100% oxygen). An endotracheal tube was first inserted and attached to a ventilator. The ventral tail artery was cannulated to monitor systemic arterial pressure. The right jugular vein was cannulated for a continuous infusion of saline (at a rate of 0.1 ml/hour) to maintain baseline blood pressure and fluid balance. Body temperature was continuously monitored and maintained at 37°C with a heating pad and external heating lamps. Asphyxia was induced by stopping mechanical ventilation and clamping the tracheal tubes at the end of expiration. Resuscitation efforts began 6 min following induced

CA. For this purpose, rats were orotracheally intubated for mechanical ventilation accompanied by chest compression delivered by a mechanical compressor at a rate of 200/min for 5 min. Once spontaneous heartbeat returned, epinephrine (2  $\mu$ g) was administered to achieve a mean arterial blood pressure of >80 mmHg. Ventilation was adjusted for animals to regain spontaneous respiration and achieve normoxia. The animals that survived this procedure were used for further interventions. For pain relief those rats received analgesic buprenorphine (0.02 mg/kg) subcutaneously immediately and every 6 hours for a 72-hour period after the asphyxial procedures. In the sham operated animals, the same surgical procedures were performed without cardiac arrest and resuscitation.

### *Study interventions*

The rats were divided randomly. Group #1 (n=20): the rats received the same surgical procedures and endotracheal intubation was performed with no asphyxia and CPR. Group #2 (n=19): CA and CPR were performed and 0.5 ml of saline (i.p., daily) was given after CA. Group #3 (n=28): CA and CPR were carried out and 3 mg/kg body weight of VCP (i.p., daily) was injected after CA. At the end of each experiment, the rats were sacrificed by an overdose of isoflurane (>5%). The brains were then taken out for biochemical measurements. Note that the cerebral cortex and hippocampus CA1 region were used in this study.

### *ELISA measurements*

Samples were taken of all the tissues from individual rats for the analysis. In brief, the cortex and hippocampus of the rats was removed. Total protein was then extracted by homogenizing the cortex and hippocampus sample in ice-cold immunoprecipitation assay buffer with protease inhibitor cocktail kit. The lysates were centrifuged and the supernatants were collected for measurements of protein concentrations using a bicinchoninic acid assay reagent kit.

The levels of PICs were examined using an ELISA assay kit (Promega Co. Madison, WI, USA) according to the provided description and modification. Briefly,

polystyrene 96-well microtiter immunoplates were coated with affinity-purified rabbit anti-IL-1 $\beta$ , anti-IL-6 and anti-TNF- $\alpha$  antibodies. Parallel wells were coated with purified rabbit IgG for evaluation of nonspecific signal. After overnight incubation, plates were washed. After this, the diluted samples and these PIC standard solutions were distributed in each plate. The plates were washed and incubated with anti-IL-1 $\beta$ , IL-6 and TNF- $\alpha$  galactosidase. The plates were then washed and incubated with substrate solution. After incubation, the optical density was measured using an ELISA reader.

#### *Western blot analysis*

After being denatured by heating at 95°C in an SDS sample buffer, the supernatant samples were loaded onto Mini-Protein TGX Precast gels and electrically transferred to a polyvinylidene fluoride membrane. The membrane was then incubated overnight with primary antibodies: rabbit anti-IL-1R, anti-IL-6R and anti-TNFR1 (Abcam Co., diluted 1:200). After being fully washed, the membrane was incubated with horseradish peroxidase-linked anti-rabbit secondary antibody and visualized for immunoreactivity. The membrane was stripped and incubated with mouse anti- $\beta$ -actin to show equal loading of the protein. The bands recognized by the primary antibody were visualized by exposure of the membrane onto an X-ray film. Then, the film was scanned and the optical densities of IL-1R, IL-6R, TNFR1 and  $\beta$ -actin bands were determined using the Scion Software. Then, values for densities of immunoreactive bands/ $\beta$ -actin band from the same lane were determined. Each of the values was afterwards normalized to a control sample.

#### *Neurological function and brain edema*

The modified method of Neurological Severity Score (mNSS) was used to examine neurological functions in this study. Note that mNSS is generally used to assess a combination of motor, sensory, and balance functions (13). Neurological function was graded on a scale of 0–18 (normal score, 0; maximal deficit score, 18). If we found that mNSS score of rats was > 0 before CA, this indicated that the rats were abnormal and they were excluded from the

experiment.

Brain edema (brain water content) was determined 72 h after CA. The brain slices (2 mm thick) of the hemispheres and cerebellum were cut. The whole brain water content was calculated from all slices (13). The brain slices were weighed to immediately obtain the wet weight and dried in an oven at 100 °C for 24 h to obtain the dry weight. The cerebellum was used as the internal control. The water content was expressed as the following formula: [(wet weight) – (dry weight)]/(wet weight)  $\times$  100%.

#### *Statistical analysis*

Experimental data were analyzed using one-way repeated measures analysis of variance (ANOVA). As appropriate, Tukey's post hoc tests were used. All values were presented as mean  $\pm$  SEM. For all analyses, differences were considered significant at  $P < 0.05$ . All statistical analyses were performed using SPSS for Windows version 20.0.

## RESULTS

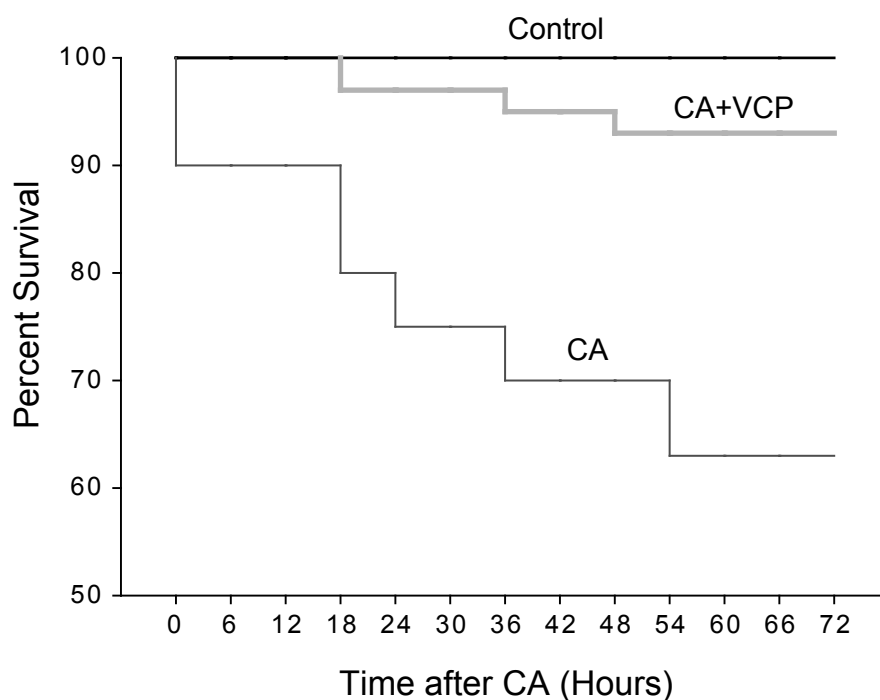
#### *Animal survival*

When CA rats received VCP treatment, the survival rate was significantly increased during 72 h compared to CA rats with saline injection ( $P = 0.0093$ ). The survival rate of 72 h was 100% (20/20 rats) in sham control rats; 63% (19/30 rats) in CA rats; 93% (28/30 rats) in CA rats injected with VCP (Fig. 1). Data obtained from those survival rats were included for the analysis in this report.

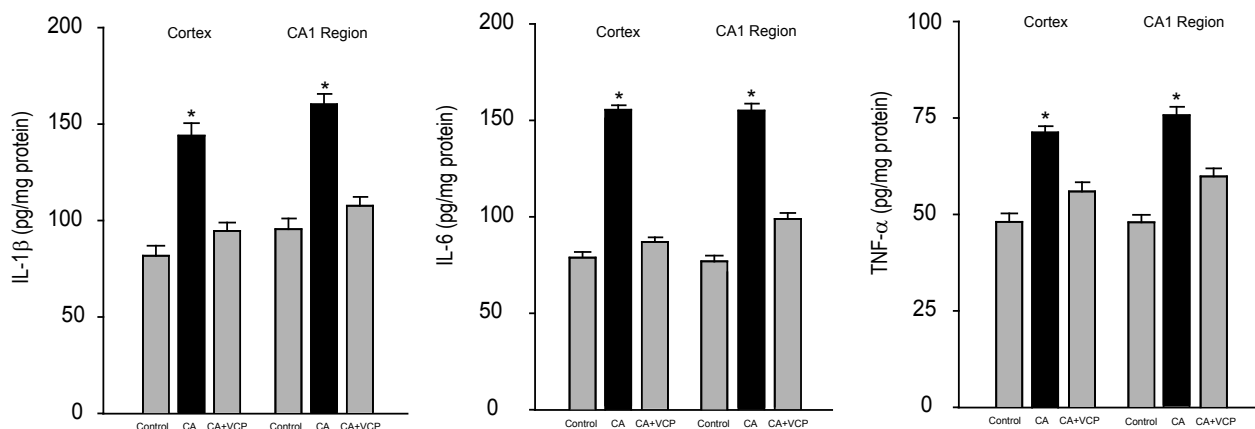
#### *Levels of PICs and their receptors expression*

As shown in Fig. 2, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  were increased in the cortex and hippocampus 72 hours after CA ( $P < 0.05$  vs control,  $n=20$  in control and  $n=19$  in CA group). Administration of VCP significantly attenuated the amplified levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  ( $P < 0.05$  vs no treatment;  $n=28$  in group: CA plus VCP). Likewise, induction of CA increased the protein expression of IL-1R, IL-6R and TNFR1 in the cortex and hippocampus ( $P < 0.05$ , control rats vs. CA rats;  $n=8-15$  in each group). When VCP was injected, enhancement in expression of these PIC receptors was significantly attenuated in CA rats (Fig. 3 A and B).

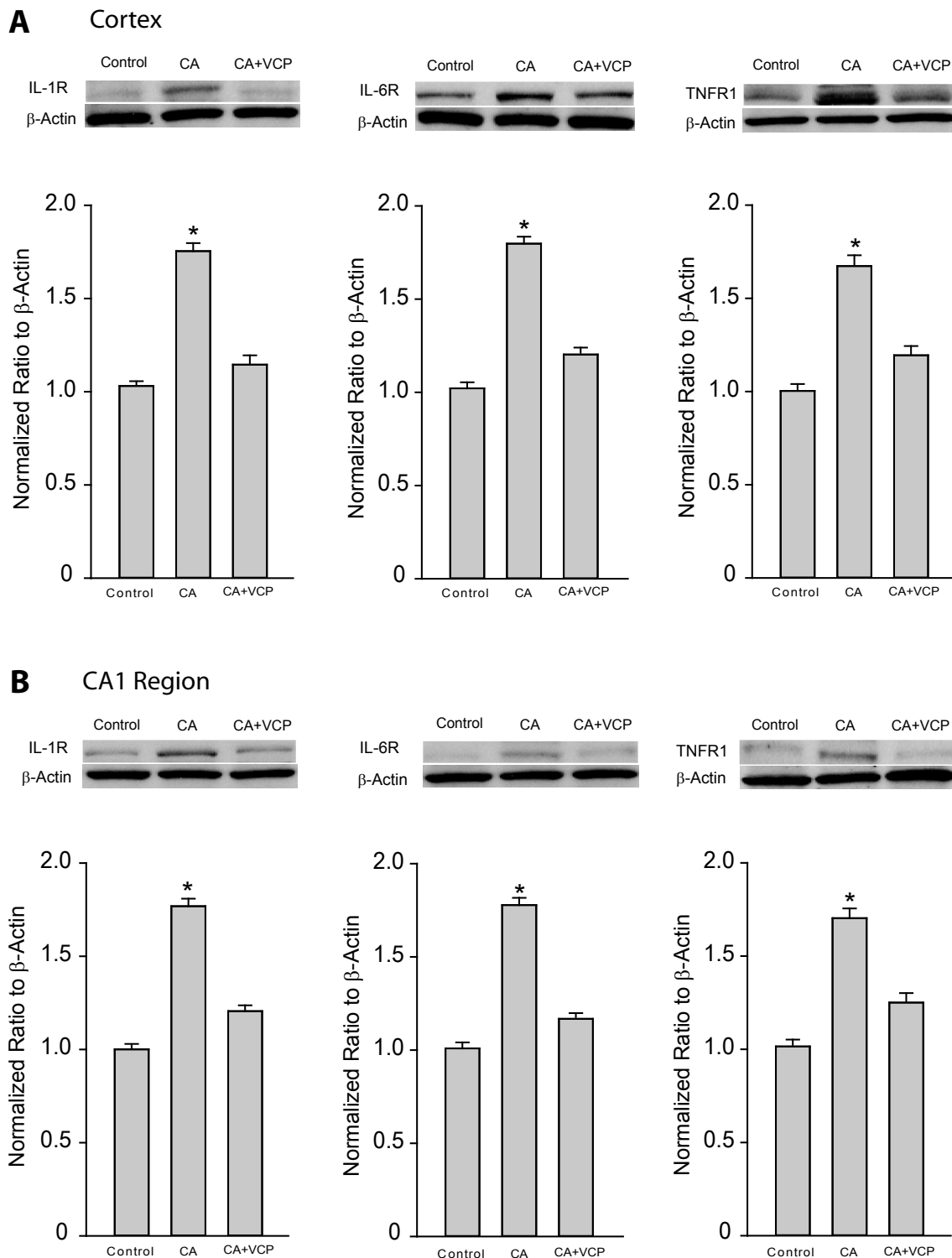




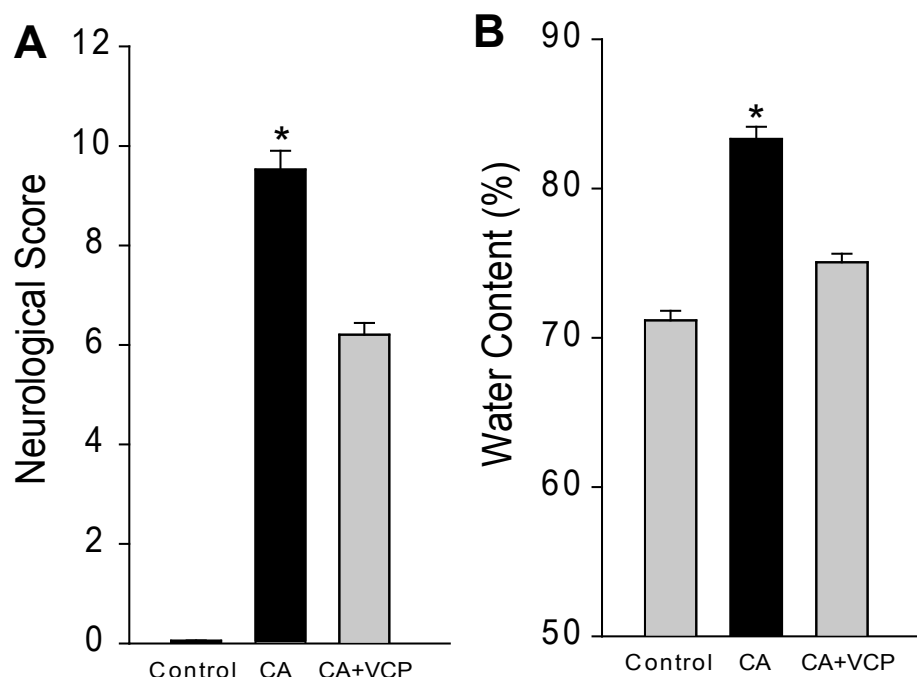
**Fig. 1.** Survival was significantly increased during 72 hours when CA rats received VCP treatment as compared with CA rat with saline injection. The survival rate of 72 hours was 63% in CA rats with no treatment as compared with 93% in CA rats injected with VCP ( $P=0.0093$  vs CA rats with no treatment).



**Fig. 2.** Induction of CA amplified the levels of PICs in the cortex and hippocampus. Systemic administration of VCP attenuated increases of PICs in rats 72 hours after CA. \* $P < 0.05$  vs control rats and CA rats injected with VCP. The number of rats = 20 in control; = 19 in CA group and = 28 in CA group with VCP.



**Fig. 3.** The protein expression of IL-1R, IL-6R and TNFR1 in the cortex (A) and hippocampus (B) tissues was increased in rats 72 hours after CA. VCP blunted the upregulation of PIC receptors induced by CA. (A, B): Top panels indicate representative bands and bottom panels indicate averaged data. \* $P < 0.05$  vs control and CA rats with administration of VCP. The number of rats = 8-15 in each group.



**Fig. 4. A, B)** The mNSS and water content of brain tissues were increased in rats 72 hours after CA and they were improved by administration of VCP. \* $P < 0.05$  vs control rats and CA rats with VCP. The number of rats = 20 in control; = 19 in CA group and = 28 in CA group with VCP.

#### Neurological function and brain edema

CA led to increases in the mNSS in rats ( $P < 0.05$ , control rats vs CA rats;  $n = 20$  in control and  $n = 19$  in CA group) (Fig. 4A). VCP significantly attenuated the impaired mNSS in animals 72 hours following CA ( $P < 0.05$ , CA rats with no treatment vs CA rats with VCP;  $n = 28$  in group: CA plus VCP) (Fig. 4A). Similarly, induction of CA amplified water content of brain tissues in rats and administration of VCP significantly attenuated increases of water content (Fig. 4B).

#### DISCUSSION

VCP is an oxidized form of camphor derivative (oxo-camphor) and camphor or its derivatives have been reported to have the regulatory effects on blood flow in the central and peripheral tissues (14-16). Recent studies also suggest that oxidized camphor is favorable to patients with heart failure

by modulating the autonomic system and it can also improve myocardial fibrosis in rats (11, 12, 17).

PICs, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  are responsive to global ischemic stress (3), which is likely to modulate the responsiveness of many cell types in a number of diseases. For instance, during diseased states, PICs can recruit cells to inflammatory sites and modulate cell survival, division, proliferation and differentiation (4). Using a rat model we have previously shown that CA increases PICs including IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in the hippocampus CA1 region (7). CA also augments a representative nucleus transcription factor indicating cell apoptosis Caspase-3, which is linked to upregulation of PIC signal pathway. In the current study, we found that systemic injection of VCP attenuates amplification of PIC levels and their receptor expression in the cerebral cortex and CA1 region of ischemic rats. Importantly, VCP improves Neurological Severity Score, brain tissue edema and survival in CA

rats. Data of the current study specifically suggest a beneficial role played by VCP in modulating transient global ischemia induced by CA via PIC signal mechanisms.

During the occurrence of CA, blood flow and oxygen delivery are abruptly halted, which lead to systemic ischemic injury in various organs including brain (18). Although CPR is applied, inadequate blood flow and tissue oxygen delivery still persist due to myocardial dysfunction, hemodynamic instability and microvascular dysfunction. In response to hypoxic stress, HIF-1 $\alpha$  is synthesized and the expression of its downstream product such as VEGF is upregulated and remains elevated (5, 19). This contributes to neuroprotection against brain ischemic conditions by improving the permeability of blood brain barrier, reducing brain edema formation and promoting the recovery of brain injuries (20). Our recent study has shown that stabilizing HIF-1 $\alpha$  significantly attenuates increases of Caspase-3 and terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL, labeling of apoptotic cell) evoked by induction of CA (9). As a result, this process improves neurological deficits and neuronal edema observed in rats after CA. We have also shown that activation and maintenance of HIF-1 $\alpha$  attenuate upregulation of central nervous PIC pathways in rats with transient global ischemia (7). Data of our current study further provide evidence suggesting that inhibition of PIC signal pathway is likely involved in neuroprotective effects and attenuating damages evoked by global cerebral ischemia.

In summary, transient global ischemia induced by CA increases the levels of PICs and their receptors in the cortex and hippocampus. Systemic administration of VCP can attenuate amplified expression of PIC signal pathway in ischemic animals and thereby improve Neurological Severity Score, brain tissue edema and survival. Our data indicate the beneficial role played by VCP in alleviating cerebral ischemia reperfusion injury via PIC mechanisms.

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## LETTER TO THE EDITOR

## EFFECTS OF TWO DIFFERENT ANESTHETIC METHODS ON CELLULAR IMMUNITY OF PATIENTS AFTER LIVER CANCER RESECTION

Y. XU<sup>1</sup>, Y. SUN<sup>2</sup>, H. CHEN<sup>1</sup>, Y. WANG<sup>1</sup> and GN. WANG<sup>1</sup>

<sup>1</sup>Department of Anesthesiology, Cancer Hospital of Harbin Medical University, Harbin, Heilongjiang, China; <sup>2</sup>Department of Anesthesiology, Heilongjiang Province Hospital, Harbin, Heilongjiang, China

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**This study aims to investigate the effects of epidural block in combination with general anesthesia and general anesthesia alone on the immune function of patients undergoing primary liver cancer resection. Twenty-four patients with liver cancer who received treatment in the Third Hospital of Harbin Medical University, Heilongjiang, China, were enrolled and randomly allocated into group A and group B, with 12 in each group. The data on the T lymphocyte subpopulation, pro-inflammatory cytokines and anti-inflammatory cytokines were recorded before, immediately after and 24 h after liver cancer resection to compare differences and changes. It was found that CD4<sup>+</sup> of patients who underwent combined anesthesia decreased after surgery and CD8<sup>+</sup> of those patients was lower than that of the general anesthesia group. The content of interleukin (IL)-10 of patients who underwent general anesthesia combined with epidural block showed a decreasing tendency immediately after surgery, but increased 24 h after surgery, and the increase was greater than that of the general anesthesia group; furthermore, those patients had lower levels of IL-1 $\beta$  and interferon (IFN)- $\gamma$ . In addition, patients who underwent epidural block and general anesthesia had a higher ratio of IFN- $\gamma$  to IL-4 (Th1/Th2). These findings suggest that general anesthesia combined with epidural block has little passive influence on the cellular immunity of the body and can be selected as an anesthetic approach for patients with liver cancer.**

To the Editor,

The occurrence of liver cancer is a complex process restrained by many factors (1). The treatment of liver cancer focuses on radical resection, however, surgery inhibits the immune function of the human body (2). Modern tumor immunology has shown that the possibility of tumors occurring is higher than usual when the immune functions are weak (3). The effects of diverse anesthetic methods on the immunity of patients vary (4).

Roggin et al. (5) believed that the type of anesthesia could affect the functions of T cells and

have long-lasting effects which have an unfavorable influence on immune functions. Two anesthetic methods, epidural block in combination with general anesthesia and general anesthesia only, were investigated in the present study.

Epidural block in combination with general anesthesia entails blocking the spinal nerve root by injecting local anesthetic through the epidural space; after injection, temporary paralysis occurs in the areas controlled and dominated by the spinal nerve root. General anesthesia involves inhaling anesthetics through the respiratory tract or injecting through

*Key words: anesthetic method, liver cancer resection, cellular immunity*

*Mailing address:*

Guonian Wang, Department of Anesthesiology,  
Cancer Hospital of Harbin Medical University,  
No. 150, Haping Road,  
Harbin, Heilongjiang, 150081, China  
e-mail: xuyang93@yahoo.com

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veins or muscles to induce temporary paralysis in the central nervous system (6, 7). Gui JB et al. (8) considered that general anesthesia combined with epidural block could effectively reduce the intensity of stress, avoid the risks of respiratory inhibition induced by excessive analgesics, and reduce the incidence of postoperative complications. The content of T lymphocyte subpopulation changes due to the inhibitory effect of tumors on the immunity of the body.

T lymphocyte subpopulation, which is composed of CD4<sup>+</sup> subpopulation and CD8<sup>+</sup> subpopulation, is an important basis for reflecting the immune level of the body; CD4<sup>+</sup>/CD8<sup>+</sup> can reflect whether cellular immunity is balanced (9). It has been found that the significant reduction of the content of CD4<sup>+</sup>, the remarkable increase of the content of CD8<sup>+</sup> and the decline of CD4<sup>+</sup>/CD8<sup>+</sup> in the peripheral blood of tumor patients suggested immunosuppression (10). Proinflammatory cytokines, including interferon-1 $\beta$  (IL-1 $\beta$ ) and IFN- $\gamma$ , and anti-inflammatory cytokines, including transforming growth factor (TGF)- $\beta$ , IL-10 and IL-4, can all reflect the immune condition of patients (11). IL-1 $\beta$ , a proinflammatory cytokine, is generated in the activated mononuclear phagocytes. IFN- $\gamma$  is produced by antigen-activated CD4<sup>+</sup> and CD8<sup>+</sup> in immune response. IL-4 and IL-10 are secreted by Th2 cells. IL-10 can inhibit the proliferation of CD4<sup>+</sup> as well as IFN- $\gamma$ .

This study randomly divided 24 patients with liver cancer into an epidural block in combination with general anesthesia group and a general anesthesia group. The cellular immune condition of the patients was analyzed through comparison. The appropriate anesthetic methods were selected for different patients, aiming to improve the immunity of patients undergoing liver cancer resection.

## MATERIALS AND METHODS

### *Research subjects*

Twenty-four patients with liver cancer who received treatment in the Third Affiliated Hospital of Harbin Medical University, Heilongjiang, China, from January 2016 to June 2016 were selected. The subjects developed no other systemic diseases and had no family history.

They were randomly allocated into two groups, i.e., group A (epidural block in combination with general anesthesia group) and B (general anesthesia), 12 in each group. All the patients underwent laparoscopic surgery. The experiments involved in this study were reviewed by the medical ethics committee. The patients signed informed consent and the study was verified by pathologists. The clinico-pathological data of the patients are shown in Table I.

### *Reagents and instruments*

Enzyme-linked immunosorbent assay kit (Shanghai Meilian Biochemical Co., Ltd., China), UniCel DxI 800 chemical luminescence immunity analyzer (Beckman Coulter Commercial Enterprise Co., Ltd., USA), Synergy H1 microplate reader (BioTek Instrument Co., Ltd., USA), E4 XLS+ multichannel pipettor (Mettler Toledo Group, Switzerland), TS-2102 constant temperature shaker (Shanghai Jiecheng Laboratory Apparatus Co., Ltd., China), Sorvall T1 series centrifuge (Thermo Fisher Scientific Co., Ltd., Germany), CytoFLEX flow cytometry (Beckman Coulter Commercial Enterprise Co., Ltd., USA), anticoagulant tube, reagent storage tank, 1L measuring cylinder and washing bottle (Suzhou Asia-Pacific Chemical Glass Instrument Co., Ltd., China) were used.

### *Experimental methods*

Patients in group A and B took no other drugs before surgery. Patients in group A underwent general anesthesia in combination with epidural block. Firstly, an intervertebral space puncture was performed. Peridural catheter was placed towards the head, and 3 mL of 2% lidocaine was injected. Temperature discrimination method was used to test whether the block plane reached T4-T12. Propofol was used as the general anesthesia in both groups, with an initial plasma targeted concentration of 4  $\mu$ g/mL by means of target controlled infusion; in addition, 3  $\mu$ g/mL of fentanyl and 0.02 mg/kg cisatracurium were injected in a single dose. After tracheal intubation, the anesthesia was maintained through intravenous inhalational anesthesia. The concentration of exhalant sevoflurane was controlled at 1-2%. The concentration of propofol in target-controlled infusion was controlled at 2-3  $\mu$ g/mL. Remifentanyl was injected constantly in a speed of 0.1  $\mu$ g/kg every minute, and cisatracurium was discontinuously supplemented.

Patients in group A were epidurally injected with 10

**Table I.** *Clinico-pathological data.*

| Group | Male : female | Age(year) | Height (cm) | Weight (kg) | Inheritance history |
|-------|---------------|-----------|-------------|-------------|---------------------|
| A     | 7:5           | 46.5±18.5 | 173±12.2    | 65±18       | None                |
| B     | 8:4           | 45±17.5   | 174±11.7    | 66±19       | None                |

mL of 0.375% ropivacaine + 1% lidocaine 20 min before surgery. The drugs were infused at a speed of 5 mL/h using a micropump. Patients in group B were intravenously injected with 0.1 g of fentanyl 5 min before surgery. The basic infusion speed of crystoloid solution during surgery was 10 mL/kg per hour.

10 ml of peripheral venous blood samples were collected using an anticoagulant tube before anesthesia, immediately after surgery, and 24 h after surgery. 5 ml was taken for the detection of CD4<sup>+</sup> and CD8<sup>+</sup>; the other 5 ml was centrifuged at 3000 r/min. The separated upper layer plasma was transferred into a reagent storage tank (-80°C) for the detection of TGF- $\beta$ , IFN- $\gamma$ , IL-4, IL-1 $\beta$  and IL-10.

#### *Detection of CD4<sup>+</sup> and CD8<sup>+</sup>*

Two fresh blood samples (100  $\mu$ l each) which had been processed by Ethylene Diamine Tetraacetic Acid were transferred to two tubes. Then 20  $\mu$ l of anti-CD4-FITC and anti-CD8-PE were added. The mixture was cultivated at room temperature for 30 min, followed by another 10 min after the addition of 2 ml of hemolysin.

The supernate was removed after centrifugation at 1000 r/min for 5 min. Then 2 ml of phosphoric acid solution was added for one time of washing. Then the solution was centrifuged again at 1000 r/min for 5 min and the supernate was removed. After the addition of 250  $\mu$ l of PBS, the mixture was mixed by shaking and analyzed by flow cytometry. Finally, the detection results of CD4<sup>+</sup> and CD8<sup>+</sup> were obtained. Before analysis, the flow cytometry was automatically corrected using FAGS Comp.

#### *Data processing*

Data obtained in the experiment were analyzed using SPSS ver. 17.0 and expressed as mean  $\pm$  standard

deviation. The comparison of ratio was processed by *Chi*-squared test. The comparison within groups at different time points was performed using one-way analysis of variance.  $p < 0.05$  suggested differences had statistical significance.

## RESULTS

### *T lymphocyte subpopulation*

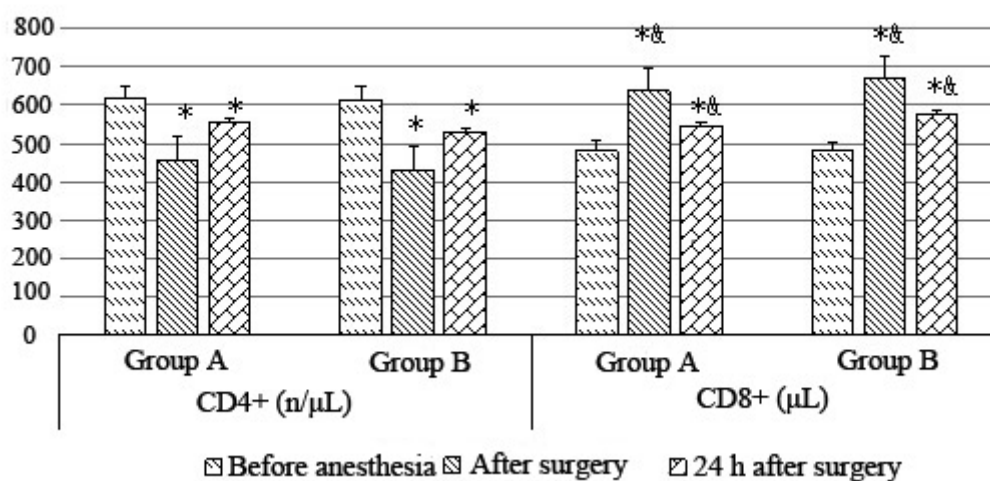
There was no remarkable difference between the two groups before anesthesia ( $p > 0.05$ ). CD4<sup>+</sup> immediately after surgery and 24 h after surgery was less than that before anesthesia ( $p < 0.05$ ). CD8<sup>+</sup> of the two groups increased compared to that before anesthesia ( $p < 0.05$ ). At the same time point, the CD4<sup>+</sup> of group A was higher than that of group B ( $p < 0.05$ ), but group A had lower concentration of CD8<sup>+</sup> compared to that of group B ( $p < 0.05$ ) (Fig. 1).

### *Pro-inflammatory factors*

IL-1 $\beta$  of group A and B firstly increased and then decreased with the passing of time, and IFN- $\gamma$  showed an increasing tendency. IL-1 $\beta$  in group B immediately after surgery was significantly different to that before anesthesia. Although IFN- $\gamma$  in group A was lower than that of group B, the difference had no statistical significance (Fig. 2).

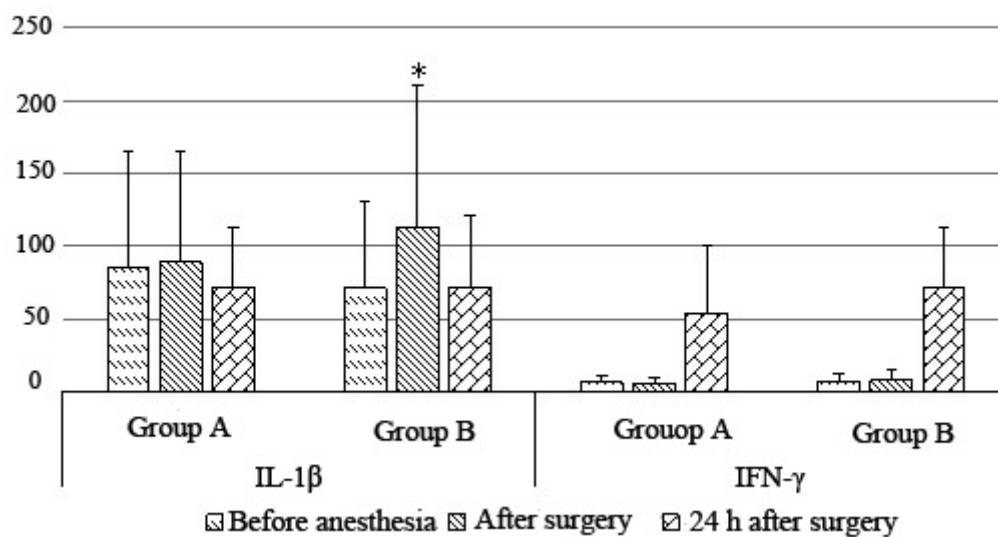
### *Anti-inflammatory factors*

As shown in Fig. 3, IL-4 firstly decreased and then increased; immediately after surgery, IL-4 in group A was significantly different to before anesthesia ( $p < 0.05$ ), and the difference of IL-4 immediately after surgery and 24 h after surgery had no statistical



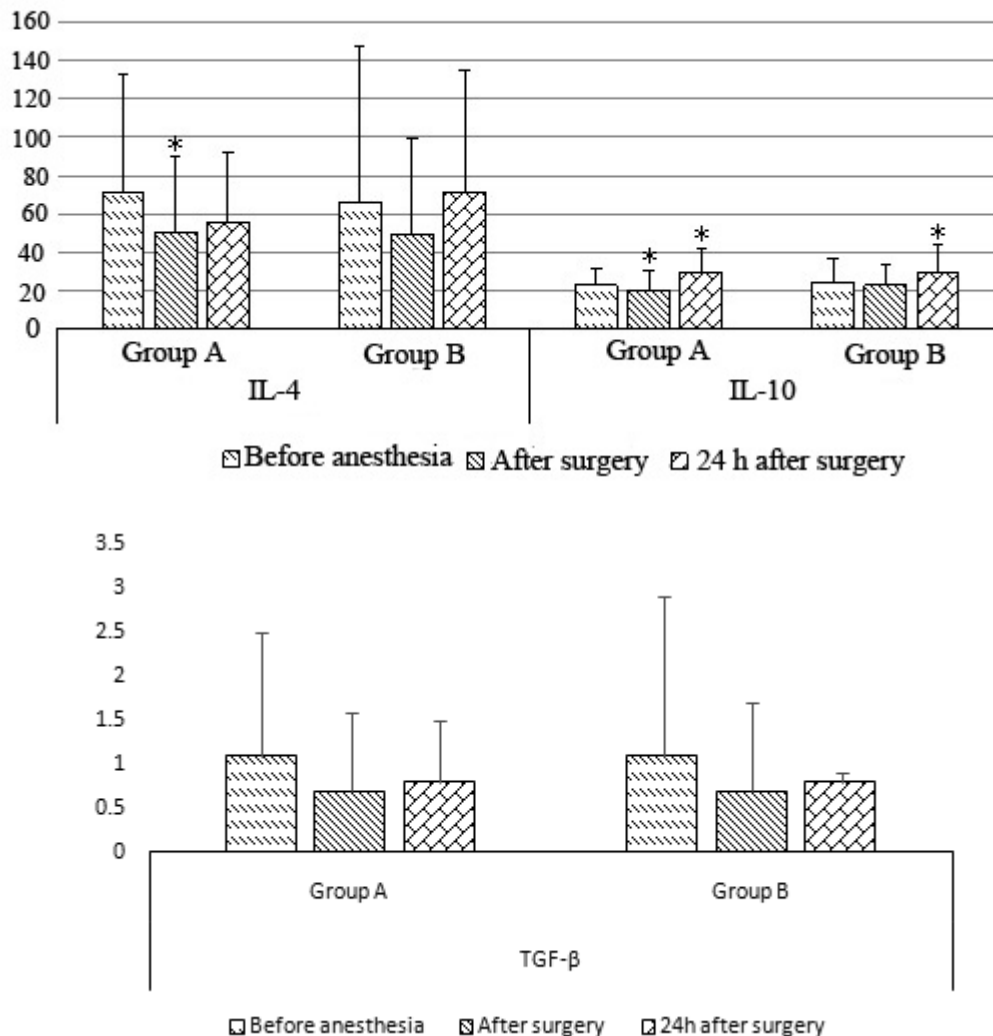
**Fig. 1.** Changes of the levels of T lymphocyte subpopulation

\*  $p < 0.05$  compared to before anesthesia; &  $p < 0.05$  compared to group A.



**Fig. 2.** Changes of the levels of proinflammatory cytokines.

\*  $p < 0.05$  compared to before anesthesia



**Fig. 3.** Changes of the levels of anti-inflammatory cytokines.

\*  $p < 0.05$  compared to before anesthesia

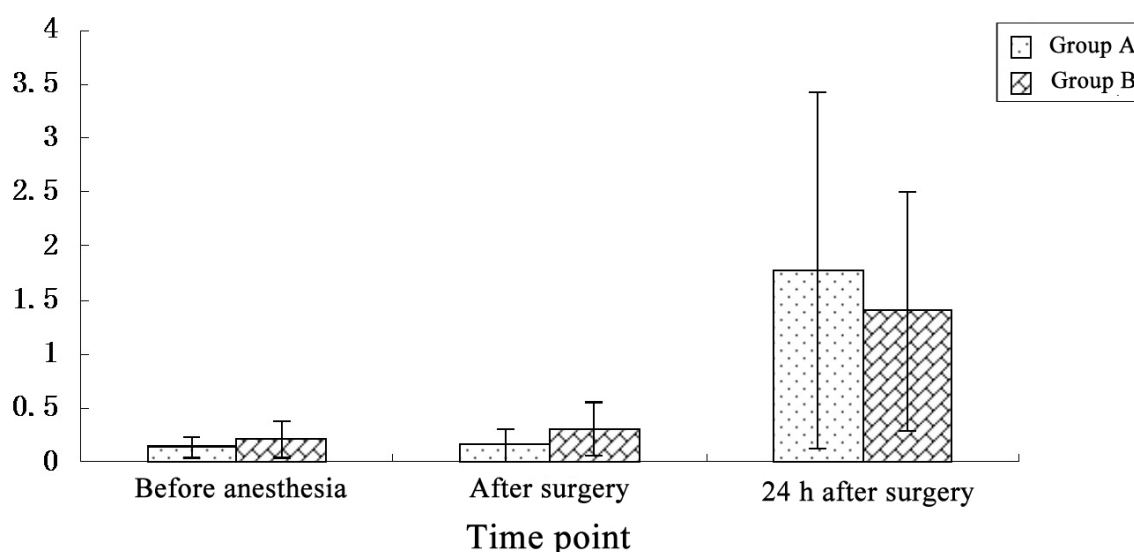
significance; IL-4 in group B before anesthesia and 24 h after surgery had no remarkable difference; the level of IL-10 of both groups declined firstly and then rose; the level of IL-10 of both groups showed an obvious increase 24 h after surgery compared to before anesthesia ( $p < 0.05$ ); the level of IL-10 of group A showed an obvious decreasing tendency after surgery ( $p < 0.05$ ), while the level of IL-10 of group B before anesthesia and after surgery had no remarkable difference. The level of TGF- $\beta$  of both groups rose firstly and then decreased. The level of two groups had no significant difference before

surgery ( $p > 0.05$ ) and there was still no significant difference immediately after surgery nor 24 h after the surgery ( $p > 0.05$ ). The level of TGF- $\beta$  before anesthesia was not remarkably different with that at the other time points, indicating that the effect of anesthetic methods on TGF- $\beta$  anti-inflammatory cytokines of patients was slight.

#### *IFN- $\gamma$ /IL-4 (Th1/Th2)*

As shown in Fig. 4, the IFN- $\gamma$ /IL-4 (Th1/Th2) of groups A and B showed an increasing tendency with the passing of time. The IFN- $\gamma$ /IL-4 (Th1/Th2)





**Fig. 4.** Changes of IFN- $\gamma$ /IL-4 (Th1/Th2).

of patients in group A was lower than that of group B, but 24 h after surgery, the IFN- $\gamma$ /IL-4 (Th1/Th2) of group A was gradually approximate to and even exceeded that of group B; the IFN- $\gamma$ /IL-4 (Th1/Th2) of group A was remarkably higher than that of group B 24 h after surgery, and 24 h after surgery was significantly different to that before anesthesia ( $p < 0.05$ ); group A and group B had no remarkable differences at the same time point.

## DISCUSSION

In operations on liver, surgery and anesthesia can both result in the decrease of coagulation function. In the process of anesthesia, the stress reaction of blood vessels is the reason for unstable circulation and is also one of the risk factors of coagulation (12). In addition, anesthesia can produce some effects on the immune system of patients, including anesthesia-induced overreaction (13), suggesting the selection of an appropriate anesthesia method is quite crucial. Opinions on whether to adopt epidural block in combination with general anesthesia or not are divided. Shontz R. et al. (14) thought that epidural anesthesia could increase the risks of coagulation

disorder as well as blood transfusion volume. Few studies concern the effect of epidural block on the immune functions of patients after surgery.

The amount of CD4<sup>+</sup> is an important index for reflecting cellular immunity level. CD8<sup>+</sup> is an incipient anti-infection immune cell. These two types of cells maintain the normal operation of the immune system through mutual regulation (15). Immune function inhibition is mainly reflected by the decrease of CD4<sup>+</sup> and the increase of CD8<sup>+</sup> (16). This study found that the patients who underwent general anesthesia combined with epidural block had reduced CD4<sup>+</sup> levels, but the CD8<sup>+</sup> levels of those patients were lower than those of patients who underwent general anesthesia, suggesting the improvement of immune function of patients of group A was better than patients in group B. In this study, only the condition 24 h after surgery was observed; hence the time when the immune function improved has not been determined.

There is an obvious relationship between IL-10 and cellular immunity suppression. The major function of IL-10 is to inhibit the growth activity of macrophage and promote the generation of IFN- $\gamma$  (17). In this study, IL-10 showed a downward

trend immediately after surgery, but increased 24 h after surgery; the increasing of IL-10 of patients undergoing combined anesthesia was higher than that of patients undergoing general anesthesia, suggesting that combined anesthesia could promote the recovery of IL-10 rather than inhibit it. Thus, epidural block in combination with general anesthesia was superior to general anesthesia, promoted the increase of IL-10 level, and accelerated the recovery of the immune system.

Isikawa M et al. (18) considered that IFN- $\gamma$ /IL-4 (Th1/Th2) of patients would decrease after liver surgery. This study found that group A had lower IFN- $\gamma$ /IL-4 (Th1/Th2) compared to that of group B before anesthesia and after surgery, but exceeded that of group B 24 h after surgery, suggesting the combined anesthesia improved IFN- $\gamma$ /IL-4 (Th1/Th2). The increase of IFN- $\gamma$ /IL-4 (Th1/Th2) indicated the improvement of immunosuppression.

To sum up, epidural block in combination with general anesthesia has little passive influence on the immune function of patients after liver cancer surgery, therefore, can be a choice for patients.

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## LETTER TO THE EDITOR

## INTERLEUKIN-6 AND INTERLEUKIN-8 IN DIAGNOSING NEONATAL SEPTICEMIA

Y.Q. WU, J. SHEN, Q.L. ZHOU, H.W. ZHAO, L.R. LIU and X. LIU

*Pediatric Medicine, the Affiliated Hospital of Chengde Medical University,  
Chengde, China**Received April 19, 2016 – Accepted November 23, 2016*

The first two authors contributed equally to this study

Neonatal septicemia (NS) is a common cause of death of newborn infants, hence early diagnosis and treatment are of the utmost importance. However, lack of specific clinical symptoms and late detection delay a correct diagnosis. It is therefore of great importance to establish auxiliary indexes for the early diagnosis of NS. To evaluate the value of interleukin (IL-6 and IL-8) in the diagnosis of NS, a prospective study was carried out. Seventy-five newborns who developed septicemia and received treatment in our hospital from January 2013 to December 2014 were selected as research subjects; also, 50 healthy newborns were set as a control group. The levels of serum IL-6 and IL-8 were compared between the two groups. Results demonstrated that levels of C-reactive protein (CRP), IL-6 and IL-8 of the septicemia group were higher than those of the control group on admission, although the difference had no statistical significance ( $P < 0.05$ ); the septicemia group had higher sequential organ failure assessment (SOFA) scores but lower pediatric critical illness scores (PCIS) compared to the control group ( $P < 0.05$ ); levels of CRP, IL-6 and IL-8 were in positive correlation to the SOFA scores and in negative correlation to PCIS. Analysis of receiver operating characteristics (ROC) curve demonstrated that the sensitivity, specificity and accuracy were 85.7%, 80.2% and 81.8%, respectively, when IL-6 level was set as 32 pg/mL, 78.1%, 64.2% and 66.9%, respectively when IL-8 level was set as 54 pg/mL, and 71.4%, 86.3% and 82.7% respectively, when detection of IL-6 and IL-8 were combined together. Hence it can be concluded that: IL-6 and IL-8 are involved in inflammatory reactions; levels of IL-6 and IL-8 were correlated to the severity of the infection; the value of IL-6 is higher than that of IL-8 in the diagnosis of neonatal septicemia and the combined detection of IL-6 and IL-8 can improve the accuracy of the diagnosis of neonatal septicemia.

To the Editor,

Neonatal septicemia (NS) refers to a systemic infection induced by the proliferation of bacteria that invade the blood and the toxicity produced in neonates is one of the main causes leading to death of newborns and is notable in neonatal intensive care units (NICU) (1). The World Health Organization (WHO) estimate that more than 1 million newborns die of severe infection every year, mostly of

septicemia or pneumonia (2). Newborns with weak immunity and poor resistance to bacterial infection easily develop systemic diffusion; hence early, rapid diagnosis and timely treatment are the keys for good prognosis (3).

Currently, diagnosis of NS mainly relies on blood culture which however, consumes time and has a low positive rate of efficacy in diagnosing septicemia (4). It has been found that C-reactive

*Key words: newborn, septicemia, interleukin-6*

Mailing address:

Dr Xia Liu,  
No.36, Nanyingzi Street,  
Shuangqiao District, Chengde,  
Hebei 067000, China  
e-mail: lliuxia@163.com

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protein (CRP) is of high specificity in the diagnosis of neonatal septicemia, but the sensitivity is low for an early diagnosis (5). Furthermore, the combination of detection of CRP level and the average value of neutrophil volume can enhance the sensitivity and specificity of the diagnosis of NS, and IL-6, which starts to increase in the early stage, is of great significance for an early diagnosis (6). Recently, it has also been found that IL-6 and IL-8 are beneficial for an early, however, those studies concentrated on a single index and few dealt with a combination of the two indexes. On account of this, this study was designed to evaluate the significance of combined detection of IL-6 and IL-8 in the diagnosis of NS.

## MATERIALS AND METHODS

### *General data*

One hundred and twenty-five newborns were selected, comprising 75 newborns with septicemia who received treatment in our hospital from January 2013 to December 2014 (septicemia group) and 50 healthy newborns (control group). Diagnosis of septicemia followed the criteria formulated by newborn cooperative group (7), i.e., culture of pathogenic bacteria based on blood sample or culture of conditioned pathogen based on double blood sample. In the septicemia group, there were 40 males and 35 females, aged 35 to 39 weeks (average  $36.2 \pm 4.1$  weeks) with 44 cases of natural delivery and 31 cases of cesarean delivery. In the control group, there were 28 males and 22 females, aged 38 to 41 weeks (average  $37.6 \pm 6.4$  weeks) with 27 cases of natural delivery and 23 cases of cesarean delivery. This study was reviewed and approved by the ethics committee of the Affiliated Hospital of Chengde Medical University, Chengde, China, and family members of all the newborns signed informed consent before the experiment. Newborns who developed had infectious diseases and had taken antibiotics before the study were excluded. Baseline data of the two groups had no statistical significance ( $P > 0.05$ ), hence the results were comparable.

### *Methods*

Blood samples were taken from bilateral lower limbs after admission and before application of antibiotics. The samples were centrifuged at 3000 r/min for 10 min. Then the supernate was collected. Levels of IL-6 and IL-8

were detected using solid phase sandwich enzyme linked immunosorbent assay (ELISA) and subpackaged reagents. CRP level was detected using immune scatter turbidimetry (Shenzhen Golsite Biological Co., Ltd). The above analyses were also carried out for the control group.

Receiver operating characteristic (ROC) curve was drawn according to levels of CRP, IL-6 and IL-8 of newborns with septicemia. Then optimal cutoff values and area under curve (AUC) of indexes were determined. Sensitivity (sensitivity = number of positive cases among newborns with septicemia / total number of cases of septicemia  $\times 100\%$ ), specificity (specificity = number of negative cases among non-septicemia cases / total number of cases of non-septicemia  $\times 100\%$ ), accuracy, positive predicted value, negative predicted value and Youden index [Youden index = (sensitivity + specificity - 1)] were calculated.

### *Determination index for general physical condition*

The general physical condition of the newborns was determined based on sequential organ failure assessment (SOFA) score formulated by National Academic Conference of Critical Care Medicine and pediatric critical illness score (PCIS) mentioned in the summary of the Fourth National Emergency Medicine Seminar held by the Emergency Team of the Academy of Pediatrics of the Chinese Medical Association.

### *Statistical analysis*

SPSS ver. 21.0 was used for statistical analysis. Measurement data conforming to normal distribution were expressed as mean  $\pm$  SD. Multi-group comparison was performed using single-factor analysis of variance; intra-group pairwise comparison was performed using Student-Newman-Keulsq (SNK) - q method. Measurement data not conforming to normal distribution were expressed as median (interquartile range) [M (P25, P75)]. Multi-group comparison was performed using Kruskal-Wallis H rank test, and intra-group pairwise comparison was performed using expanded *t*-test. Enumeration data were expressed by rate (%); multi-group comparison was performed using *chi*-squared test. Diagnostic performance was analyzed using ROC curve. Correlation was analyzed by carrying out single-factor regression analysis taking levels of IL-6, IL-8 and CRP as independent variables. Difference was considered statistically significant if  $P < 0.05$ .



## RESULTS

*Comparison of levels of serum IL-6, IL-8 and CRP between two groups*

Levels of IL-6, IL-8 and CRP of the septicemia group were higher than those of the control group, and the difference had statistical significance ( $P < 0.05$ ) (Table I).

IL-6, an inflammatory factor generated by endometrial stromal cells, amniotic membrane, chorion and decidual cells, plays an important role in the anti-infection process of the host. Stimulation of bacteria can result in a rapid increase of IL-6. Moreover, IL-6 can induce liver cells to generate acute phase protein such as CRP (8).

IL-8, a chemotactic factor, is generated by mononuclear macrophage and epithelial cells and is correlated to the severity of infection. In normal conditions, the content of IL-8 is low and free from the effects of gestational age and time of birth (9). When inflammation occurs, the level of IL-8 increases within 1~3 h and the half-life period is shorter than 4 h. In

recent years, IL-6 and IL-8 have shown outstanding value in the early diagnosis of NS. Through meta-analysis, Su et al. (10) found levels of white cord blood cells and CRP could not be used for accurate early diagnosis NS and that IL-6 was a reliable index for the early diagnosis. In this study, levels of IL-6 and IL-8 of the septicemia group were much higher than those of the control group, suggesting a high positive rate of IL-6 and IL-8 which can be taken as the index for early diagnosis of NS.

*Analysis of correlation between levels of IL-6, IL-8 and CRP and systemic condition*

In the diagnosis and treatment of NS, accurate determination of the general physical conditions of newborns is beneficial for the timely adjustment of treatment (11). SOFA and PCIS are the commonly used methods for evaluating the disease condition of children. SOFA is used for determining the general physical condition, which is of great significance in the prediction of death risk, determination of prognosis and evaluation of the efficiency of the intensive care

**Table I.** Comparison of levels of IL-6, IL-8 and CRP between two groups.

| Group                   | IL-6*    | IL-8**    | CRP***          |
|-------------------------|----------|-----------|-----------------|
| Septicemia group (N=75) | 38.8±9.1 | 65.3±17.7 | 23.1(8.7, 59.9) |
| Control group (N=50)    | 11.8±7.7 | 32.8±21.2 | 3.2(2.9, 8.0)   |
| F (H) value             | 88.811   | 32.423    | (77.629)        |
| P value                 | <0.05    | <0.05     | <0.05           |

Note: \*: mean±SD, pg/mL; \*\*: (mean±SD, pg/mL); \*\*\*: [M ( $P_{25}$ ,  $P_{75}$ ), mg/L]

**Table II.** Correlation between levels of IL-6, IL-8 and CRP of newborns with septicemia and systemic condition.

|             | SOFA score |                |       | PCIS   |                |       |
|-------------|------------|----------------|-------|--------|----------------|-------|
|             | b          | r <sup>2</sup> | P     | b      | r <sup>2</sup> | P     |
| IL-6(pg/mL) | 1.879      | 0.482          | <0.05 | -2.181 | 0.446          | <0.05 |
| IL-8(pg/mL) | 1.947      | 0.512          | <0.05 | -1.994 | 0.527          | <0.05 |
| CRP(mg/L)   | 2.184      | 0.558          | <0.05 | -2.392 | 0.583          | <0.05 |

**Table III.** Analysis results of ROC curve of levels of IL-6, IL-8 and CRP in diagnosis of neonatal septicemia.

| Index | COV       | AUC (%) | Sensitivity (%) | SPE (%) | PPV (%) | NPC (%) | Youden index | Accuracy (%) |
|-------|-----------|---------|-----------------|---------|---------|---------|--------------|--------------|
| IL-6  | 32 pg/mL  | 86.7    | 85.7            | 80.2    | 57.8    | 95.2    | 0.67         | 81.8         |
| IL-8  | 54 pg/mL  | 73.4    | 78.1            | 64.2    | 50.1    | 89.6    | 0.41         | 66.9         |
| CRP   | 26.4 mg/L | 66.1    | 45.2            | 83.7    | 46.6    | 82.6    | 0.29         | 73.8         |

Note: COV: Cut-off value; AUC: Area under the curve; SPE: Specificity; PPV: Positive predicted value; NPV: Negative predicted value

**Table IV.** Evaluation results of diagnosing neonatal septicemia with two or more indexes.

| Index         | Sensitivity (%) | SPE (%) | PPV (%) | NPV (%) | Youden index | Accuracy (%) |
|---------------|-----------------|---------|---------|---------|--------------|--------------|
| IL-6+IL-8     | 71.4            | 86.3    | 62.5    | 90.3    | 0.58         | 82.7         |
| IL-6+CRP      | 37.2            | 90.6    | 56.3    | 81.8    | 0.27         | 78.2         |
| CRP+IL-8      | 33.1            | 89.1    | 48.6    | 80.2    | 0.21         | 76.5         |
| CRP+IL-6+IL-8 | 30.5            | 92.7    | 59.9    | 80.6    | 0.23         | 78.3         |

Note: SPE: Specificity; PPV: Positive predicted value; NPV: Negative predicted value

unit (12). PCIS is used for reflecting the infection severity of organs in the body of children; higher scores indicated more severe disease condition (13). The results of this study demonstrated that, the average SOFA score of the septicemia group was  $(9.1 \pm 1.4)$  points, much higher than the control group  $(2.2 \pm 0.6)$ ; PCIS of the septicemia group was  $71.8 \pm 11.2$  points, much lower than the control group  $(95.7 \pm 12.8)$ ; the differences had statistical significance ( $t=12.742$  and  $5.857$ ,  $P<0.05$ ).

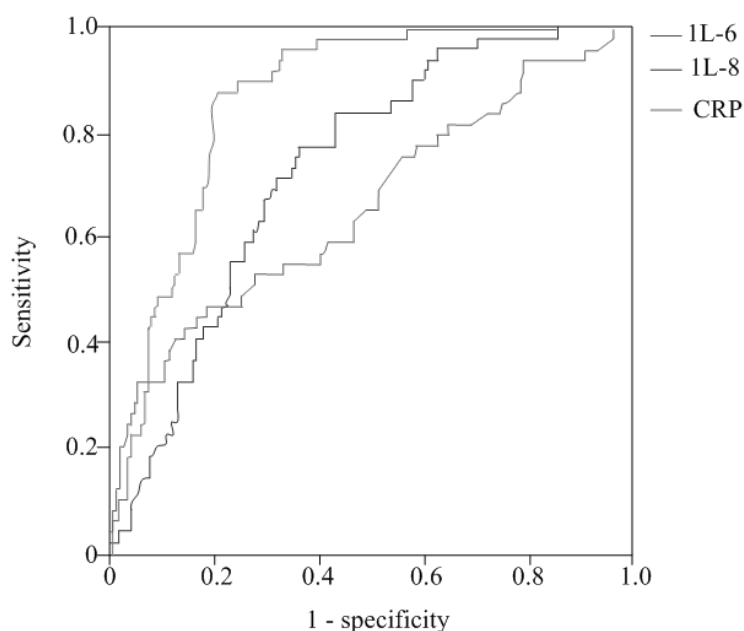
Single-factor regression analysis was carried out, taking levels of IL-6, IL-8 and CRP as independent variables and SOFA score and PCIS as dependent variables. The analysis suggested that levels of IL-6, IL-8 and CRP were in a positive correlation to SOFA score and in a negative correlation to PCIS (Table II).

#### Analysis of ROC curve of levels of IL-6, IL-8 and CRP in the diagnosis of neonatal septicemia

Analysis of ROC curve suggested that, AUC of IL-6, IL-8 and CRP was 86.7 %, 73.4% and 66.1%

respectively (Fig. 1). When cutoff value of serum IL-6 was set as 32 pg/ml, the sensitivity and specificity were 85.7% and 80.2% ,respectively; when cutoff value of serum IL-8 was set as 54 pg/ml, the sensitivity and specificity were 78.1% and 64.2%, respectively; when cutoff value of serum CRP was set as 26.4%, the sensitivity and specificity were 45.1% and 83.8%, respectively. The accuracy of IL-6 in the diagnosis of NS was the highest (Table III).

Study results demonstrated that when CRP threshold value was 26.4 mg/L, the sensitivity was only 45.2%, much lower than that of IL-6 and IL-8. ROC curve suggested that AUC of CRP was 66.1, suggesting CRP is involved in the occurrence of NS but the accuracy of CRP is low. Through meta-analysis, Shahkar et al. (14) found that the sensitivity and specificity of IL-6 were 79% and 84% and AUC was 89% (95% confidence interval: 84%~94%) and assumed IL-6 was valuable in the early diagnosis of NS. Boskabadi et al. (15) found that, IL-6 had higher sensitivity (92.5%) and negative predicted



**Fig 1.** ROC curve of levels of IL-6, IL-8 and CRP in diagnosis of neonatal septicemia.

value (93.1%), which was consistent with the results of this study. Table III shows that the sensitivity and specificity of IL-8 in the diagnosis of NS were 77.6% and 63.8% respectively, suggesting the higher value of IL-8 in the diagnosis, which is consistent with most studies. Taking 80 newborns as research subjects, Boskabadi et al. (15) found the sensitivity, specificity, positive predicted value and negative predicted value of IL-8 were 95%, 10%, 97% and 10% respectively when cutoff value of IL-8 was set as 60 pg/mL, suggesting the higher value of IL-8 in systemic infection. Hence IL-8 is considered as the sensitive index for the early diagnosis of NS.

#### *Evaluation results of diagnosing neonatal septicemia with combined indexes*

Diagnosis with combined indexes suggested that IL-6 in combination with IL-8 had the highest sensitivity, positive predicted value, negative predicted value and Youden index, i.e., 71.4%, 62.5%, 90.3% and 0.58 respectively; the accuracy and specificity of diagnosing NS with IL-6 combined with IL-8 were higher than the accuracy and specificity of diagnosing NS with only IL-6 (83.1% vs 81.8%; 86.3% vs 80.2%); the specificity

of diagnosing neonatal septicemia with CRP, IL-6 in combination with IL-8 was the highest (92.7%), but the sensitivity was only 30.5%. Therefore, diagnosis using IL-6 in combination with IL-8 has the highest value. Details are shown in Table IV.

## DISCUSSION

NS is a notable cause of disability and death in newborns; early diagnosis and timely treatment are the key measures for improving the survival rate of (16). However, the onset of NS is not obvious as it has no specific symptoms. Besides time consuming, diagnosis based on blood culture may obtain negative false results, thus delaying targeted treatment (17). Clinically, white blood cell, CRP and blood sedimentation are frequently used as auxiliary examination indexes. Most patients are observed with significant changes of those indexes during onset; however, diagnosis based on these shows low sensitivity and specificity (18, 19).

Taking IL-6, IL-8 and CRP as auxiliary examination indexes, this study analyzed the value of IL-6, IL-8 and CRP in early diagnosis of neonatal NS. Research results shows that, levels of IL-6, IL-8

and CRP of the septicemia group were higher than those of the control group, and the sensitivity and specificity of IL-6 in combination with IL-8 were 71.4% and 86.3% respectively, suggesting levels of serum IL-6, IL-8 and CRP of newborns with septicemia changed and IL-6 in combination with IL-8 was of high sensitivity and specificity.

In conclusion, IL-6 and IL-8 are involved in an inflammatory reaction in the early stage of infection and the levels are correlated to the severity of the infection. Hence, IL-6 and IL-8 are of great significance in the early diagnosis of NS, determination of infection severity and guidance for the administration of antibiotics.

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## A PLACEBO-CONTROLLED TRIAL OF A PROPRIETARY LIPID-LOWERING NUTRACEUTICAL SUPPLEMENT IN THE MANAGEMENT OF DYSLIPIDEMIA

M. HOUSTON<sup>1</sup>, R. ROUNTREE<sup>2</sup>, J. LAMB<sup>3</sup>, S. PHIPPS<sup>2</sup>, S. MENG<sup>4</sup> and B. ZHANG<sup>2</sup>

<sup>1</sup>Hypertension Institute of Nashville, Saint Thomas Hospital, Vanderbilt University School of Medicine, Nashville, Tennessee, USA; <sup>2</sup>Thorne Research, Inc., Sandpoint, Idaho, USA;

<sup>3</sup>Hypertension Institute of Nashville, Saint Thomas Hospital, Nashville, Tennessee, USA;

<sup>4</sup>Department of Epidemiology, Harvard School of Public Health, Boston, Massachusetts, USA

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There is an ever growing emergence in the popularity of patient-driven care. As this health and wellness model grows, inquiries into diet, lifestyle, and supplemental approaches will continue to become a focal point for the healthcare consumer. Because of this, the aim of this study is to determine the tolerability, and overall effectiveness of a proprietary multi-ingredient lipid-lowering supplement in subjects with dyslipidemia. Forty participants were recruited for a single-center, double-blind randomized, placebo-controlled trial. Study participants were recruited between December 2014 and March 2015. Initial screening included a physical examination, renal and hepatic function, serum lipid, serum electrolytes, complete blood counts, and urine analysis. The 40 participants were randomly assigned to receive either the proprietary multi-ingredient lipid-lowering supplement (PMILLS) n= 20 or placebo n= 20. The trial consisted of a screening visit, a two-week run-in, and a four-month treatment period. Samples were taken at baseline, one month and four months of treatment. Results from the trial showed that the PMILLS significantly reduced total cholesterol (TC), low density lipoprotein (LDL-C), very low density lipoprotein (VLDL-C), oxidized LDL (oxLDL), Apo-lipoprotein B, triglycerides (TG), LDL particle number (LDL-P), heart rate, and diastolic blood pressure compared to placebo at one month and four months. The PMILLS significantly increased high density lipoprotein (HDL) particle number (HDL-P), and low density lipoprotein (LDL) particle size from dense type III and IV to larger type I and II LDL particle, compared to placebo at one month and four months. In addition, the PMILLS significantly reduced high sensitivity C-reactive protein (hs-CRP), tumor necrosis alpha (TNF- $\alpha$ ), and interleukin 6 (IL-6) within the treatment group from baseline. There were no adverse effects noted in the treatment group after four months of supplementation. The present study demonstrates this PMILLS improves all relevant lipid parameters, such as particle numbers and particles sizes, as well as showing a significant reduction in inflammatory markers linked to cardiovascular health. With such combined changes in lipids, lipid sub-fractions, and inflammation, which are considered among the most effective means of reducing coronary heart disease (CHD), this PMILLS represents a new addition to safe and effective lipid-modifying strategies.

*Key words: dyslipidemia, nutraceutical supplements, coronary heart disease, cardiovascular disease, vascular inflammation, LDL cholesterol, HDL cholesterol, triglycerides*

*Mailing address:*

Dr Bodi Zhang,  
152 W, 57<sup>th</sup> St,  
NYC, NY, 10019, USA  
Tel.: +1 208 320 9744 - Fax: +1 800 7471950  
e-mail: bzhang@thorne.com

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To the Editor,

Dyslipidemia is considered to be one of the top five risk factors for coronary heart disease (CHD) and cardiovascular disease (CVD) along with hypertension, diabetes mellitus (DM), smoking, and obesity (1). Although there are an infinite number of vascular insults, there are only three finite responses of the cardiovascular tissue to these insults. The mechanisms by which certain lipids induce vascular damage are complex, from a pathophysiologic viewpoint; but still can be easily categorized into responses of inflammation, oxidative stress, and vascular immune dysfunction (2-4). These endpoints lead to endothelial dysfunction (EnD), vascular smooth muscle dysfunction (VSMD), cardiac myocyte dysfunction (CMD), and lastly CHD. Management of dyslipidemia with supplemental nutrients has been infrequently reviewed (5-7). Further preclinical and clinical studies are required to understand the present role of these natural agents in the management of dyslipidemia and subsequent sequelae (5-7). To date, there have been clinical trials that have shown significant improvement in both CHD and serum lipids with supplementation. Smaller studies involving nutrient therapies have also demonstrated reductions in surrogate vascular markers with decreases in carotid intimal medial thickness, carotid artery obstruction, plaque progression, and coronary artery calcium score as well as improvement in generalized atherosclerosis and EnD (5-9). It is important that the treatments for dyslipidemia, and subsequent vascular disease, address not only clinical lipid parameters, but also the various mechanisms by which lipids induce CVD. The combination of diet and mechanistically targeted nutritional supplementation have the potential to significantly modulate lipoprotein particle characteristics, improve HDL functionality, reverse cholesterol transport (RCT), and cholesterol efflux capacity. In addition, inflammation, oxidative stress, and vascular immune responses can be favorably influenced. In several clinical trials, surrogate cardiovascular markers, CHD and CVD have been reduced by optimal nutrition and/or administration of several nutritional supplements, including red yeast rice (RYR), curcumin, berberine,

and phytosterols. Many of the ingredients in the PMILLS formula have been shown to reduce inflammation, and ingredients like RYR have been shown to reduce CHD and myocardial infarction (6). With that in mind, a combined regimen of dietary modification and nutritional supplements represents a viable alternative for patients who are statin-intolerant, cannot take other drugs for the treatment of dyslipidemia, or prefer non-drug therapies. Moreover, this combined approach targets numerous molecular pathways and networks known to be involved in the development of atherosclerosis and CHD.

## MATERIALS AND METHODS

### *Study design*

This clinical trial was designed to examine the overall effects of the proprietary lipid-lowering nutraceutical on various lipids and inflammatory markers (Fig. 1). The study was a randomized, double-blind, randomized, placebo-controlled, single-center trial consisting of a screening visit, a two-week run-in, and a four-month treatment period. Written informed consent was obtained from each patient. The study was conducted in accordance with the principles of the Declaration of Helsinki. Study participants were recruited between December 2014 and March 2015 from the Hypertension Institute in Nashville, Tennessee, USA. Initial screening included taking a medical history, physical examination, renal and hepatic function, serum lipid concentrations, serum electrolytes, complete blood counts, and urine analysis. The 40 patients were randomly assigned to receive (double-blind) either proprietary multi-ingredient lipid-lowering supplement (PMILLS) or placebo. Randomization was performed centrally and was concealed. All 40 participants completed the study and none were lost after randomization.

### *Study goal*

Primary aim: Reduction of LDL-P, LDL-C, apolipoprotein B (ApoB), TG, and total cholesterol level.

Secondary aim: Reduction of VLDL particle size and number, increases in HDL, HDL size, and HDL-P, changes in lipoprotein(a) (Lp(a)), oxLDL, changes in hs-CRP, myeloperoxidase (MPO), other inflammatory markers, cardiac and vascular function, evaluation of endothelial

function, heart rate variability (HRV), augmentation index (AI), small and large arterial compliance, and central arterial pressure. All vascular testing was non-invasive.

#### Participants

Subjects eligible for enrollment were either male or female, ages 18 to 80 years, with fasting dyslipidemia (LDL 130-250 mg/dL) based on initial screening with direct LDL measurements. Participants who were statin-intolerant or who refused to take statins or any other lipid-lowering drug for any reason were primarily recruited.

The exclusion criteria were: myocardial infarction, coronary artery bypass grafting, percutaneous transluminal coronary angioplasty, or stent within five years, known clinical CHD symptoms, or clinical angina; history of cerebrovascular accident; creatinine over 2.5 mg/dL; known allergy or sensitivity to any components of the nutritional supplement used; (v) chronic liver disease with AST, ALT, alkaline phosphatase over 1.5 x normal; known cancer within two years; clinical congestive heart failure (systolic or diastolic CHF); type I or type 2 DM; pregnancy or nursing; and women of child-bearing age not on an approved and accepted contraceptive.

Participants were randomized to receive PMILLS (one double packet, twice daily; for detailed ingredients see Table I), or a placebo prepared in indistinguishable

capsules. A dose reduction to one double packet daily was suggested whenever constipation lasting more than two weeks occurred. The treatment capsules and placebo capsules were provided by Thorne Research, Inc. (Sandpoint, Idaho, USA).

#### Clinical and biochemical measurements

Blood samples were taken from the study participants after a 12-hour fast, except for the consumption of water and regular medications, which were allowed to be taken prior to each visit. Laboratory tests were performed at baseline and variable times depending on the lab specified. Date of birth, smoking, alcohol consumption, and past medical history were assessed. Height and weight (light clothes and without shoes), and seated blood pressure (measured on the patient's non-dominant arm supported at heart level) were determined by a senior physician or a qualified clinical nurse research specialist, using the American Heart Association (AHA) criteria for accurate blood pressure measurement. All patients were required to maintain current weight, nutrition program, diet, exercise regimen, alcohol intake, smoking, caffeine intake, and prescription medications (excluding lipid-lowering agents) during the study. This was to help reduce variability of effect in the placebo arm, given that alterations in lifestyle during the trial could potentially have positive effects on

**Table I.** Ingredient list of the proprietary lipid supplement.

| Each double packet contains:                                                                       |        |
|----------------------------------------------------------------------------------------------------|--------|
| Ingredients                                                                                        | Dosage |
| Phytosterol esters                                                                                 | 800 mg |
| Aged Garlic Extract (bulb) ( <i>Allium sativum</i> ) (Kyolic®)                                     | 600 mg |
| Red Yeast Rice ( <i>Monascus purpureus</i> )                                                       | 500 mg |
| Curcumin Phytosome ( <i>Curcuma longa</i> extract (root)/<br>Phosphatidylcholine complex)          | 250 mg |
| Green Tea Phytosome ( <i>Camellia sinensis</i> extract (leaf) /<br>Phosphatidylcholine complex)    | 250 mg |
| N-Acetyl-L-Cysteine                                                                                | 250 mg |
| Berberine HCL (from Indian Barberry extract (root)<br>( <i>Berberis aristata</i> )                 | 200 mg |
| Deglycyrrhized Licorice (DGL) extract (root)                                                       | 100 mg |
| Trans-Resveratrol                                                                                  | 40 mg  |
| Quercetin Phytosome ( <i>Sophora japonica</i> concentrate<br>(leaf) / Phosphatidylcholine complex) | 50 mg  |
| Lycopene (from tomato, <i>Lycopersicon esculentum</i> )                                            | 4 mg   |

lipid and inflammatory parameters. Potential medication interactions with the treatment arm were monitored and, if present, were documented in the adverse event case report. Medications known to effect serum lipids were prohibited. Nutritional supplements known to effect lipids were not permitted. All such medications or supplements were required to be discontinued at least one month prior to study initiation and a steady state lipid profile was obtained and repeated prior to study entry.

### Statistical analysis

This study was designed in accordance with a predetermined statistical analysis plan. The statistical analysis was performed in a blinded fashion by an independent third party that was not involved in the design, implementation or data collection.

A sample size of 20 patients in each of the two study groups, with a dropout rate up to 10%, was planned to provide a 90% power to detect a 15% or greater (with 95% confidence intervals,  $p < 0.05$  for significance) reduction in the primary end point variables in the PMILLS compared with the placebo group after one and four months at the end of the study. Statistical analysis was performed using the SAS 4.0 system (SPSS Inc., Chicago, IL), and data are presented as mean standard error (SE). Logarithmic transformation was used for interleukin-6 (IL-6) and hs-CRP because of the high degree of skewing.

Within-group comparisons were performed with paired sample *t*-tests to evaluate differences from baseline in each group. Independent-sample *t*-test and the analysis of covariance (ANCOVA) analysis with a model that included the baseline value of the dependent variable as a covariate were also used for comparison between groups.

## RESULTS

### Baseline characteristics

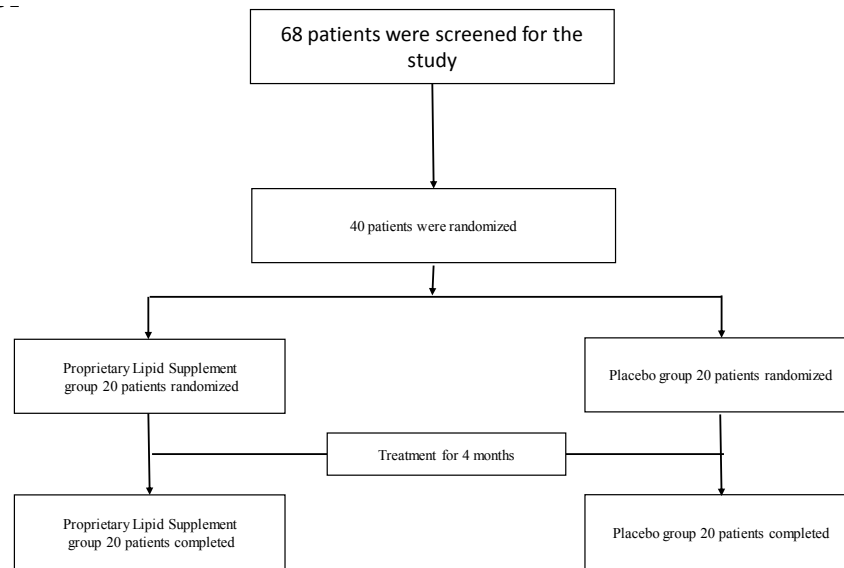
The data of 40 participants were analyzed, as shown in Table II. The baseline variables are not significantly different between the PMILLS and placebo groups.

### Primary outcomes

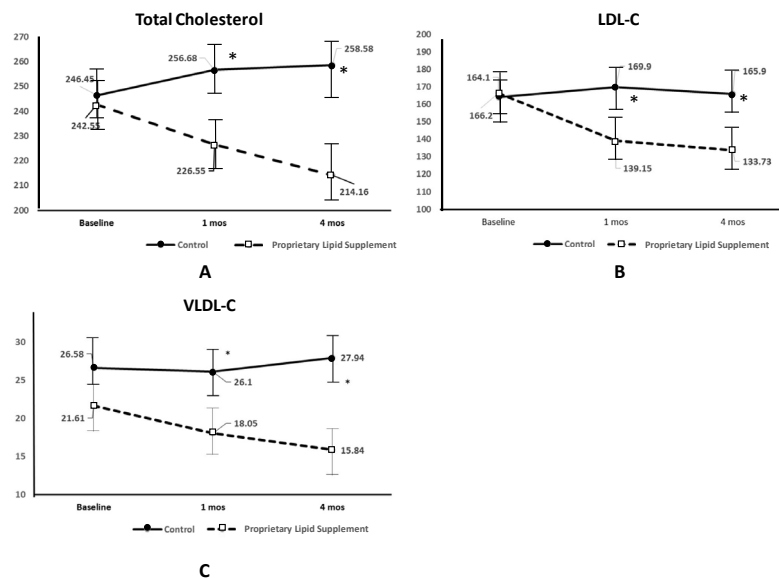
Compared to the participants who received placebo ( $n=20$ , female=9, male=11), those receiving the PMILLS ( $n=20$ , female=9, male=11), had a significant reduction in total cholesterol, LDL-C, and VLDL-C at both one and four months. Total cholesterol decreased from 242.55 mg/dL to 214.16 mg/dL at four months with the PMILLS but increased from 246.65 mg/dL to 258.58 mg/dL with placebo (Fig. 2A). LDL-C fell from 166.2 mg/dL to 133.73 mg/dL with the PMILLS group, versus a slight increase from 164.1 mg/dL to 165.9 mg/dL with placebo (Fig. 2B). VLDL-C decreased from 21.62 mg/dL to 15.84 mg/dL with the PMILLS group, and increased from 26.58 mg/dL to 27.94 mg/dL with placebo (Fig. 2C). The ANCOVA analysis showed that serum total cholesterol, LDL-C, and VLDL-C concentrations in the proprietary PMILLS were significantly reduced compared to the changes in the placebo group ( $p < 0.0001$ ,  $p < 0.001$ ,  $p < 0.0001$ , respectively). Serum total cholesterol, triglycerides, and LDL-C concentrations were reduced from one month ( $p < 0.0001$ ) compared with the baseline and maintained significance at four months, indicating that the PMILLS becomes effective as early as one

**Table II.** Baseline and 4-month clinical characteristics of the proprietary lipid supplement and placebo groups. *a*= between group comparisons *b*- within group comparisons. \* $p < 0.05$

|                                  | Proprietary Lipid Supplement |              |                      | Placebo      |              |                      | P Value <sup>a</sup> |
|----------------------------------|------------------------------|--------------|----------------------|--------------|--------------|----------------------|----------------------|
|                                  | Before                       | After        | P Value <sup>b</sup> | Before       | After        | P Value <sup>b</sup> |                      |
| No. (Male/Female)                |                              | 20 (11/9)    |                      |              | 20 (11/9)    |                      |                      |
| Age (Yr)                         |                              | 62 ± 6       |                      |              | 58 ± 7       |                      |                      |
| Body Weight (lb)                 | 168.4 ± 26.5                 | 165.3 ± 24.7 | 0.87                 | 170.2 ± 31.5 | 171.4 ± 29.7 | 0.79                 | 0.84                 |
| BMI                              | 26.76 ± 2.41                 | 26.49 ± 2.53 | 0.91                 | 26.98 ± 3.05 | 27.01 ± 2.99 | 0.81                 | 0.75                 |
| Systolic blood pressure (mm Hg)  | 135.7 ± 4.92                 | 130.9 ± 3.62 | <b>0.098*</b>        | 136.6 ± 4.57 | 135.1 ± 5.72 | 0.82                 | 0.41                 |
| Diastolic blood pressure (mm Hg) | 72.3 ± 2.09                  | 69.4 ± 1.79  | <b>0.001*</b>        | 75.05 ± 2.01 | 74.78 ± 2.14 | 0.79                 | <b>0.04*</b>         |
| Heart beat                       | 66.5 ± 2.24                  | 63 ± 1.99    | <b>0.001*</b>        | 67.5 ± 3.1   | 70.8 ± 2.4   | 0.38                 | <b>0.009*</b>        |
| HbA1C (%)                        | 5.59 ± 0.41                  | 5.63 ± 0.62  | 0.92                 | 5.81 ± 0.57  | 5.77 ± 0.48  | 0.65                 | 0.69                 |



**Fig. 1.** Clinical study design.



**Fig. 2.** Comparison of cholesterol parameters in the proprietary lipid supplement and placebo group. **A)** Total cholesterol (1-month, \*:  $p=0.039$ ; 4-month, \*:  $p=0.0041$ ); **B)** LDL-C (1-month, \*:  $p=0.029$ ; 4-month, \*:  $p=0.0016$ ); **C)** VLDL-C (1-month, \*:  $p=0.016$ ; 4-month, \*:  $p=0.0057$ ).

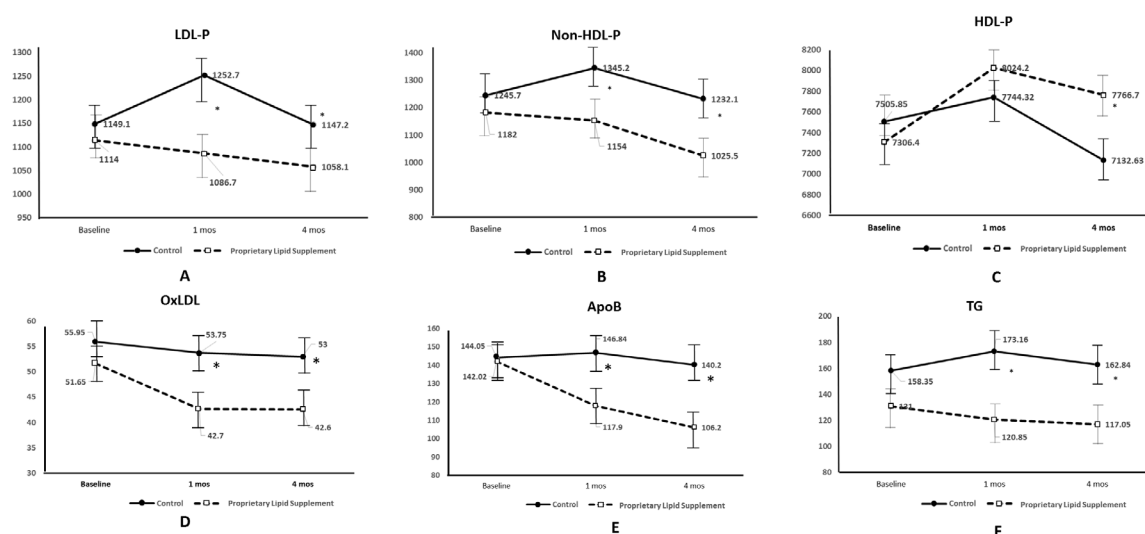
month and has continuing effects.

LDL-P decreased from 1,114/dL to 1,058.1/dL ( $p=0.0492$ ; Fig. 3A) at four months in the PMILLS group, while no change was observed in the placebo group (1,149.1/dL vs 1,147.2/dL).

Total LDL-III and IV particle number (LDL-P), an important indicator of LDL particle size, decreased to  $392\pm44$  nmol/L in the PMILLS group, significantly

lower than the  $576\pm59$  nmol/L in the placebo group ( $p=0.0027$ ).

After treatment, oxLDL was significantly decreased in the PMILLS group, (51.65 mg/dL versus 42.6 mg/dL,  $p=0.021$ ); whereas, no change was observed in the placebo group (55.95 mg/dL vs 53 mg/dL,  $p=0.89$ ) (Fig. 3D). Similar between-group changes were also observed for Apo-lipoprotein



**Fig. 3.** Comparison of lipid particle parameters in the proprietary lipid supplement and placebo group. **A)** LDL-P (1-month,  $^*p=0.0059$ ; 4-month,  $^*p=0.0372$ ); **B)** non-HDL-P (1-month,  $^*p=0.0031$ ; 4-month,  $^*p=0.0421$ ); **C)** HDL-P (4-month,  $^*p=0.0018$ ). **D)** oxLDL (1-month,  $^*p=0.0019$ ; 4-month,  $^*p=0.0323$ ); **E)** ApoB (1-month,  $^*p=0.0055$ ; 4-month,  $^*p=0.021$ ); **F)** TG (1-month,  $^*p=0.014$ ; 4-month,  $^*p=0.0019$ ).

B and TG (Fig. 3 E and F), ( $p=0.0029$  and  $0.014$ , respectively).

### Secondary outcomes

Diastolic blood pressure decreased from  $72.4 \pm 9$  mmHg to  $68.1 \pm 11$  mmHg in the PMILLS group, while increasing from  $72.3 \pm 8$  mmHg to  $73.9 \pm 9$  mmHg with placebo (see Table II) ( $p=0.047$ ). No changes in systolic blood pressure were observed. Supplementation with the PMILLS group, was also associated with decreased heart rate, from  $67.5 \pm 6$  to  $62.4 \pm 7$  ( $p=0.0093$ ), which was significantly lower compared to the placebo group (Table II).

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), hs-CRP, and IL-6 were measured in serum. Between-group analysis showed no significant difference between the PMILLS group, and the placebo group, which may be due to the high standard deviations. Pearson correlation was conducted, comparing baseline values of inflammatory markers with the change in inflammatory markers over time. In the PMILLS group, participants with the highest inflammatory markers at baseline showed the greatest response to intervention, which was highly significant (hs-CRP;  $p<0.001$ ; TNF- $\alpha$ ;  $p<0.001$ ; IL-6;  $p<0.001$ ) (Fig. 4). These effects were not observed in the placebo group. Myeloperoxidase (MPO) is another

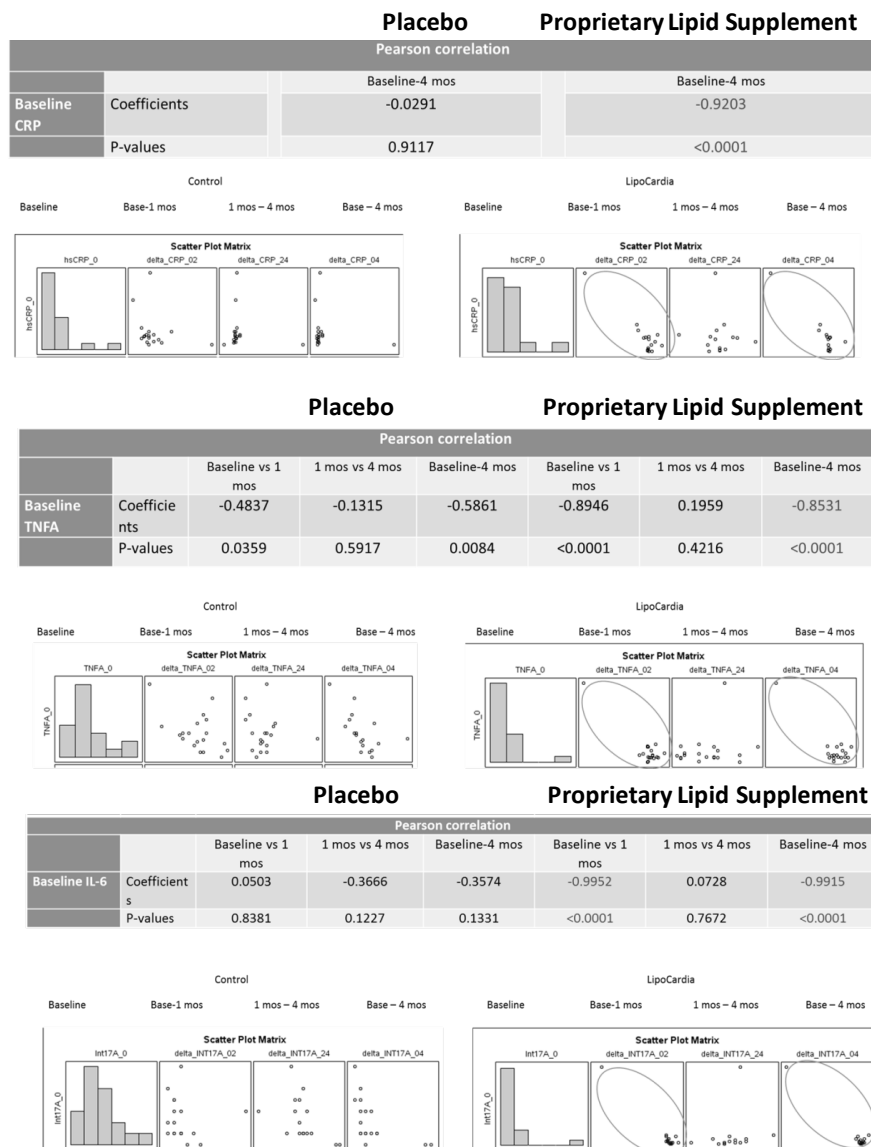
critical marker for oxidative stress and less so with inflammation. However, the MPO change was not significant between PMILLS and placebo groups or within the PMILLS group. This may be in part due to the relatively normal oxidative stress in the study patients. In addition, the duration of the study may have been too short to affect MPO. Using the EndoPAT medical device, endothelial cell function was measured in both the PMILLS group, and the placebo group.

Heart rate variability (HRV), augmentation index (AI) and small and large arterial compliance with Computerized Arterial Pulse Wave Analysis (CAPWA) were also measured, since they are major indicators of cardiovascular events. No significant changes were observed in any of the indicators, which may also be due to the short duration of the study, as changes in arterial compliance of both large and small arteries, as well as pulse wave velocity may take 6 to 12 months to improve. No significant changes in the EndoPAT score, a measure of endothelial dysfunction, occurred due to the relatively brief trial time.

### Safety

During the study period, safety parameters including renal and hepatic function, serum electrolytes, blood counts, and urinary analyses





**Fig. 4.** Comparison of inflammatory markers in the proprietary lipid supplement and placebo group. A) CRP; B) TNF- $\alpha$ ; C) IL-6.

were assessed. Adverse events were reported and recorded in case report form for each patient. No adverse events occurred in the treatment group or in the placebo group. No episodes of hypoglycemia were reported. Two of the recognized side effects related to HMG-CoA inhibitor-based therapy are myopathy and serum coenzyme Q10 (CoQ10) reduction. None of the study participants reported any symptoms of myalgias or myopathy. Nor was there any significant reduction in serum CoQ10 levels at any time in the PMILLS group compared to the control group.

## DISCUSSION

This PMILLS appears to be a safe, effective nutritional supplement for the management of dyslipidemia and its attendant inflammation. It offers an alternative to individuals who are statin intolerant or who refuse to take statins/other lipid-lowering drugs. This PMILLS significantly reduced all of the atherogenic particles implicated in CHD, such as LDL, LDL-P, Apo-lipoprotein B, VLDL, TG and oxLDL. LDL and LDL-P, the driving risks for CHD (6, 10), and achieved significant reductions of

19.5% ( $p < 0.001$ ) and 5% ( $p = 0.0492$ ), respectively, at one and four months. Small dense LDL particles were also decreased. These are considered a major risk factor for CHD when the LDL-P is also elevated (6, 10). Further contributing to a reduced CHD risk, HDL-P significantly increased by 6.3 percent. In addition, the PMILLS group had a significant reduction in primary inflammatory markers associated with vascular disease. This observation was more pronounced in participants with higher baseline levels ( $p < 0.001$ ). Finally, there was a reduction in diastolic blood pressure and heart rate in the PMILLS group.

Lipoprotein particle numbers are being viewed as emerging indicators of cardiovascular health (10), however, LDL-P has been largely ignored in previous studies. LDL particle size is also a critical indicator of cardiovascular risk. It has been shown that LDL particles of smaller size, especially LDL-III and LDL-IV, have a stronger association with stroke, CHD, and myocardial infarction. The present study demonstrates that PMILLS successfully reduces lipoprotein particle numbers, and increases LDL particle size. These results further indicate the PMILLS protective role and opens a new avenue to explore the underlying molecular mechanism.

The limitations of this research can be resolved in future studies. Future study design should increase the number of participants alongside the duration of the trial, given the chronicity of dyslipidemia, and to ensure that the safety profile of the formulation continues to produce minimal adverse events. Future studies should also explore participants with a more complicated CVD picture, and if there are other effects to the standard of care (SoC) that could potentially lower therapeutic dosage, further optimizing the risk benefit profile for the SoC. This PMILLS was developed to target a number of molecular pathways and networks important in lipid-induced vascular disease, with ingredients considered to be safe, especially in the doses utilized (11-13). In this study, this PMILLS was found to improve all relevant lipid particle numbers and particles sizes, as well as improving diastolic blood pressure and inflammatory markers. Such combined changes in lipids, lipid sub-fractions, and inflammation are considered among

the most effective means of reducing CHD, based on large clinical trials that have used HMG Co-A reductase-based therapy. This PMILLS represents a new addition of safe and effective lipid-modifying strategies.

## ACKNOWLEDGEMENTS

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DISCLOSURE: Drs Zhang, Rountree and Phipps are employed by Thorne Research Inc.

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## LETTER TO THE EDITOR

# **NASAL IRRIGATION WITH NASIR® IN CHILDREN: A PRELIMINARY EXPERIENCE ON NASAL CYTOLOGY**

M. GELARDI<sup>1</sup>, S. TALIENTE<sup>1</sup>, K. PICCININNI<sup>1</sup>, G. SILVESTRE<sup>2</sup>, N. QUARANTA<sup>1</sup>  
and G. CIPRANDI<sup>3</sup>

<sup>1</sup>Department of Otolaryngology, University of Bari, Bari, Italy; <sup>2</sup>Private Practice, Pescara, Italy;

<sup>3</sup>Department of Medicine, IRCCS-AOU San Martino, Genoa, Italy

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Allergic rhinitis (AR) and upper airway respiratory infections are frequent in children, and both have a relevant impact on some social aspects, including school attendance and performance, sleep, quality of life (also of the parents), and costs. Saline nasal irrigation is widely employed to reduce nasal congestion and mucopurulent secretion, to stimulate cleansing of the nasal and paranasal cavities, and to induce restoration of mucociliary clearance. The present study evaluated the effects of nasal irrigation on nasal cytology, using the new device Nasir® in 66 children (40 males, 26 females, mean age  $7.31 \pm 1.7$  years, age range 4-17 years) with allergic rhinitis. The patients were treated with nasal irrigation with warm (36°C) Nasir® (250 mL sacs of premixed solution): one sac twice daily for 12 days. Nasal irrigation significantly reduced the neutrophilic infiltrate (baseline median value  $2.8 \pm 0.7$ ; post treatment value  $2 \pm 0.5$ ;  $p < 0.05$ ). In addition, there was a reduction of eosinophil infiltrate ( $T_0 = 3.2 \pm 1.1$ ;  $T_1 = 2.6 \pm 1.2$ ;  $p = < 0.05$ ). There was no significant change with regard to bacteria ( $T_0 = 2.7 \pm 0.9$ ;  $T_1 = 2.3 \pm 1.02$ ;  $p = 0.17$ ). In conclusion, this pilot study reports that nasal irrigation with Nasir® might be useful to attenuate upper airway inflammation.

To the Editor,

Allergic rhinitis (AR) and upper airway respiratory infections are frequent in children, and both have a relevant impact on some social aspects, including school attendance and performance, sleep, quality of life (also of the parents), and costs (1).

The immunopathology of allergic disorders is characterized by an allergen-specific defect of T regulatory cells, promoting Th2 polarization and consequent Th1 impairment (1). Th2-dependent cytokines are relevant for driving allergic inflammation. In particular, IL-5 promotes recruitment and activation of eosinophils that sign allergic inflammation (2). On the other hand, defecting Th1 function lessens defence against infections. Respiratory infections,

mainly if bacterial, are characterized, from a cytological point of view, by abundant neutrophilic infiltrate and bacterial colony (3).

It has been reported that allergic children have more numerous and severe respiratory infections than non-allergic children (4). AR may therefore facilitate a vicious circle characterized by mucosal inflammation and susceptibility to respiratory infections. An adequate control of allergic inflammation in children with AR may consequently be an important strategy for improving symptoms, lessening drug use, and possibly preventing infections (5). In this regard, numerous studies on topical treatments have been performed to demonstrate reduction of the side effects of systemic therapy as well as improvement

*Key words: nasal irrigation, Nasir®, allergic rhinitis, respiratory infection, 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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of nasal symptoms (6, 7). For treating rhinosinusitis, the latest guidelines of the European Academy of Allergology and Clinical Immunology (8) and of the International Rhinology Society (9) suggest also the use of saline nasal irrigation in addition to various therapeutic options (including antibiotics, topical corticosteroids, topical decongestants, etc.). A recent meta-analysis (10) reviewing this topic evaluated several studies, focusing on two (11, 12). In addition, a recent position paper of the European Academy of Allergy and Clinical Immunology included nasal irrigation among the therapeutic options for the treatment of pediatric AR (13).

Saline nasal irrigation is widely employed to reduce nasal congestion and mucopurulent secretion, to stimulate cleansing of the nasal and paranasal cavities, and to induce restoration of mucociliary clearance (8, 9). A huge variety of systems are currently available to cleanse the upper airways (such as drops, sprays, aerosols, nasal douche, and irrigation) as well as the types of solution used in treatment (isotonic and hypertonic saline solutions, with the addition of copper, magnesium, sulfur, etc). A previous study reported that nasal irrigation with isotonic saline solution (as ancillary treatment), carried out by the Lavonase® system, was able to reduce signs and symptoms of acute rhinosinusitis in children (14). Given that inflammation control may be an interesting target, the present preliminary study evaluated the possible effect of nasal irrigation with the Nasir® system, an evolution of Lavonase®, on cytological features in children with AR and frequent respiratory infections. Nasir® differs from Lavonase® as it allows to regulate the flow and pressure of nasal irrigation.

The study cohort consisted of 66 children (40 males, 26 females, mean age  $7.31 \pm 1.7$  years, age range 4-17 years). Inclusion criteria were: i) age between 4 and 17 years, ii) documented diagnosis of AR, iii) more than 5 respiratory infections per year. AR diagnosis was performed if there was consistency between sensitization and symptom occurrence after exposure to sensitizing allergens. Previous respiratory infections were documented by medical history. Exclusion criteria were: presence of acute respiratory infection, anatomic alterations,

concomitant use of interfering drugs (including corticosteroids, antihistamines, antibiotics, and mucolytics), interfering chronic and systemic disorders. The study was performed according to Good Clinical Practice rules and an informed written consent was signed by parents of all the children.

The children then underwent a complete otorhinolaryngological examination, including endoscopy. Endoscopic examination was performed with a flexible fiber optics endoscope (ENT 2000; Vision Sciences, Orangeburg, NY, USA) with a diameter of 3.4 mm. Endoscopy served to identify exclusion criteria, evaluating: i) presence of intranasal anatomic alterations (septal deviation, hypertrophy of turbinates, cartilaginous spurs, concha bullosa, intranasal tumors); ii) mucosal appearance (hyperemia, edema, atrophy, areas of de-epithelialization); and iii) type of secretions (serous, mucous, pus, hematic, exudate clotted scabs).

Nasal cytology was performed by anterior rhinoscopy using a nasal speculum and good lighting. Scrapings of the nasal mucosa were collected from the middle portion of the inferior turbinate, using a Nasal scraping® (IRmedical, Italy) cytology curette. Samples were placed on a glass slide, fixed by air drying and then stained by the May-Grunwald Giemsa method (Carlo Erba, Milan, Italy). The slide was observed under a Nikon E600 light microscope (Nikon, Canada) equipped with a digital camera (Nikon "Coolpix 3:34", Nikon, Tokyo, Japan) for the acquisition of microscopic images. For the rhinocytogram analysis, 50 microscopic fields were read at a magnification of  $\times 1000$  to assess the presence of normal and abnormal cellular elements, along with any microscopic features (spots, special inclusions, etc.) important for diagnosis. Cell counts and bacterial and fungal analysis were carried out by a semi-quantitative grading. In particular, bacterial and inflammatory cells (e.g. eosinophils and neutrophils) was determined as follows: grade 0 (not visible); grade 1+ (occasional groups); grade 2+ (moderate number); grade 3+ (easily visible); and grade 4+ (many cover the entire field of view), according to validated criteria (2, 3).

The patients were treated with nasal irrigation with warm (36°C) Nasir® (250 mL sacs of premixed



solution): one sac twice daily for 12 days. The Nasir<sup>®</sup> system consists of a sac (250 mL) of premixed sterile saline isotonic solution and a delivery system with a 60-cm tube to which an irrigator is attached (as sketched in Fig. 1). The irrigator tip is placed at the nostril to dilate the valve and the solution is directed into all portions of the nasal cavity.

Children were visited and treated at the ENT outpatient clinic and at a family pediatric practice by qualified and well-trained specialists. The study was conducted during the summer season. Visits were carried out at baseline (T0), and at the end of the treatment (T1). During each visit, nasal cytology was performed.

Statistical analysis was performed by the Wilcoxon's test. P values <0.05 were taken as indicating statistical significance. Statistical analysis was carried out using the SPSS software package. Most of the children were attending primary school, only 4 subjects were adolescents. All subjects completed the treatment and no relevant side effects were reported.

Nasal irrigation significantly reduced the neutrophilic infiltrate (baseline median value  $2.8 \pm 0.7$ ; post treatment value  $2 \pm 0.5$ ;  $p < 0.05$ ), as shown in Fig. 2. In addition, there was a reduction of eosinophil infiltrate ( $T_0 = 3.2 \pm 1.1$ ;  $T_1 = 2.6 \pm 1.2$ ;  $p = < 0.05$ ) as reported in Fig. 3. There was no significant change

with regard to bacteria ( $T_0 = 2.7 \pm 0.9$ ;  $T_1 = 2.3 \pm 1.02$ ;  $p = 0.17$ ).

Cleansing nasal cavities is a main step in the treatment of nasal diseases (allergic and non-allergic rhinitis) and infections of the sinus cavities, such as rhinoadenoiditis, rhinosinusitis, nasal polyposis with bacterial or fungal over-infection (15). Nasal irrigation is indicated as adjuvant treatment, as excessive accumulation of catarrhal or mucopurulent secretion, sometimes presenting as crust, is a known predisposing factor to the development of local, such as otitis, rhinosinusitis, and distant inflammation, such as rhinobronchial syndrome, bronchitis, pneumonia, and asthma (16). Removal of secretions allows reactivation of the nasal cavity defense systems and restoration of mucociliary clearance by which endonasal bacterial load is eliminated or reduced (17).

The present preliminary uncontrolled study shows that nasal irrigation with the Nasir<sup>®</sup> system was able to affect nasal inflammation, mainly with regard to neutrophil infiltrate. The pressure at which irrigation is delivered and the distribution of the spray inside the nasal cavity may be factors involved in the positive effects. The delivery system, which consists of a 60-cm tube, generates a standard irrigation pressure of 0.058 Pa, but it is possible to reduce the spill pressure by a flow

## NASAL IRRIGATION

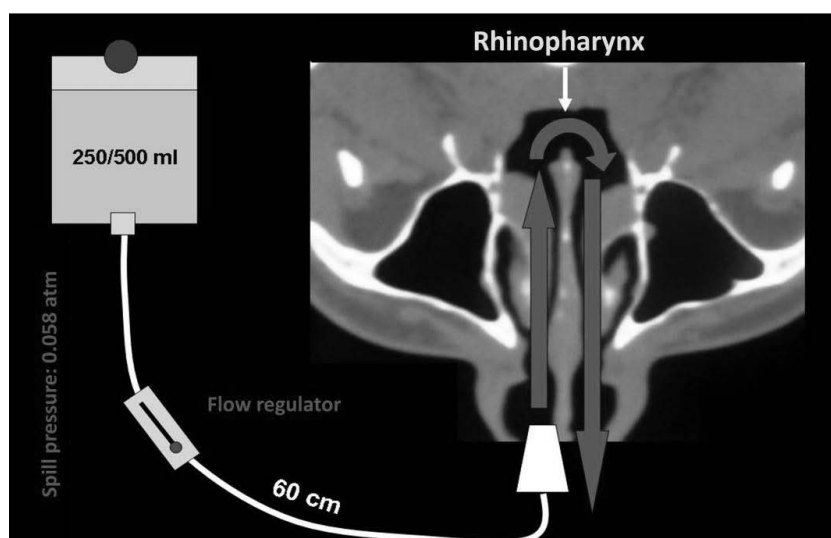
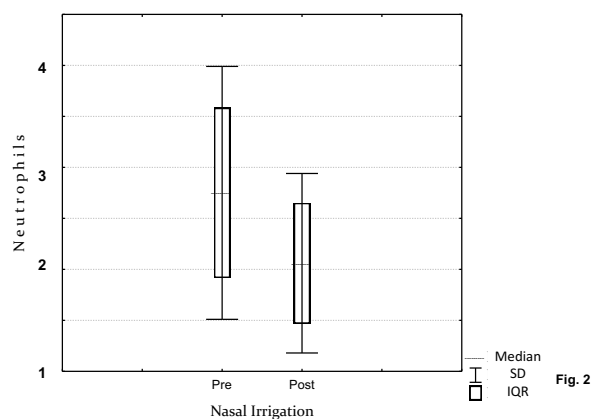
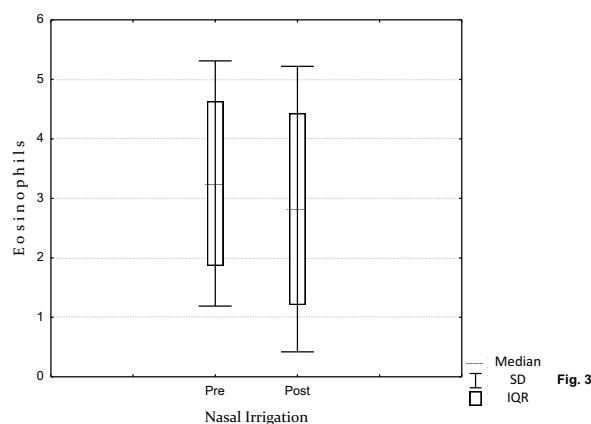


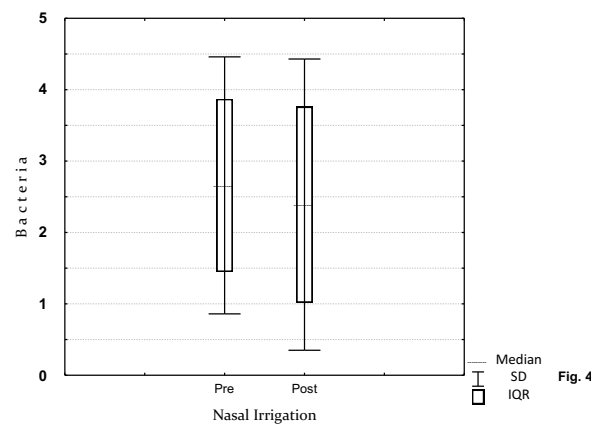
Fig. 1. Sketch of the Nasir<sup>®</sup> device.



**Fig. 2.** Nasal neutrophil count assessed before (T0) and after treatment (T1). Data are expressed as median, interquartile range, and standard deviation.



**Fig. 3.** Nasal eosinophil count assessed before (T0) and after treatment (T1). Data are expressed as median, interquartile range, and standard deviation.



**Fig. 4.** Nasal bacteria assessed before (T0) and after treatment (T1). Data are expressed as median, interquartile range, and standard deviation.

regulator: this aspect is relevant for the tolerability of the device. In addition, also the higher pressure is still too low to possibly damage the nasal mucosa or the middle ear through the Eustachian tube. The irrigator tip fits snugly and adapts to the nasal vestibule, permitting a fan-shaped spray of the solution inside the nasal cavity. By keeping the head bent forward during irrigation, the solution first flows through the nasal cavity closed off by the irrigator, and after reaching the rhinopharynx, crosses postero-anterior to the contralateral cavity, thus washing out with the saline solution not only pathogenic secretions but also dust, allergens and contaminants.

Moreover, it is noteworthy that it has been recently evidenced that nasal irrigation needs the use of an abundant quantity of liquid (18). Consistently, it has been reported that large-volume low-pressure nasal irrigation using undiluted seawater seems, in the present state of knowledge, to be the most effective protocol (19).

The main limitations of the present study were that: i) it was performed as an open trial, and the observed differences might be related to allergen exposure variations or changes in weather conditions; and ii) symptoms were not evaluated, thus definite conclusions could not be drawn by the current study. Therefore, there is the need to design and conduct further study to confirm these preliminary outcomes.

In conclusion, this pilot study reports that nasal irrigation with Nasir® might be useful to attenuate upper airway inflammation.

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## LETTER TO THE EDITOR

**HUMAN HERPESVIRUSES-6 AND -7 ENCEPHALITIS IN IMMUNOCOMPETENT INFANTS: ARE THEY REALLY SO UNCOMMON?**

L. MARSEGLIA, S. MANTI, G. D'ANGELO, G. STROSCIO, D. IMPOLLONIA, S. ARENA,  
C. SALPIETRO and E. GITTO

*Department of Human Pathology in Adult and Developmental Age "Gaetano Barresi"  
University of Messina, Italy*

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**Human herpesviruses-6 and -7 (HHV-6 and 7) are considered uncommon causes of central nervous system infection and may occasionally cause encephalitis in young infants, however, the clinical syndrome and incidence are not well defined. In immunosuppressed hosts, reactivation is associated with a worse outcome such as encephalitis, hepatitis, or graft rejection. In immunocompetent hosts, this persistent infection is generally of no consequence. We report 4 cases of immunocompetent critically ill children, affected by HHV-6 and -7 encephalitis, admitted to our Pediatric Intensive Care Unit. In three patients, herpesvirus polymerase chain reaction in blood and cerebrospinal fluid was positive for HHV-6, while one patient was positive for HHV-7. In our cases, a typical clinical picture of viral infection was not present but neurological symptoms were predominant. In all 4 children, neurological involvement rapidly regressed after acyclovir therapy. In this report, we offer evidence that HHV-6 and -7 primary infections can cause several clinical manifestations, such as encephalitis, also in immunocompetent hosts. In our experience, children with neurological symptoms suggestive of viral encephalitis should be fully investigated for these two viruses.**

To the Editor

The role of human herpesvirus (HHV)-6 and -7 in central nervous system (CNS) disease is an area of ongoing investigation. The range of CNS manifestations ascribed to these viruses includes asymptomatic infection, febrile convulsions, seizure disorders, meningitis, meningoencephalitis, and, rarely, fatal encephalitis (1). HHV-6 and HHV-7 are closely related *b-herpesviruses* that are universally acquired in early childhood (2, 3). Although HHV-6 affects over 90% of the population silently or with only minor symptoms, the infection is associated

with several diseases in immunosuppressed patients. However, in recent years, some cases of primary HHV-6 infection and neurological disease in young immunocompetent children have been reported (4). The key finding linking HHV-6 to these cases was the detection of viral DNA in cerebrospinal fluid (CSF) by polymerase chain reaction (PCR) (5), leading to the assumption that HHV-6 was replicating in the CNS. HHV-7 is closely related to HHV-6 and together they form the *Roseolovirus* genus in *Betaherpesvirinae* subfamily among *Herpesviridae* (6). HHV-7 is ubiquitous in the population, and more

*Key words: human herpesvirus, central nervous system infection, immunocompetent children, pediatric intensive care*

*Mailing address:*

Lucia Marseglia, MD  
Neonatal and Pediatric Intensive Care Unit,  
Department Of Human Pathology  
in Adult and Developmental Age "Gaetano Barresi"  
University of Messina, Italy  
Via Consolare Valeria, 1 - 98125 Messina, Italy  
Tel.: +390902213100 - Fax: +390902213876  
e-mail: lmarseglia@unime.it

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than 85% of individuals are infected, usually early in childhood (7). The virus remains latent in its host throughout life. The CD4 molecule is the essential cellular receptor for HHV-7 (8). HHV-6 and HHV-7 encephalitis are rare in immunocompetent patients, and clinical syndrome and incidence are not well defined (9). To our knowledge, cases have mainly been reported in adult patients. In this study, we examined the incidence of HHV-6 and -7 encephalitis in children admitted to our 2-bed Pediatric Intensive Care Unit (PICU) from August 2011, when HHV-6 and -7 virus detection by routine PCR in CSF was initiated, to August 2013. We identified 15 patients with meningo-encephalitis, 4 of whom were affected by HHV-6 and HHV-7 encephalitis.

#### *Cases 1 and 2 of HHV 6 encephalitis*

An 18-month-old, Italian male child, was admitted to the PICU for decreasing levels of consciousness (Gaslow Coma Scale -GCS- 6), headache, fever, speech difficulties, and seizures. Laboratory test values, including clotting and inflammatory index, were normal. Lumbar puncture revealed increased cell count (28 cells/mmc) with predominance of mononuclear cells, glucose level in cerebrospinal fluid (CSF) of 89 mg/dL, and total protein level of 17 mg/dL. The presence of viral genome in CSF and blood was investigated by PCR.

A 36-month-old, Italian female child, presented in the PICU for the sudden appearance of headache, fever, and decreasing levels of consciousness (GCS 7). Values of laboratory tests were normal except for a slight increase in inflammatory markers, including erythrocyte sedimentation rate and C-reactive protein. Lumbar puncture detected a CSF cell count of 42 cells/mmc, glucose level of 153 mg/dL, and total protein level of 77 mg/dL. PCR was used to detect viral genome in CSF and blood.

In both patients, herpesvirus PCR in CSF was positive for HHV-6, 359 viral copies/ml and 524 viral copies/ml, respectively, and negative for HSV-1, HSV-2, varicella-zoster virus, Epstein-Barr virus and cytomegalovirus. IgG titers for HHV-6 in serum specimens did not indicate past infection. Thus, the HHV-6 DNA finding in both patients' CSF was ascribed to primary viral infection.

Electroencephalography revealed changes consistent with herpes simplex virus encephalitis, while a brain magnetic resonance imaging (MRI) did not reveal abnormal findings. For MRI scan, melatonin was used to sedate the child (10, 11). Complete blood count with manual differential to count neutrophils, lymphocytes and platelets, lymphocyte subpopulations, quantitative serum immunoglobulin (IgG, IgA, IgM and IgE) levels, titers to protein and polysaccharide vaccines were performed in these patients to exclude immunodeficiency. In both children, neurological symptoms rapidly regressed after acyclovir therapy, carried out for 21 days. They showed no neurological sequelae after 6 months of follow-up.

#### *Case 3*

A 13-year-old Italian female, previously healthy, was admitted to the PICU for unconsciousness (GCS 5), status epilepticus, muscle hypertonicity and trismus. The patient was intubated and mechanically ventilated. Initial CSF investigation revealed pleocytosis with 115 cells/mmc, glucose 135 mg/dL, and total protein level 82 mg/dL. Herpes simplex viruses 1 and 2, Epstein-Barr virus, cytomegalovirus, HHV-6 and HHV-7 DNA were investigated in CSF and blood with a qualitative multiplex PCR. HHV-7 DNA was detected in CSF (12,783 viral copies/ml) and blood samples (21,684 viral copies/ml). The high IgG titers for HHV-7 suggested a virus reactivation. Immunodeficiency was excluded, as previously specified. Brain MRI was normal. Following initiation of acyclovir therapy, neurological symptoms rapidly regressed. The patient was extubated after 36 hours of mechanical ventilation and received intravenous acyclovir for 21 days, showing no neurological sequelae during 6 months of follow-up.

#### *Case 4*

A previously healthy 15-month-old boy was admitted to the intensive care unit for a typical, acute encephalopathy characterized by seizures, decreased level of consciousness (GCS 10), and ataxia. Due to further deterioration of consciousness, the patient required intubation and mechanical ventilation. A moderate elevation of serum aminotransferases



was found, without any other basic biochemical abnormalities. Serum and CSF antibodies for Epstein-Barr virus, herpes simplex virus 1 and 2, varicella-zoster virus, influenza A and B, and cytomegalovirus, were negative. HHV-6 was isolated from both CSF (477 viral copies/ml) and serum (851 viral copies/ml) with serial serum titers compatible with primary infection. Brain MRI demonstrated characteristic lesions of acute necrotizing encephalopathy of childhood (ANEC). Axial and coronal T1-, T2-, and coronal fluid-attenuated inversion recovery (FLAIR) weighted images showed hyperintensity in the thalami (Fig. 1), white matter of internal and external capsules, bilateral frontal, temporal lobes and insula. Hyperintensity was also detected in medium cerebellar peduncles, ponte and mesencephalus partly. After intravenous acyclovir for 21 days, neurological symptoms rapidly improved. However, during 6 months of follow-up the child presented lower extremity mild weakness with rigidity.

## DISCUSSION

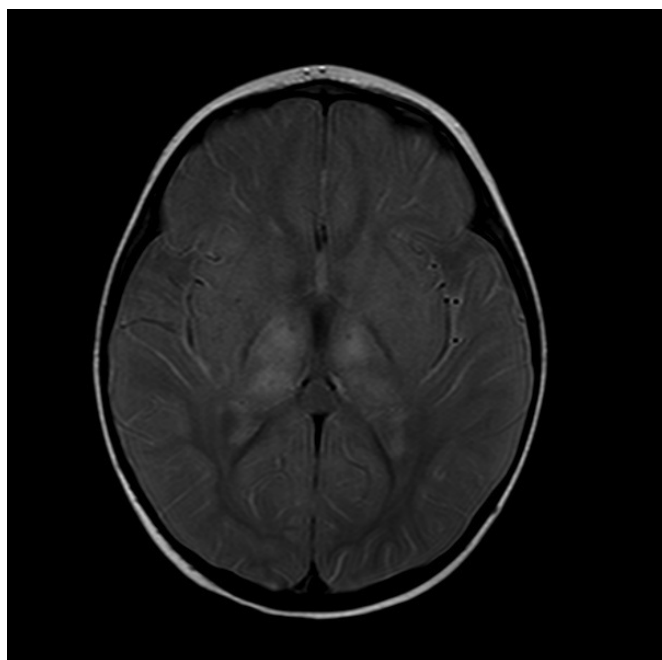
We describe 4 cases of immunocompetent children suffering from HHV-6 and HHV-7 encephalitis, one of whom was affected by ANEC. HHV-6 and -7 infections are usually self-limiting illnesses occurring in early childhood (12). Primary infection with HHV-6 was identified as the etiological cause for *Exanthema subitum* (13). HHV-7 infection is also common in children and has been found as a cause of fever and maculopapular rash without respiratory symptoms (14). In immunocompetent hosts, this infection is generally of no consequence. Our cases differ in the absence of rash and febrile seizures with the dominance of neurological symptoms suggestive of viral encephalitis.

Meningitis and encephalitis are reported in literature as common complications in immunosuppressed patients with reactivated HHV-6 and HHV-7 (14). In our children, specific investigations excluded immunodeficiency, and the absence of IgG titers for HHV-6 in serum indicated no past infection, while the HHV-7 encephalitis was associated with a viral reactivation. Several studies reported either basal ganglia involvement or diffusion abnormalities in

the cerebral white matter in brain MRI of patients with encephalopathy associated with HHV-6 (15). However, MRI did not reveal abnormal finding in 3 of our patients. Conversely, in the youngest of our patients, MRI showed characteristic lesions of ANEC that represent an extremely infrequent form of acute encephalopathy of unknown aetiology. Although influenza A is the major associated virus, influenza virus, herpes simplex virus, and HHV-6 have been reported (16). In our patient, extensive clinical and laboratory evaluation yielded evidence of acute infection with HHV-6. The clinical course of ANEC is frequently fulminant, with rapid onset of convulsions, and variable degrees of hepatic dysfunction. Affected patients have high mortality and severe neurologic sequelae. In our case, the neurologic function decline occurred rapidly and then gradually improved. Ganciclovir and foscarnet have proven to be more effective than acyclovir for some immunosuppressed patients with HHV-6 induced encephalitis (17). In most of the reported cases involving immunocompetent patients, a specific treatment regimen was not given or was not available (9). Our cases were treated with intravenous acyclovir for 21 days. To perform the MRI scan, children were sedated using oral melatonin (18, 19). Moreover, as high endogenous melatonin levels have been found in critically ill children (20), we evaluated serum nocturnal melatonin levels in patients 1 and 2. In both children, melatonin levels at 01.00 am were higher than published age matched controls (21): 675 pg/ml and 462 pg/ml, vs  $210 \pm 35$  pg/ml, respectively, of published data (21).

The children were followed-up for 6 months and showed no neurological sequelae, except for the child with ANEC who continued to show motory disorders. To our knowledge, HHV-7 encephalitis is a rare event in non-immunodeficient subjects. It has been reported that the clinical manifestations of primary and reactivated HHV-7 infection are similar (7). However, in our case, no skin rash was present and neurological symptoms were predominant.

In conclusion, HHV-6 and HHV-7 are considered uncommon causes of CNS infection in children (7). We report both cases of primary infections and reactivation, and, as viral load was heterogeneous and patients were all immunocompetent, we can speculate



**Fig 1.** Axial T1-weighted brain MRIs showing hyperintensity of the thalami.

that interindividual genetic differences could explain the seriousness of the neurological picture in these patients. However, there are no studies reporting the absolute incidence of HHV-6 and -7 infections. HHV-6 primary infection is associated with 10-20% of febrile convulsions in infants (22). Primary infection with HHV-6 is frequently related with febrile convulsion, predisposing to subsequent possible development of epilepsy. Mamishi et al. detected HHV-6 DNA in 6% of CSF specimens obtained from 100 immunocompetent children under 2 years of age admitted for febrile convulsion (23). Therefore, the authors recommend the evaluation of CSF (detecting HHV-6 DNA by PCR) in children with febrile convulsion. Kawamura and Colleagues, first assessed viral copy number in CSF samples collected from HHV-6 encephalitis patients to investigate the relationship between primary infection or reactivation and viral load in CSF. They found that DNA copies were significantly higher in patients with post-transplant HHV-6 encephalitis than in patients with primary infection. Our data support this finding, as viral load was lower in children with primary infection (cases 1, 2, and 4) than in the patient with reactivation (24).

In our population of critically ill children admitted to PICU, HHV-6 and HHV-7 DNA were detected in 19.8%, and 6.6%, respectively. The evidence associating HHV-6 and -7 with cases of encephalitis of unknown cause has grown increasingly persuasive and the scale of the contribution of HHV-6, and even more of HHV-7, infection to serious neurological disease and encephalitis is probably not well known. Thus, it is reasonable to consider these viruses when testing individuals for encephalitis. In our experience, children with neurological symptoms suggestive of viral encephalitis should be fully investigated for these two viruses. In addition, the diagnosis of ANEC should be considered in children with rapid neurologic deterioration after a short viral illness. It is clear that a great deal more is known on the behaviour and role of HHV-6 than on HHV-7, and it is therefore necessary to concentrate on the latter virus for future studies.

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## LETTER TO THE EDITOR

**PARASYMPATHETIC NERVOUS SYSTEM INVOLVEMENT IN FOOD ALLERGY:  
DESCRIPTION OF A PAEDIATRIC CASE**

G. VITALITI<sup>1</sup>, P. PAVONE<sup>1</sup>, G.R. GIUGNO<sup>2</sup>, M. VECCHIO<sup>3</sup>, S. COCUZZA<sup>4</sup>, A. SERRA<sup>4</sup>,  
L. MAIOLINO<sup>4</sup>, P. DI MAURO<sup>4</sup>, R. LUBRANO<sup>5</sup> and R. FALSAPERLA<sup>1</sup>

<sup>1</sup>*Paediatric Operative Unit and Acute and Emergency, Vittorio-Emanuele University Hospital, University of Catania, Italy;* <sup>2</sup>*Paediatric and Neonatal Operative Unit, Gravina Hospital, Caltagirone, Italy;* <sup>3</sup>*Centre of Physical Medicine and Rehabilitation, Policlinico-Vittorio Emanuele Hospital, University of Catania, Italy;* <sup>4</sup>*ENT Department G.F. Ingrassia, Policlinico-Vittorio Emanuele University Hospital, University of Catania, Catania, Italy;* <sup>5</sup>*Pediatric Department, Pediatric Nephrology Operative Unit of La Sapienza University of Rome, Rome, Italy*

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The latest research data emphasize the interaction between the nervous and the immune systems. It has been demonstrated that the central nervous system (CNS) can be involved secondarily due to blood brain barrier (BBB) disruption via pro-inflammatory cytokines released in allergy. More recently it was demonstrated that the parasympathetic nervous system (PNS) could also be equally involved in models of peripheral inflammation such as food allergy; although this last clinical presentation has rarely been described. Herein, the authors report the case of a five-year-old Caucasian female who was admitted to our Pediatric Acute and Emergency Operative Unit for cyclic vomiting. Her vomiting, which was preceded by objective torque vertigo, headache and weakness, had been recurring with constant frequency every two months since she was 3 years old. After a complex diagnostic flow-chart, it was found that this spectrum of neurologic symptoms was due to a food allergy syndrome, which postulates some etiopathogenic hypotheses to explain the relationship between the two mentioned diseases.

To the Editor,

Recent research data have shown the interaction between the nervous system and immune system. It has been demonstrated that disruption of the blood brain barrier (BBB) secondary to peripheral inflammation may play a key role in this relationship. This assumption is linked to recent findings suggesting that constituent units of BBB are not only neurological components, but have been reconsidered as “neurovascular” elements in which immune system molecules are transferred within the central nervous system (CNS).

From a clinical point of view, several authors have tried to demonstrate this link, describing the CNS involvement in allergy (1-4), and highlighting a clinical correlation between seizures and allergy to both inhalants and food.

More recently, some reports in murine experimental models have also shown a link with the parasympathetic nervous system (PNS). This association confirms that inflammation involves the nervous system in a multidimensional fashion. Even though the involvement of the CNS in allergy has been established, the role of PNS remains unclear

*Key words: parasympathetic nervous system, immune system, paediatric, case report, peripheral inflammation*

## Mailing address:

Dr Giovanna Vitaliti,  
AOU Policlinico-OVE,  
Via Plebiscito n. 628,  
95100 Catania, Italy  
Tel.: +39 0957435254  
e-mail: giovitaliti@yahoo.it

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and has to be more extensively studied.

This study reports the case of a five-year-old Caucasian female, who was admitted to the Pediatric Acute and Emergency Operative Unit for cyclic vomiting. Her symptoms were recurring with a constant frequency every two months, and preceded by objective torque vertigo, headache and weakness since she was 3 years old. After a complex diagnostic flow-chart, it was found that this spectrum of neurologic symptoms was occurring due to a food allergy syndrome. This case represents some etiologic hypotheses to explain the association between the two mentioned diseases.

### *Case report*

A five-year-old female Caucasian child was admitted to the Paediatric Acute and Emergency Operative Unit, Vittorio-Emanuele Hospital, Catania, Italy. She had suffered from cyclic vomiting, mostly preceded by objective torque vertigo, headaches and weakness, recurring with a constant frequency of every two months, since she was 3 years old.

The child was the second-born of non-consanguineous parents, born on term by caesarean section; and had favourable gestational age. Her familial history showed a high prevalence of allergy in her parents (her mother and father suffered from inhalant allergy), and her brother (who suffered from both inhalant and food allergy).

Physical examination on admission suggested within normal range developmental parameters; and her neurological examination was also normal. Cardiac, respiratory and abdomen examinations were also normal. Routine blood analyses were within the normal range, except for total IgE dosage that was higher than normal (340 UI/Lit, normal value: 70 - 100 UI/Lit).

Due to persistence of her symptoms, a cardiac evaluation by Doppler-echocardiogram and tilt test were performed, in addition to an EEG, cerebral MRI, nerve conduction study, ophthalmologic and ENT consulting, including visual and auditory evoked potentials. All the mentioned tests were normal. The patient was therefore discharged with a diagnosis of cyclic vomiting. In spite of this diagnosis, her symptoms persisted and were associated with the

onset of recurrent abdominal pain between the cyclic vomiting episodes. Thereafter, the child was readmitted to the hospital almost 8 months after her first hospitalization.

Routine blood analysis and BGA were still within the normal range. During her second admission, the patient underwent a specialist consultancy for allergy in which skin prick tests (SPT) for inhaled and food allergens were carried out. The skin prick tests resulted in a slight positivity for casein and tomato (the diameter of her wheal was 3.5 mm). Thereafter, she underwent a Radioallergosorbent Test (RAST) evaluation for food allergens, which confirmed the positive results of skin prick tests (casein > 3.45 UI/ml and tomato > 3.80 UI/ml). Considering these results, she was started on a casein and tomato-free diet which was subsequently followed by complete resolution of her symptoms.

The patient did not express any more cyclic vomiting episodes, headache or weakness during follow-up. She is currently following a phase of reintroduction of casein and tomato according to the standard desensibilization protocols (2); and on low dosages is showing no further neurological symptoms.

## DISCUSSION

Herein, the authors report the case of a five-year-old female child with cyclic vomiting, recurring with a constant frequency every two months, and preceded by objective torque vertigo, headache and weakness since she was 3 years old, which was caused by food allergy.

This case is an example of association between food allergy and secondary involvement of the parasympathetic nervous system (PNS). This is a novel literature finding, as the only clinical report on the association between allergy and PNS involvement in childhood was described by Wu H et al. (5), in a 13-year-old boy with frequent episodes of vertigo and auditory symptoms. In that case, Ménière's disease (MD) was diagnosed, but failed to respond to conventional treatment. Allergy testing revealed serious reactions to several allergens and autonomic tests showed that the patient was suffering



from autonomic neuropathy. An allergen-restricted diet and treatment of dysautonomia were effective, and the patient was vertigo-free within 2 months. The authors therefore concluded that the autonomic nerves and the immune system can interact (4). To our knowledge, this is the only clinical case described in literature on the association between allergy and PNS disorders. The case in the present study is the second one, with an earlier onset of symptoms since early infancy.

From a laboratory point of view, immunochemistry data have demonstrated the expression of immunoglobulin E (IgE) receptor on the Vagus nerve in the mouse intestine. In this regard, in 2011 Liang H et al. isolated Vagus ganglion neurons (VGN) from mice and cultured them *in vitro*. They examined the IgE receptor/IgE complex on VGN in mouse models of food allergy (6). The authors found a high-affinity IgE receptor on the intestinal Vagus nerve, and demonstrated an increased expression of the IgE receptor on the Vagus nerve in the intestines of mice with intestinal immune inflammation. Isolated mouse VGN expressed IgE receptor I, which could form complexes with IgE. Re-exposure to specific antigens activated the sensitized VGN, manifesting the release of transmitter glutamate that could activate dendritic cells by increasing the expression of CD80 and major compatibility complex class II and suppressing interleukin-12. The partial removal of the Vagus nerves suppressed Th2 intestinal inflammation (6).

More recently, in 2014, Yamamoto et al. (7) investigated the cholinergic anti-inflammatory pathway in murine models as a regulatory system to recover disrupted mucosal immune homeostasis in the intestines. In this study, BALB/c mice sensitized with ovalbumin received repeated oral ovalbumin to determine the onset of food allergy (FA). FA mice exhibited severe allergic diarrhea, and demonstrated enhanced type 2 helper T (Th2) cell immune responses in both systemic immunity and mucosal immunity, which was accompanied with hyperplasia of colon mucosal mast cells. However, p85a corrupted phosphoinositide-3 kinase-deficient mice that lacked mast cells in the gut did not represent these symptoms. Vagal stimulation by 2-deoxy-D-glucose and drug

treatment with nicotinic ACh receptor (nAChR) agonists (nicotine, and  $\alpha 7$  nAChR agonist GTS-21), ameliorated allergic symptoms of FA mice. Nicotine treatment suppressed mucosal mast cell hyperplasia and upregulated mRNA expression of Th1 and Th2 cytokines in colon of FA mice. Mucosal mast cells, which were negatively regulated by  $\alpha 7$ -nAChRs, were shown to be often located in close proximity to cholinergic CGRP-immunoreactive nerve fibers in the FA mice colon. The authors concluded that the cholinergic neuro-immune interaction via  $\alpha 7$ -nAChRs on mucosal mast cells is largely involved in preserving intestinal immune homeostasis, an indication that could be target for a novel treatment against mucosal immune diseases (7).

In general, these studies highlight the relationship between the immune system and the parasympathetic nervous system and represent a new research scenario revolving the association between PNS and immune system, possibly targeting the PNS for those clinical disorders involving the two mentioned systems. The present case report highlights this relationship, and the authors believe that healthcare assistants should exclude the presence of allergy in case of autonomic symptoms such as vertigo or Vagal impairment.

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## LETTER TO THE EDITOR

**FAVOURABLY EFFECTIVE FORMULATION OF SODIUM IODIDE AND SALICYLIC ACID PLUS PROFESSIONAL HYGIENE IN PATIENTS AFFECTED BY DESQUAMATIVE GINGIVITIS**

P. CARCIERI, R. BROCCOLETTI, S. GIACOMETTI, A. GAMBINO,  
D. CONROTTO, M. CABRAS and PG. ARDUINO

*Department of Surgical Sciences, Cir-Dental School, University of Turin, Italy*

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The aim of this prospective pilot study was to evaluate the efficiency of an oral hygiene protocol, in combination with a solution of sodium iodide associated to salicylic acid (SISA), in patients affected by desquamative gingivitis (DG). Twenty patients not totally responding to conventional topical therapies, were selected. They received oral hygiene instructions with non-surgical periodontal therapy in a 21-day cohort study (during 3 weekly appointments). The SISA was used at the end of each session, with an impregnated gauze (with 5 ml of the solution) applied for 15 minutes for the upper jaw, and for a further 15 minutes with a new gauze for the lower. Evaluated clinical outcome variables included the full mouth plaque (FMPS) and bleeding (FMBS) scores, probing depth, patient related outcome and clinical gingival signs. Two months after concluding the planned protocol, a statistically significant reduction was observed for FMPS ( $P=0.032$ ), FMBS ( $P=0.038$ ), reported pain ( $P=0.000$ ) and gingival clinical improvement ( $P=0.005$ ). Topical application of SISA and professional oral hygiene procedures are connected with improvement of gum status, and decrease of related pain in subjects affected by severe DG.

To the Editor,

Desquamative gingivitis (DG) is neither a specific disorder nor recognizes a single aetiopathogenesis, it simply represents the gingival manifestation associated with some heterogeneous mucocutaneous disorders (e.g. oral lichen planus, mucous membrane pemphigoid, pemphigus vulgaris) and is usually characterized by epithelial desquamation, erythema and erosive and/or vesiculo-bullous lesions (1-5). DG has no association with loss of attachment and alveolar bone destruction, nonetheless, it can significantly compromise the patient's approach to correct oral hygiene and this could denote a possible risk factor for long-standing periodontal health (6-9). There have been limited studies regarding

therapy for DG (6). We recently demonstrated that detailed oral hygiene procedures and correct non-surgical periodontal therapy could be associated with improvement of clinical and patient related outcomes in distinctive cases (10-12).

Fertomcidina-*U*<sup>®</sup> is a novel formulation, composed of a salsobromiodic solution containing salicylic acid and magnesium bisphosphate, having a strong bactericide and fungicide action, applicable on human skin and mucosae for tissue reparation and re-epithelization (13, 14). Because of its action, which is related to the sodium-iodide that in the presence of oxygen acts as iodophor, developing small amounts of iodine, this medication should be contraindicated in patients with thyroid disorders.

*Key words: desquamative gingivitis, oral hygiene, non-surgical periodontal treatment, sodium iodide associated to salicylic acid*

*Mailing address:*

Dr. Paolo G. Arduino,  
Department of Surgical Sciences, Oral Medicine Section,  
CIR-Dental School, University of Turin,  
Via Nizza 230, 10126 Turin, Italy  
Tel.: +39 0116331522 - Fax: +39 011618639  
e-mail: paologiacomo.arduino@unito.it

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The aim of this study was to evaluate the clinical efficiency of an oral hygiene protocol, in combination with a solution of sodium iodide associated to salicylic acid, in patients affected by DG not completely responding to conventional topical therapies.

## MATERIALS AND METHODS

Subjects with histologically proven DG oral lichen planus (OLP), plasma cell gingivitis (PCG), mucous membrane pemphigoid (MMP) and pemphigus vulgaris (PV) were selected among individuals regularly visited at the Oral Medicine Unit of the CIR – Dental School of the University of Turin, Italy, between January 2012 and September 2015. The patients had been treated topically (and also systemically in cases with a diagnosis of PV) in the preceding 12 months.

Exclusion criteria included: i) history of periodontal therapy (surgical and non-surgical) in the previous 6 months; ii) less than 18 teeth, iii) pregnancy and iv) diabetes mellitus.

All eligible candidates for this study were informed about the experimental protocol, and signed a consent form. The ethics review board of the CIR - Dental School approved the study (prot. N° II-pato-2011CD).

A prospective case series protocol was designed. Patients received a complete periodontal examination at baseline visit, including full mouth plaque scores (FMPS), full mouth bleeding upon probing scores (FMBS) and probing pocket depth (PPD). Activity scores [modified from Escudier and co-workers (15)], including extent and severity of the gingival lesion (site score), were documented as previously reported (10). Scores ranged from 1 to 12, with higher results indicating a worse clinical aspect. Patient related outcomes included pain perception assessed at each visit by Visual Analogue Scale (VAS). The VAS consisted of a 10-cm horizontal line marked with 0 (=no pain) to 10 (=most severe pain experienced). Total resolution of all clinical symptoms was defined as the absence of any discomfort, corresponding to a zero VAS score. Partial response, worsening, or persistence of the patient's condition meant a decrease, increase, or no change at all, respectively, in the patient's score.

An experienced dental hygienist provided thorough supra- and sub-gingival scaling over 3 weeks (once

weekly). During each visit, subjects were instructed about correct oral hygiene maintenance at home; such instructions were reinforced at each visit and were personalised when necessary. Fertomcidina-*U*<sup>®</sup> (Theriaca S.r.l., Rome, Italy) was used at the end of each of the 3 sessions: a gauze impregnated with 5 ml of the solution was applied for 15 min for the upper jaw and for a further 15 min for the lower using a new impregnated gauze. Patients were advised not to swallow the medication, and to avoid eating and drinking for at least 3 hours after the treatment.

The technical procedures provided in the protocol are fully detailed in Table I.

Comparative statistics were performed between T0 and T5. Paired samples test was used to test the difference in FMBS, FMPS and PPD. Wilcoxon's signed rank was used to calculate the significance of the patient related outcomes (VAS) and clinical outcome (activity score). P-values  $\leq 0.05$  were considered to be statistically significant. SPSS (SPSS for windows, version 11, SPSS inc, Chicago, IL, USA) statistical software was utilized.

## RESULTS

Twenty patients were included in the study: 9 with OLP, 6 with PV, 4 with PCG and 1 with MMP. The mean age at baseline was 56.95 ( $\pm 16.75$ ) years. Three subjects were male (15%).

Table II shows the comparison of selected data during the protocol period. A reduction in FMBS ( $P=0.032$ ) and FMPS ( $P=0.038$ ) were observed. Moreover, a statistically significant reduction in symptoms reported was observed with a reduction in VAS scores ( $P=0.000$ ). Also, interestingly, the clinical gingival involvement improved after the proposed therapy ( $P=0.005$ ).

All subjects were initially advised of the possible unpleasant taste of the medication, and 60% of them still reported this problem at the end of the follow-up period, but none of them were unable to complete the treatment. No patient complained of other adverse effects.

## DISCUSSION

This is the first prospective case series of DG

patients treated with a solution of sodium iodide associated to salicylic acid in combination with non-surgical periodontal therapy. This initial pilot analysis suggests that the proposed protocol could be a possible means for reducing clinical gingival inflammation and improving outcomes in patients with different forms of DG.

It has been reported that several systemic diseases, affecting the gingiva, could have an inflammatory profile because of two main reasons: one may be a non-specific inflammatory response to plaque (thus a plaque-induced inflammation); another response may be due to a specific disease or agent, today not well understood (5, 6).

No conclusive standard of care has been set for DG patients and there is no prospective literature on long-term management of these subjects (4). The present report was developed by taking motivation from conclusions achieved by earlier works reporting that patients affected by different DG conditions and treated with non-surgical periodontal therapy are connected with improvement of gingival status and decrease in gingival-related pain (10-12). However, plaque removal alone cannot induce severe DG regression. For this reason it was decided to add Fertomcidina-*U*<sup>®</sup> topically to accelerate the healing process. In fact, it has been reported that the salicylic acid acts by environment acidification, with different

**Table I.** *Clinical protocol used.*

---

*Time 0 (T0):*

- Oral evaluation and comprehensive measurements\*
- 

*Time 1\_Day 7 (T1):*

- Scaling and root planing
- “Fertomcidina-U” topical application
- Oral hygiene instruction:
  - a) use a ultra-soft toothbrush<sup>1</sup> for manual brushes, placing the bristles at a 45° angle to the tooth surface at the gum edge and then move the bristles back and forth in short (tooth-wide) strokes or small circular movements

*Time 2\_Day 14 (T2):*

- Scaling and root planing
  - “Fertomcidina-U” topical application
- 

*Time 3\_Day 21 (T3):*

- Scaling and root planing
  - “Fertomcidina-U” topical application
  - Oral hygiene instruction:
    - a) use a super-soft toothbrush<sup>2</sup> for manual brushes, placing the bristles at a 45° angle to the tooth surface at the gum edge and then move the bristles back and forth in short (tooth-wide) strokes or small circular movements
- 

*Time 4\_Day 49 (T4\_1 month after finishing the proposed protocol):*

- Oral hygiene instruction:
    - a) use a soft toothbrush<sup>3</sup> for manual brushes, placing the bristles at a 45° angle to the tooth surface at the gum edge and then move the bristles back and forth in short (tooth-wide) strokes or small circular movements
  - Oral evaluation; VAS measurement and activity score
- 

*Time 5\_Day 77 (T5\_2 months after finishing the proposed protocol):*

- Oral evaluation and comprehensive measurements\*
- 

\* *Comprehensive measurements include:* - VAS (visual analogue scale), a chromatic scale graduated from 0 (no pain) to 10 (unbearable pain), where it defines intensity of pain strong or very strong. - FMBS (full mouth bleeding score). - FMPS (full mouth plaque score). - PPD (probing pocket depth). - Activity Score: to value the extent and severity of the gingival lesion.

<sup>1</sup>Curaprox CS 5460; <sup>2</sup>Curaprox CS 3960; <sup>3</sup>Curaprox CS 1560

**Table II.** Comparison of selected data at time 0 (T0), and at one (T4) or two (T5) months after the proposed protocol.

|                                      | <b>T0</b>     | <b>T4</b>   | <b>T5</b>     | <b>p*</b> |
|--------------------------------------|---------------|-------------|---------------|-----------|
| <b>Full mouth bleeding score (%)</b> | 59.00 ± 16.25 | -           | 23.88 ± 11.11 | 0.032     |
| <b>Full mouth plaque score (%)</b>   | 45.02 ± 13.63 | -           | 29.20 ± 2.73  | 0.038     |
| <b>Probing depth (mm)</b>            | 2.48 ± 0.51   | -           | 2.29 ± 0.67   | 0.791     |
| <b>Referred symptoms (VAS score)</b> | 5 ± 1.32      | 3.38 ± 1.19 | 2.44 ± 1.42   | 0.000     |
| <b>Activity score</b>                | 6.48 ± 1.20   | 3.08 ± 1.77 | 2.81 ± 1.79   | 0.005     |

\*Comparative statistics were performed between T0 and T5. Paired samples test was used to test the difference in FMBS, FMPS and PPD. Wilcoxon's signed rank was used to calculate the significance of the patient related outcomes and in gingival clinical outcome (activity score).

anti-inflammatory assets, sometimes comparable to corticosteroids (16). The keratoplastic properties are shown by studies reporting both an increased thickness of epithelial tissues and mitotic index of the basal layer (17, 18). This is the rationale to use this compound in treating epithelial inflammatory diseases. Moreover, this medication could be used to treat dissimilar kind of oral pathogens, not only the typical periodontal ones but also others quite different, normally resident in the oral cavity as well as introduced in other ways (19, 20).

Non-surgical periodontal therapy is the most common therapeutic procedure for patients with periodontal diseases, eliminating or reducing the microbial counts of subgingival flora following improvements in clinical parameters and oral health of patients (21). Furthermore, it is now clear that the previous statement is effective also for DG. Oral hygiene improved in all participants. This is bound to the time devoted to oral hygiene enactment and home directives. Patients were favourably motivated to perform their routine home maintenance and, above all, their cooperation was indispensable.

The positive clinical results obtained with appropriate professional oral hygiene could serve as

a basis for recommending this as a complementary therapeutic intervention, especially in patients with pure gingival involvement, in order to decrease gingival inflammation and related pain and help affected patients in maintaining a good oral hygiene. Moreover, the simple use of a topically applied solution of sodium iodide associated to salicylic acid could improve the healing process, without adverse effects. However, it is important to remember that the main limitations are the lack of a control group, and that there were no intra-reliability analysis of the examiner and no blinding included in the design. For these reasons, additional randomized trials, with different approaches and a larger sample size, are needed.

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## LETTER TO THE EDITOR

PLASMA ENDOCANNABINOID BEHAVIOUR IN TOTAL KNEE  
AND HIP ARTHROPLASTY

R. OTTRIA<sup>1</sup>, L. CAPPELLETTI<sup>2</sup>, A. RAVELLI<sup>3</sup>, M. MARIOTTI<sup>3,4</sup>, F. GIGLI<sup>3</sup>,  
S. ROMAGNOLI<sup>5</sup>, P. CIUFFREDA<sup>1</sup>, G. BANFI<sup>6</sup> and L. DRAGO<sup>2,7</sup>

<sup>1</sup>Department of Biomedical and Clinical Sciences “Luigi Sacco”, University of Milan, Milan, Italy;

<sup>2</sup>Laboratory of Clinical Chemistry and Microbiology, IRCCS Galeazzi Orthopaedic Institute, Milan, Italy; <sup>3</sup>Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy;

<sup>4</sup>IRCCS Galeazzi Orthopaedic Institute, Milan, Italy; <sup>5</sup>Department of Joint Replacement, IRCCS Istituto Ortopedico Galeazzi, Milan, Italy; <sup>6</sup>Scientific Direction, IRCCS Galeazzi Orthopaedic Institute, Milan, Italy; <sup>7</sup>Biomedical Sciences for Health, University of Milan, Milan, Italy

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Endocannabinoids are a class of lipid mediators involved in a wide range of physiological pathways including pain perception, and immunological defences. In particular, the involvement of endocannabinoids in bone metabolism and bone resorption has recently been studied. Moreover, one study on total knee arthroplasty describes the probable role of endocannabinoids in pain perception after surgery. The aim of the present study was to evaluate variations of endocannabinoid concentrations in patients undergoing total hip or total knee arthroplasty before and after surgery. Sera from 23 patients were collected at three different times: before surgery and at two different times during rehabilitation, and endocannabinoids were quantified by HPLC-MS/MS analysis. Mean values of endocannabinoids in presurgical serum samples were:  $6.11 \pm 0.5$  ng/ml for N-palmitoylethanolamide,  $1.39 \pm 0.08$  ng/ml for N-stearoylethanolamide,  $4.84 \pm 0.04$  ng/ml for N-oleoylethanolamide,  $0.44 \pm 0.03$  ng/ml for N-arachidonylethanolamide,  $0.84 \pm 0.05$  ng/ml for N-linoleoylethanolamide,  $0.17 \pm 0.01$  ng/ml for N- $\alpha$ -linolenylethanolamide. Statistical analysis showed a significant decrease of all the endocannabinoids after surgery, while there were no remarkable differences between total hip and total knee arthroplasties or between genders. Moreover, the results show no significant correlation between endocannabinoid concentrations and C-reactive protein and Erythrocyte sedimentation rate. The present study shows for the first time a specific and univocal behaviour of six endocannabinoids and N-acylethanolamides in orthopaedic surgery, suggesting the endocannabinoid system as a possible pharmacological target for presurgical therapeutics.

To the Editor,

Endocannabinoids (eCBs) belong to the superfamily of long chain fatty acid amide derivatives. The different saturation of carboxylic aliphatic chain of the various eCBs is crucial for determining the

effects on cannabinoid receptors (CB1, CB2) and also to additional ones as the vanilloid receptor type 1 (TRPV1) (1, 2). The effects of eCBs are evident in various physiological pathways, as appetite control, energy balance, pain perception, and

*Key words: endocannabinoids, pain, total knee arthroplasty, hip arthroplasty*

Mailing address:

Dr P. Ciuffreda,  
Department of Biomedical and Clinical Sciences “Luigi Sacco”,  
University of Milan, Via G.B. Grassi 74, 20157 Milano, Italy  
Tel.: +390250319695 - Fax: +390250319695  
e-mail: pierangela.ciuffreda@unimi.it

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immunological defences. Moreover, their effects on bone metabolism have been recently studied: the data are indicating the modulation of osteoblasts and osteoclasts (3). However, the reported effects on bone metabolism and on the balance between bone resorption and bone formation are quite contentious, probably due to different quantities and mixes of molecules evaluated, methodological pitfalls, and different physiopathological conditions where the eCBs were studied (4-6). We recently demonstrated in an animal model represented by *Danio rerio* (zebrafish) that AEA and L- $\alpha$ -linoleoylethanolamine (LEA) are increasing alkaline phosphatase activity in fish scales, where bone cells are active, through the activation of CB2 receptor (7). Thus, a direct effect of some eCBs on bone is demonstrated: the role of specific molecules should be elucidated, while some of these could stimulate bone formation, some do not modify the bone metabolism. A previous study on total knee arthroplasty (TKA) described the link and probable role of eCBs with pain perceived by patients after surgery (8). Azim and co-workers evaluated the levels of four eCBs in serum, cerebrospinal and synovial fluids of patients with painful osteoarthritis undergoing TKA, finding higher levels of 2-arachidonoyl glycerol in patients with higher postoperative pain. Moreover, local increase of the anti-inflammatory palmitoylethanolamide correlated with functional disability. The aim of our study was to evaluate variations of seven eCB concentrations in patients undergoing total hip arthroplasty (THA) or TKA before and after surgery to understand their behaviour in orthopaedic surgery. The comprehension of eCB involvement in bone metabolism and pain management could allow to identify the endocannabinoid system as a new target for presurgical therapies.

## MATERIALS AND METHODS

### *Patients*

Patients undergoing THA or total TKA in the IRCCS Galeazzi Orthopaedic Institute, Milan, Italy over a two-week period were consecutively enrolled in the study during. The study was approved by the Ethics Committee of the Institution, and at the pre-surgery visit all participants

gave informed consent to participate. Blood samples were included in the patient workup. All procedures performed in the study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1975 Declaration of Helsinki and its later amendments or comparable ethical standards. Twenty-three patients were enrolled, 10 male and 13 female aged between 55-85 years with a mean age of 68, eight of whom were to undergo hip replacement, and fifteen to knee arthroplasty. The patient group was homogeneous, while the surgical procedures, including instruments and drugs, the surgeon, and the surgical staff (anaesthesiologist, nurses) were identical for all the patients. Drugs used for controlling pain, blood and plasma transfused or recovered to maintain the correct blood/plasma volumes were registered. Patients did not receive analgesic drugs on post surgical days (11-22 days).

Dill and Costill equation was applied to evaluate possible influence of plasma volume differences, due to blood lost during surgery, on eCB quantification. Blood samples were taken before surgery, during presurgical controls, approximately two weeks before admission to the hospital, and 24 hours after surgery, and, finally, during the rehabilitation period, from 11<sup>th</sup> to 22<sup>nd</sup> day after arthroplasty. Two specimens were collected during rehabilitation, the first at the beginning (11<sup>th</sup> -14<sup>th</sup> days), the second at the end (19<sup>th</sup> - 22<sup>nd</sup> days). The average values calculated on these two specimens are pictured in results, to simplify the behaviour of the parameters during the entire process of THA/TKA.

### *Biochemical and haematological parameters measurements*

Biochemical and haematological parameters were strictly controlled. Haematological parameters were measured by XE-2100 (Sysmex, Tokyo, Japan), a flow cytometer that uses a separate channel for haemoglobin analysis, minimizing interference from high leukocyte concentrations. The haematocrit (HCT) is directly determined, based on the count and volume detection of each individual red blood cell. Imprecision of biochemical and haematological parameters was <3%. C-reactive protein (CRP) was measured by Architect c4000 (Abbott Diagnostics, Illinois, USA). The quantitation of CRP in serum was made by a latex particle enhanced turbidimetric immunoassay. The test is based on the photometric measurement of the agglutination between anti-human-

CRP coated latex particles and CRP present in the sample. The agglutination was detected as an absorbance change, which allows for the quantification of CRP in the sample via a calibration curve. Erythrocyte sedimentation rate (ESR) was measured by Test1 SDL (Alifax, Polverara (PD), Italy), an automated haematology system that uses the technology based on the aggregation capability of red blood cells while changing the optical density (photometric measurement of the kinetics of aggregation of erythrocytes). Sera for eCB quantification were collected from dry tubes (Vacutainer, Becton Dickinson, Rutherford, NJ, USA) after centrifugation, and they were stored at -80°C until analysis.

N-palmitoylethanolamide (PEA, C16:0), N-stearoylethanolamide (SEA, C18:0), N-oleoylethanolamide (OEA, C18:1), Anandamide (N-arachidonylethanolamide, AEA, C20:4), N-linoleoylethanolamide (LEA, C18:2), N- $\alpha$ -linolenylethanolamide (LNEA, C18:3) and N-eicosapentaenoylethanolamide (EPEA, C20:5) were quantified by HPLC-MS/MS using the previously reported method (9). Quantification was carried out on an ABSciex 5600 TripleTOF mass spectrometer (AB Sciex, Foster City, CA, USA) coupled with an Agilent 1200 Infinity pump LC system equipped with an Agilent 1290 Infinity autosampler (Agilent Technologies, Waldbronn, Germany). Briefly, 0.5 ml of serum were spiked with internal standard solution [ $d_4$ -SEA and  $d_4$ -LNEA synthesized as previously described (10)] and 800  $\mu$ l of cold acetone were added for protein precipitation. After

centrifugation, supernatants were purified by solid phase extraction on solid phase extraction cartridges OASIS HBL (30 mg/mL) purchased from Waters (Etten-Leur, the Netherlands). The extraction was performed with 750  $\mu$ l of acetonitrile (HPLC grade, Sigma-Aldrich Munich, Germany), containing 1% of formic acid, and eluates were collected and maintained at -80°C until the analyses. Quantifications were performed using Multiquant 1.2.1 software by AB Sciex. All extraction and purification experiments were performed in duplicate and all eluates were injected (20  $\mu$ l) and analysed two times, mean of the obtained four values were used for statistical analyses.

Statistical analyses were performed with GraphPad Prism 5: significance of eCBs levels variations were assessed by Student's *t*-test while correlation matrix was built to evaluate correlation between eCBs concentrations and CRP and ESR levels.

## RESULTS

Quantification of eCBs in presurgical serum samples (pr) and in samples collected during rehabilitation, p1 (11<sup>th</sup> -14<sup>th</sup> days) and p2 (19<sup>th</sup> - 22<sup>nd</sup> days), data corrected by plasma volume modifications are expressed in ng/ml as mean  $\pm$  standard deviation in Table I.

Only EPEA levels were under the limit of detection of the method in all samples. All the eCBs showed a significant decrease after surgery, when the values were calculated after correction by plasma

**Table I.** Quantification of eCBs. Levels of eCBs in presurgical serum samples (pr), and in samples collected during rehabilitation.

|             | pr              | p1              | p2              |
|-------------|-----------------|-----------------|-----------------|
| <b>PEA</b>  | 6.11 $\pm$ 0.49 | 2.05 $\pm$ 0.28 | 2.60 $\pm$ 0.50 |
| <b>OEA</b>  | 4.84 $\pm$ 0.40 | 1.57 $\pm$ 0.20 | 2.03 $\pm$ 0.31 |
| <b>SEA</b>  | 1.39 $\pm$ 0.08 | 0.55 $\pm$ 0.06 | 0.69 $\pm$ 0.10 |
| <b>AEA</b>  | 0.44 $\pm$ 0.03 | 0.16 $\pm$ 0.02 | 0.20 $\pm$ 0.03 |
| <b>LEA</b>  | 0.84 $\pm$ 0.04 | 0.56 $\pm$ 0.08 | 0.66 $\pm$ 0.11 |
| <b>LNEA</b> | 0.17 $\pm$ 0.01 | 0.09 $\pm$ 0.01 | 0.11 $\pm$ 0.02 |

pr: presurgical serum samples; p1 (11<sup>th</sup> -14<sup>th</sup> days); p2 (19<sup>th</sup> - 22<sup>nd</sup> days) expressed in ng/ml as mean  $\pm$  standard deviation

volume modifications. The comparison between presurgical values and postsurgical ones, after 11-22 days from arthroplasties, always showed significant differences. The decrease of eCBs was maintained during the period. eCB levels in samples at 24 h from surgery did not display differences from the presurgical ones. The absolute values of eCBs, i.e. not corrected by plasma volume, were very similar, showing the decrease of all the molecules after surgery. However, some differences between the presentation of the two modes of values could be remarked. LEA was apparently increased in absolute value, SEA and AEA were not significantly different after surgery (Fig. 1).

The time course analysis of eCBs concentrations highlights the slow and quite linear decrease of LNEA concentration, while all other eCB concentrations show a profound decrease in the first 15 days from surgery and an adjustment in

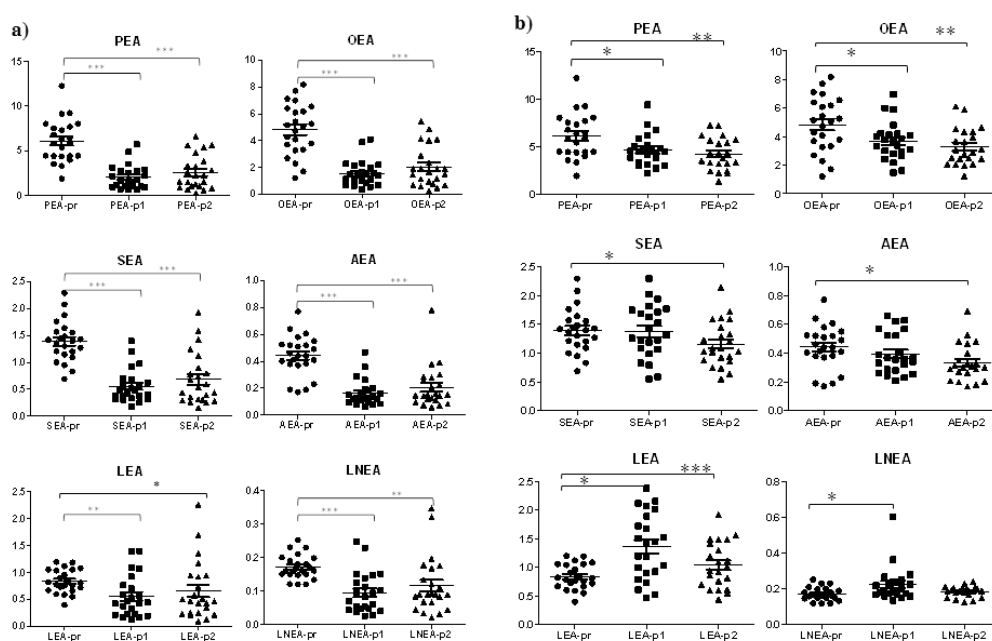
the second period (Fig. 2).

No difference was remarked between THAs and TKAs or between genders. A Parsons correlation between eCB concentrations and CRP and ESR levels after surgery did not show significant values.

## DISCUSSION

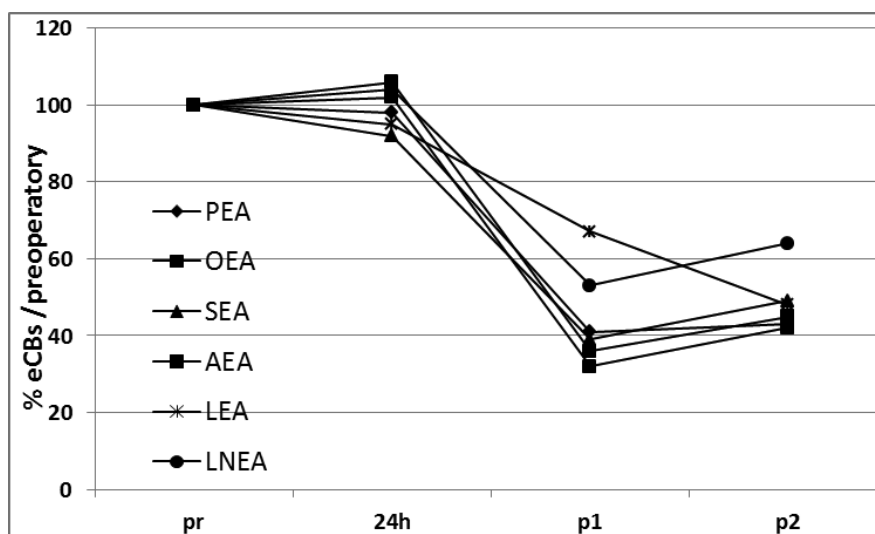
One of the principal therapeutic targets of eCBs is analgesia but it is well documented that chronic pain, that occasionally follows tissue injury, differs fundamentally from inflammatory pain (11). Therefore, the study of eCBs in orthopaedic surgery could allow a better understanding of their involvement in tissue injury and associated pain. To date, the literature reports only one study on TKA that considered few molecules (AEA, 2-AG, PEA and OEA) and described a possible link between eCBs and pain perceived by patients after surgery (8).

In the present study, the behaviour was evaluated



**Fig. 1.** Levels of the eCBs expressed in ng/ml: PEA (*N*-palmitoylethanolamide), SEA (*N*-stearoylethanolamide), OEA (*N*-oleoylethanolamide), AEA (*N*-arachidonylethanolamide), LEA (*N*-linoleoylethanolamide), LNEA (*N*- $\alpha$ -linolenylethanolamide). **a)** Data corrected by plasma volume modifications (application of Dill and Costill equation) **b)** Data without corrections. EPEA levels were under the limit of detection of the method in all samples. eCBs quantified by HPLC-MS/MS analysis in pr, presurgical serum samples, and in samples collected during rehabilitation, p1 (11th -14th days) and p2 (19th - 22nd days). Statistical significance of differences was evaluated by Student *t*-test and \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$





**Fig. 2.** Relative decrease of the seven eCBs analysed concentrations during time in respect to presurgical value. PEA (*N*-palmitoylethanolamide), SEA (*N*-stearoylethanolamide), OEA (*N*-oleoylethanolamide), AEA (*N*-arachidonylethanolamide), LEA (*N*-linoleoylethanolamide), LNEA (*N*- $\alpha$ -linolenylethanolamide).

of six different eCBs during TKA and THA and rehabilitation. All the eCBs showed a decrease after surgery that ameliorates its statistical significance when the values were calculated after correction by plasma volume modifications. This kind of correction is very important in orthopaedic studies because of the significant blood loss during orthopaedic surgical procedures. The comparison between presurgical values and postsurgical ones, 11-22 days after arthroplasties, showed a significant decrease of eCBs maintained during the period (Figs. 1 and 2), suggesting postsurgical values as the real basal ones. Values of samples at 24 h from surgery are not reported because they displayed no differences from presurgical ones. However, the basal values are quite different in the current literature; in the present study, postsurgical values were very similar to those reported by Fanelli (12) for PEA, OEA, and AEA, and in Hauer D et al. (13) for the same molecules and SEA. Few descriptions of eCB modifications after surgery, i.e. cardiac (14) or bariatric (15) surgery, have been reported and the role of eCBs in chronic inflammation is well established (16). This feature could explain higher values of eCBs before arthroplasties: arthrosis and connective tissue inflammation can stimulate the release of eCBs, which

are reduced by elimination of the inflamed tissues. In fact, a correlation between eCBs and classical inflammatory markers, such as CRP and ESR, was not found. The inflammatory markers were measured after surgery and were still high owing to stressful stimulus of the surgery itself, added to chronic inflammatory basis of the joint disease.

This study is the first devoted to eCB behaviour before and after hip and knee arthroplasties. The different eCB molecules have similar responses while they decreased after surgery, although quantitatively some differences among molecules exist. The role of eCBs in joint disease is not elucidated and few studies have been performed on this topic and only few molecules, namely AEA and 2-AG, were generally evaluated. However, data from this preliminary study are picturing a specific and univocal behaviour of eCBs in orthopaedic surgery. Thus, a possible pharmacological target for presurgical therapies could be identified on the eCB system. Further investigations on a larger number of patients and in different experimental settings will be necessary to establish a possible role of eCBs as target for pain modulation or as specific marker for bone modelling.

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## LETTER TO THE EDITOR

**MINOXIDIL DOSE RESPONSE STUDY IN FEMALE PATTERN HAIR LOSS PATIENTS DETERMINED TO BE NON-RESPONDERS TO 5% TOPICAL MINOXIDIL**J. McCOY<sup>1</sup>, A. GOREN<sup>1,2</sup>, M. KOVACEVIC<sup>2</sup> and J. SHAPIRO<sup>3</sup>

<sup>1</sup>*Applied Biology, Inc., Irvine, CA, USA;* <sup>2</sup>*Department of Dermatology and Venereology, University of Rome "G.Marconi", Rome, Italy;* <sup>3</sup>*Department of Dermatology, New York University Langone Medical Center, New York City, New York, USA*

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Topical minoxidil is the only US FDA approved drug for the treatment of female pattern hair loss (FPHL). 5% minoxidil foam is only effective at re-growing hair in a minority of women (approximately 40%). Thus, the majority of FPHL patients remain untreated. Previously, we demonstrated that non-responders to 5% minoxidil have low metabolism of minoxidil in hair follicles. As such, we hypothesized that increasing the dosage of topical minoxidil to low metabolizers would increase the number of responders without increasing the incidence of adverse events. In this study, we recruited FPHL subjects that were identified as non-responders to 5% topical minoxidil utilizing the previously validated assay for minoxidil response. Subjects were treated for 12 weeks with a novel 15% topical minoxidil solution. At 12 weeks, 60% of subjects achieved a clinically significant response based on target area hair counts (>13.7% from baseline), as well as significant improvement in global photographic assessment. None of the subjects experienced significant hemodynamic changes or any other adverse events. To the best of our knowledge, this is the first study to demonstrate the potentially beneficial effect of a higher dosage of minoxidil in FPHL subjects who fail to respond to 5% minoxidil.

To the Editor,

Minoxidil is the only US FDA approved drug for the treatment of female pattern hair loss (FPHL). Topical minoxidil is currently approved by the US FDA as a 2% solution and as a 2% and 5% foam. A moderate increase in hair regrowth is observed in up to 40% of subjects following 16 weeks of daily application of minoxidil (1). In clinical studies, the 5% topical foam demonstrated non-inferiority to the 2% solution (2).

Topical minoxidil has a good safety profile with few adverse events in both the 2% and 5% concentrations. Events such as allergic contact dermatitis, irritant dermatitis, hypertrichosis and cardiac events in

hypertensive patients following withdrawal of the drug have been reported (3, 4). 5% minoxidil foam showed significantly lower rates of local intolerance especially in pruritus and dandruff compared with 2% minoxidil solutions (2).

Although the exact mechanism of action of minoxidil is not completely understood, it is known that minoxidil sulfate is the active form of minoxidil, required for both the promotion of hair regrowth and the vasodilatory effects of minoxidil (5). Several studies have demonstrated a correlation between sulfotransferase expression in the scalp and minoxidil response (6, 7). Goren et al. describes a novel colorimetric sulfotransferase assay that identifies non-

*Key words: female pattern hair loss, minoxidil, alopecia*

Mailing address:

Andy Goren, MD

Applied Biology,

17780 Fitch, Suite 192, Irvine, CA 92614, USA

Tel: +1 (949) 387-4526

e-mail: andyg@appliedbiology.com

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responders to 5% topical minoxidil utilizing plucked hair follicles (7, 8). The assay predicted minoxidil treatment response with 95% sensitivity and 73% specificity in a mixed cohort of male and female patients (8). In addition, McCoy et al. demonstrated a correlation between the change in scalp perfusion within 60 minutes following topical minoxidil application and sulfotransferase enzyme activity in plucked hairs (9). Taken together, these studies support the hypothesis that increasing the dosage of minoxidil in low metabolizers could increase the number of responders to topical minoxidil without increasing the rate of adverse events.

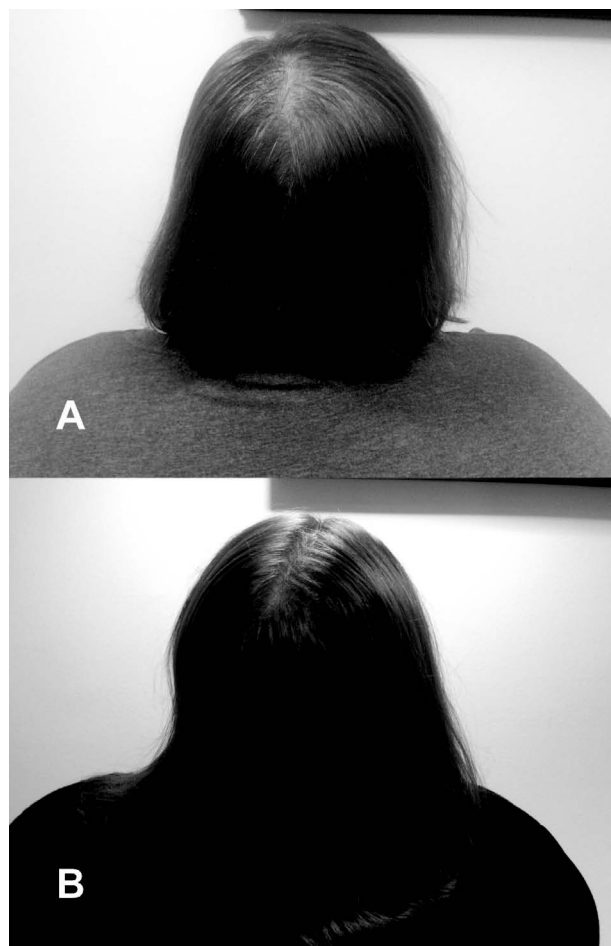
## MATERIALS AND METHODS

Twenty-five subjects with female pattern hair loss were enrolled in the study. The study was conducted with approval from an ethics committee and informed consent was obtained from each participant. None of the subjects suffered from cardiac disease, thyroid disease, diabetes, or any other chronic disease. None of the subjects were taking medication that could affect FPHL or had a prior adverse event to topical minoxidil. None of the subjects were pregnant or lactating during the study. Subjects were screened for minoxidil response utilizing the plucked hair sulfotransferase activity assay reported by Goren et al. (8). Of the twenty-five subjects, five were identified as low minoxidil metabolizers, i.e., non-responders to 5% topical minoxidil. The average age of the subjects included in the study was 36.2 years. During the initial visit, subjects underwent hemodynamics measurement for 60 min after application of 1 mL of the novel 15% topical minoxidil solution. For assessing the change in target area hair counts (TAHC), a 1 cm<sup>2</sup> area on the top of the scalp was shaved and tattooed with a permanent dye. Trichograms and global photographs were collected at baseline and week 12. Hemodynamic measurements and adverse events assessments were conducted at baseline, weeks 4, 8, and 12. During the study, subjects self-applied 1 mL of the novel 15% topical minoxidil solution once daily.

## RESULTS

Table I summarizes the target area hair counts

(TAHC) at baseline and week 12, as well as, hemodynamic measurements at baseline, week 4, and week 12 for the subjects enrolled in the study. 60% of the subjects identified as non-responders to 5% topical minoxidil exhibited an increase in TAHC greater than 13.7% following 12 weeks of treatment with the novel 15% topical minoxidil solution. The 13.7% value was previously demonstrated by Goren et al. (8) as the threshold for significance over placebo in topical minoxidil hair loss studies. Global photographs assessed by an independent expert correlated with TAHC ( $r=0.8$ ). No significant changes were noted in hemodynamic parameters. Fig. 1 shows the global photographs of patient 1 at baseline and week 12.



**Fig. 1.** Global photographs of patient 1 at baseline (A) and week 12 (B).

## DISCUSSION

Minoxidil is a pro-drug converted to its active form, minoxidil sulfate, in the outer root sheath of the hair follicle (6). In previous studies, our group demonstrated the clinical utility and validity of the minoxidil sulfotransferase assay in identifying non-responders to 5% topical minoxidil (8). Utilizing data obtained from multiple studies applying the minoxidil sulfotransferase assay (7, 8), we determined that the average non-responder has approximately 50% of the sulfotransferase (SULT1A1) enzyme activity required to surpass the threshold to become a responder. Based on analysis of SULT1A1 kinetics (Michaelis-Menten), we determined that in order to double the bioavailable minoxidil sulfate, a 15% topical minoxidil formulation would be needed. Safety was a paramount concern as the increased minoxidil dosage could result in significant hemodynamic changes; however, the vasodilatory effects of minoxidil are also dependent on the conversion of minoxidil to minoxidil sulfate by the SULT1A1 enzyme (9, 10). While the expression of SULT1A1 in the scalp and in blood platelets are not identical, a strong correlation has been reported (11); as such, we hypothesized that increasing the dosage of topical minoxidil would be safe for patients determined to have a low level of sulfotransferase activity in the scalp. Although the small sample size of our cohort is a limitation of this study, the results lend support to the efficacy and safety of the novel 15% topical minoxidil formulation in subjects identified as non-responders to 5% topical minoxidil due to low scalp SULT1A1 activity.

**DISCLOSURE:** Applied Biology, Inc. developed the diagnostic test for minoxidil response used in this study.

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## LETTER TO THE EDITOR

**HIGH LEVEL LASER THERAPY FOR THE TREATMENT OF LOWER BACK PAIN:  
CLINICAL EFFICACY AND COMPARISON OF DIFFERENT WAVELENGTHS**

A. NOTARNICOLA<sup>1,2</sup>, G. MACCAGNANO<sup>2</sup>, S. TAFURI<sup>3</sup>, M.F. GALLONE<sup>3</sup>,  
L. MORETTI<sup>2</sup> and B. MORETTI<sup>1,2</sup>

<sup>1</sup>*Department of Basic Medical Sciences, Neurosciences and Organs of Sense, Course of Motor and Sports Sciences, Faculty of Medicine and Surgery, University of Study of Bari, Bari, Italy;*

<sup>2</sup>*Department of Basic Medical Sciences, Neurosciences and Organs of Sense, Orthopedics Section, Faculty of Medicine and Surgery, University of Study of Bari, General Hospital, Bari, Italy;*

<sup>3</sup>*Department of Biomedical Sciences and Human Oncology, Faculty of Medicine and Surgery, University of Study of Bari, Bari, Italy*

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High energy laser therapy (HELT) could be a new alternative treatment for lower back pain (LBP), which is a significant public health problem. Nevertheless, differences between the various light waves of HELT have not yet been fully researched. Sixty-six patients with lower back pain were treated using a high energy laser therapy. They were randomized into three different protocols which differed according to wave length (650 nm, 810 nm and TRIAX, which is simultaneous emission of 810 nm, 980 nm e 1064 nm). The other parameters remained constant (5 W and 50 J/cm<sup>2</sup> for ten daily sessions). The visual analogue scale (VAS), the Roland Scale, and the Oswestry Score were measured before treatment (T0), and at end of the treatment session (T1) and 1 month (T1), 2 months (T2) and 4 months of follow-up (T4). In each group we verified a statistically significant improvement over time and that there was a relationship between the time and treatment ( $p < 0.01$ ). At T1 for all wavelengths we found a statistically significant improvement of three scores ( $p < 0.01$ ), which was maintained up to T4. The group treated with 810 nm HELT, showed a better remission of pain on the VAS scale, and disability on the Oswestry Scale at T4 ( $p = 0.01$ ). Comparing T0-T1 the variation in the Roland Score was significant in the patients treated with 810 nm ( $p < 0.01$ ). All the wavelengths analyzed proved to be efficacious for LBP. The greater efficacy of 810 nm in promoting nerve regeneration and in modulating the nociception transmission could explain the better outcomes.

To the Editor,

Lower back pain (LBP) is a common musculoskeletal problem, with an estimated prevalence of approximately 23% (1). Oxidative stress that develops after peripheral neuropathic lesion leads to the activation of an inflammatory

pathway involving the whole peripheral nerve (distal and proximal) up to the spinal dorsal horn, where a subsequent activation of microglia occurs (2). This process plays an active part in spine sensitization and in chronic neuropathic pain. These mediators of inflammation affect the other

*Key words: lower back pain, laser therapies, high energy, light waves, leg pain*

*Mailing address:*

Angela Notarnicola, MD, Ph.D., Orthopedics Section,  
Department of Basic Medical Sciences,  
Neurosciences and Organs of Sense,  
Faculty of Medicine and Surgery,  
University of Study of Bari, General Hospital,  
Piazza Giulio Cesare 11, 70124 Bari, Italy  
e-mail: angelanotarnicola@yahoo.it

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structures in the vicinity, causing painful spasms of the muscles. Also muscular pain can become a cause of LBP.

When there is no indication for surgery, LBP is commonly treated with drugs and rehabilitation, which includes exercise therapy, back schools, transcutaneous electrical nerve stimulation (TENS), superficial heat or cold, low level laser therapy (LLLT), individual patient education, massage, behavioral treatment, lumbar supports, traction, and multidisciplinary rehabilitation (3).

Laser therapy (LT) is a light source treatment that emits no heat, sound, or vibration, but may act via non-thermal or photochemical reactions in cells (4). Laser therapy takes advantage of the biological effects induced by electromagnetic radiation concentrated in a straight ray culminated through stimulated emission characterized by the coherence of the monochromaticity and brightness. There is evidence of laser inducing anti-inflammatory, anti-nociceptive, central nervous and lymphatic effects (4).

More recently, high energy laser therapy (HELT), that uses higher intensity laser irradiation and causes minor and slow absorption of light by chromophores, has been utilized. This preliminary clinical experience verified the effectiveness of HELT in the treatment of musculoskeletal disorders (5). The primary aim of this study is to verify the efficacy of HELT for LBP. To the authors' knowledge, no study has yet compared the efficiency of different wavelengths of HELT on LBP. The secondary aim of this study is to compare the effects of the most important wavelengths administered: 650-nm in single administration and 810-nm, 980-nm and 1064-nm, in single or combined administration.

## MATERIALS AND METHODS

A prospective, randomized study design was used to assess the clinical efficiency of HELT for LB. The study was conducted according to the Declaration of Helsinki and the guidelines for Good Clinical Practice. The study was approved by the local Ethics Committee (authorization n.325, 19<sup>th</sup> June 2014). Prior informed consent to take part was given by all participants. Each

subject's medical history was examined for exclusion and inclusion criteria.

### *Patients*

Sixty patients were recruited from the Orthopedic Unit of the General Hospital, Bari, Italy. All the patients enrolled in the study had been taking NSAIDs or pain killer drugs, and these therapies were suspended at the beginning of the study. Inclusion criteria were age >18 years of age, a diagnosis of chronic LBP lasting at least 12 weeks, made on the basis of clinical symptoms and instrumental tests, and a functional visual analogic scale (VAS) score  $\geq 4$ ; computed tomography (CT)- or nuclear magnetic resonance (MRI)-documented discopathy or disk hernia. Patients were excluded from the study if they had contraindications to high level laser therapy, neoplasia or current or previous infections of the affected area, a cardiac pacemaker, or pregnancy. They were also excluded if they had the presence of active vertebral fractures, a history of previous spine surgery, rheumatic diseases such as rheumatoid arthritis, infections and systemic diseases that could affect pain perception (diabetes, etc.), or local injections (local anesthetic and/or corticosteroids; O2O3, etc.) or physiotherapy administered within the previous 4 weeks.

### *Enrolment and follow-up visits*

On enrolment, the study protocol included the collection of medical history and clinical visits. The following epidemiological, clinical and treatment characteristics of patients were analyzed: age, gender, weight, height, BMI, smoking, side, LBP with or without leg pain, total joule. Patients with leg pain were also included to see the effects in the presence of a radicular pain.

The clinical results were evaluated using: i) VAS (11 points total) which is widely adopted to measure pain. At the time of recruitment (T0) and at the follow-up (FU) visits on 10th–15th days (end of the complete session of treatment) (T1) and 1, 2 and 4 months (FU) (T2, T3 and T4) each patient was asked to indicate his/her perceived pain intensity along a 10 point/cm horizontal line (10=severe pain; 0=no pain); ii) the Roland Morris Disability Questionnaire (24 points total) which is a self-administered disability questionnaire composed of 24 items. The scores should be between 0 and 24. Greater levels of disability are reflected by higher numbers (24=

disability); and iii) and the Oswestry Low Back Pain Disability Questionnaire (100 points total) which is used to measure a patient's permanent functional disability. The score is expressed as a percentage between 0% (no disability) to 100% (important disability).

The patient recruitment, monitoring processes and all HELT treatments were conducted in a blinded manner.

#### *Treatment*

The patients were treated with HELT (THEAL therapy, Ixyon, Mectronic Medicale, Italy) and were randomly allocated into 3 groups, in which the wavelength of HELT differed. To avoid selection bias the randomization criteria were sex (male/female) and age ( $\leq 50$  or  $> 50$  years). Each group was formed of 20 patients. Group 1 (650-nm HELT,  $n = 20$ ) received HELT at 650-nm wavelength; group 2 (810-nm HELT,  $n = 20$ ) received HELT at 810-nm wavelength; group 3 (TRIAx,  $n = 20$ ) received HELT at 810-nm, 980-nm and 1064-nm wavelength concurrently.

The protocol was defined on the basis of the knowledge of biological effects and on the basis of the previous experience of the authors treating patients with lower back pain. The wave length was selected in accordance with the group: 650 nm, 810 nm or TRIAX. We chose a super-pulse stochastic emission ( $E^2C$ ), characterized by the randomness of the impulses emitted with a frequency varying between 20-70 Hz. Each patient was treated five times a week, for a total of 10 sessions. The power was set constantly at 5 Watt. The spot size was 1 cm<sup>2</sup>. The patient was placed in a prone position on the treatment couch with hip and knee extended and the ankle in maximal extension.

The treatment surface area was lumbar paravertebral zone in correspondence with the vertebra affected by discal disease. We measured the two sides of this zone and we calculated the corresponding surface. In a single session, each patient was treated with HELT using a dosage of 50 J per each cm<sup>2</sup> of this painful area at the level of the column.

The time required for the delivery of 50 Joules is 22 seconds. We used manual scanning for laser therapy. During the treatment the scanner was placed on the surface area of the skin. The spinal region was scanned with a 45° inclination lateral to facet articular joints and moving from cranio-caudal to caudo-cranial direction. The remaining area was treated carrying out the treatment following the direction of the muscle fibers with an inclination of 90° of the handpiece. For eye protection from the laser beam, all

subjects wore protective darkened glasses. Patients were asked to report immediately the onset of collateral effects such as skin hyper-sensibility, burns, severe pain.

#### *Statistical evaluation*

For each patient a standardized schedule was compiled. The file included the following data: sex, age, weight, height, body mass index (BMI), joule, time, smoking habit, vertebral region involved in discal disease (from L1 to S1), pain surface (LBP with or without leg pain), pain side (right/left/bilateral), type of treatment, scores for each scale. The compiled schedules were put into a database built with FileMaker Pro software and the data were analyzed using Stata MP12 statistic software. For categorical variables we evaluated absolute frequency and proportion. The proportions in the three treatment groups were compared using the *Chi*-squared test.

The scores, obtained in different scales at different times and determinates made up of continuous variables, were described as average according to standard deviation and range; the averages of different times were compared using the student's *t*-test for paired sample analyzing two time points at a time (in the study population: T0 vs T1; T1 vs T2; T2 vs T3; T3 vs T4; T0 vs T4; to compare the groups in relation to wave length and to compare LBP vs LBP with leg pain: T0 vs T1).

The scores stratified according to treatment type and time of measurements were described as average within the range; the averages were compared of the three groups at the same time using Kruskal-Wallis test for independent samples. The ANOVA test was carried out for repeated measurements to compare the scores at the beginning of the different FUs in the different groups.

To evaluate the association between the score and first follow-up in three functional scores, a multiple regression model was built which included the following characteristics: sex, age, BMI, type of treatment, side, score obtained at the beginning of the study. The results were described according to coefficient and confidence range at 95% of the test and *p* value. For each test we considered a statistical significant value of  $p < 0.05$ .

## RESULTS

The study population was made up of 14 males (23.3%) and 46 females (76.7%) (Table I). The

average age was 60 (DS: 10.8; range: 13-54), weight of 73.5 Kg (DS: 11; range: 20-76), height of 1.70 m (DS: 0.1; range: 1.5-1.8), BMI of 26.8 (DS: 2.7; range: 19-30); 20% were smokers. The average time of LBP onset was 20.5 weeks (DS: 10.8; range: 13 – 54). The main site of hernia disease was L2-L3 for 14 patients, L3-L4 for 2 patients, L4-L5 for 30 patients, L5-S1 for 14 patients. In 53.3% of patients the localization was the right side, 30% left side, 16.7% bilateral. In 30% the LBP was only in the lumbar region (local LBP only), whilst in the others also a leg (LBP with leg pain) was observed. For each session we administered an average 894 Joule (DS: 2.7; range 300-2000).

Each treatment group in relation to wave length was made up of 20 patients. Between the three groups we did not detect statistically significant differences with regard to epidemiological and clinical features, and number of Joule administered (Table I). There were no drop-outs. The patients did not refer any side effects.

#### VAS

In the study population, the VAS scores were reduced significantly from T0 (7.5±1.5) to T1 (3.5±2.6; univariate analysis  $t=8.7$ ,  $p<0.01$ ), then there was a slight increase at the following FUs. (T2: 3.4±2.6; T3: 3.8±2.4; T4: 4.1±2.5) (comparison of average of scores at different FUs by univariate analysis T1 vs T2:  $t=0.3$ ,  $p<0.73$ ; T2 vs T3:  $t=-0.8$ ,  $p=0.4$ ; T3 vs T4:  $t=-0.7$ ,  $p=0.44$ ; T0 vs T4:  $t=6.3$ ;  $0<0.01$ ). The trend for the patients with LBP without leg pain was similar to patients with LBP and leg pain (comparison of the average of the scores by Kruskal-Wallis test for independent sample, T0 vs T1:  $\chi^2=0.4$ ,  $p=0.5$ ).

The comparison of the scores between the groups at different follow-ups showed a significant difference at T4 for the group 810 nm (comparison of median by Kruskal-Wallis test for independent sample  $X^2=8.7$ ;  $p=0.01$ ) (Table II).

#### Roland Score

Roland score decreased significantly at T0 (14.6±5) and T1 (8.9±6.1; univariate analysis:  $t=7.1$ ,  $p<0.01$ ), remaining more or less the same at subsequent FUs (T2: 9±7; T3: 9±6.7; T4: 8.8±6.4)

(comparison of the average of the scores at different FUs by univariate analysis T1 vs T2:  $t=-0.1$ ,  $p=0.9$ ; T2 vs T3:  $t=0$ ,  $p=1$ ; T3 vs T4:  $t=0.3$ ,  $p=0.76$ ; T0 vs T4:  $t=5.3$ ;  $p>0.01$ ) (Table III).

The trend of clinical improvement for patients with LBP without leg pain and those with leg pain was similar (comparison of median of scores according to Kruskal-Wallis test for independent sample, T0 vs T1:  $\chi^2=1.9$ ,  $p=1.7$ ).

At different time points the comparison between the three groups did not show any significant differences (Table III). However, there did emerge a significant difference in the 810 nm group with regard to the difference of values at T0 and T1 (comparison of median according to Kruskal-Wallis test for independent sample  $X^2=10.6$ ;  $p<0.01$ ).

#### Oswestry Score

The Oswestry score improved significantly from T0 (43.2±15.4) to T1 (28.4±18;  $t=6.9$ ;  $p<0.01$ ), and continued to improve (T2: 26.9±19.7; T3: 25.8±19.4; T4: 26.3±18) (comparison of median of scores at different follow-ups by univariate analysis T1 vs T2:  $t=0.9$ ,  $p=0.39$ ; T2 vs T3:  $t=0.7$ ,  $p=0.46$ ; T3 vs T4:  $t=-0.3$ ,  $p=0.8$ ; T0 vs T4:  $t=5.8$ ;  $p<0.01$ ). No difference emerged between the patients with and without leg pain (comparison of median of scores according to Kruskal-Wallis test for independent sample, T0 vs T1:  $\chi^2=2.3$ ,  $p=0.1$ ).

The comparison of values between the groups at different times showed a significant difference at T4 for the 810 nm group (Kruskal-Wallis test for independent sample  $X^2=8.7$ ;  $p=0.01$ ) (Table II).

The ANOVA test for repeated measurements showed that VAS, Roland and Oswestry values were modified in all groups and these modifications were statistically significant in time, therefore there is a direct correlation between time and treatment ( $p<0.01$ ).

The multiple regression model did not show any correlation between VAS score and T1 as regards any of the variables (sex, age, BMI, group, score at T0 and affected side); both the Roland score (coef.=0.8; IC95%: 0.3–1.2;  $t=3.8$ ;  $p<0.01$ ) and Oswestry score (coef.=0.8; IC95%: 0.5–1.1;  $t=5.1$ ;  $p<0.01$ ) at T1 were correlated to the scores at T0.

**Table I.** Comparison of epidemiological features of the enrolled population, expressed as average and range or as percentage, in the three different groups according to wave length by univariate analysis.

| Epidemiological features  | 650                   | 810                   | TRIAx                 | $\chi^2$ | p   |
|---------------------------|-----------------------|-----------------------|-----------------------|----------|-----|
|                           | median (range)        | median (range)        | median (range)        |          |     |
| Age                       | 59.5<br>(20 - 75)     | 60.5<br>(45 - 70)     | 66.5<br>(50 - 76)     | 1.2      | 0.5 |
| Joule Tot                 | 750<br>(300 - 2000)   | 1000<br>(300 - 2000)  | 700<br>(300 - 1500)   | 1.3      | 0.5 |
| Weight                    | 70.5<br>(55 - 88)     | 76.5<br>(60 - 90)     | 78.5<br>(54 - 89)     | 0.9      | 0.6 |
| Height                    | 1.62<br>(1.56 - 1.72) | 1.63<br>(1.55 - 1.82) | 1.67<br>(1.54 - 1.78) | 0.6      | 0.7 |
| BMI                       | 26.7<br>(19.0 - 30.0) | 27.3<br>(25.0 - 29.7) | 26.3<br>(22.8 - 29.8) | 0.4      | 0.8 |
|                           |                       |                       |                       |          |     |
|                           | 650                   | 810                   | TRIAx                 | $\chi^2$ | p   |
|                           | %                     | %                     | %                     |          |     |
| Male gender               | 20                    | 20                    | 30                    | 0.4      | 0.8 |
| Smoking                   | 20                    | 20                    | 20                    | 0.0      | 1.0 |
| Right side                | 60                    | 70                    | 80                    | 0.9      | 0.6 |
| Left side                 | 60                    | 30                    | 50                    | 1.8      | 0.3 |
| Bilateral involvement     | 40                    | 0                     | 60                    | 4.3      | 0.4 |
| Only LBP without leg pain | 40                    | 30                    | 20                    | 1.0      | 0.6 |

Significant value WAS for  $p < 0.05$ .

## DISCUSSION

The main aim of the study was to verify the efficacy of high energy laser in LBP treatment. We verified that the benefits of the therapy at the end of treatment are maintained for medium-long term for 4 months. These clinical results are coherent with the known effects of bio-stimulation (6), of inflammation (7) and pain modulation (8-10) and angiogenesis of lasertherapy (11). These data

confirm the appropriateness of HELT in LBP.

The second aim of our study was to compare the clinical effects of HELT at different wavelengths. The different wavelengths did not show significant differences in short and medium term (T0-T3) as regards pain remission measured on the VAS scale. 810 nm wavelength demonstrated greater efficacy in terms of recovery disability at short term (comparison of Roland Score variation at T1) and medium-long term (comparison of Oswestry Score at T4) and pain



**Table II.** Comparison of the average scores of the different scales in the three treatment groups in relation to wave length at the different FUs (Kruskal-wallis test for independent sample).

| Scores at different times for each scale | 650               | 810               | TRIAX             | $\chi^2$ | p     |
|------------------------------------------|-------------------|-------------------|-------------------|----------|-------|
|                                          | median (range)    | median (range)    | median (range)    |          |       |
| Vas T0                                   | 7.0<br>(4 - 10)   | 7.75<br>(5 - 10)  | 7.0<br>(6 - 10)   | 0.7      | 0.7   |
| Vas T1                                   | 3.5<br>(0 - 10)   | 3.0<br>(0 - 5)    | 3.0<br>(0 - 10)   | 0.3      | 0.9   |
| Vas T2                                   | 3.0<br>(0 - 6)    | 2.5<br>(0 - 7)    | 3.0<br>(0 - 10)   | 0.9      | 0.6   |
| Vas T3                                   | 4.0<br>(0 - 8)    | 2.5<br>(0 - 8)    | 3.0<br>(1 - 8)    | 1.4      | 0.5   |
| Vas T4                                   | 3.5<br>(2 - 9)    | 2.0<br>(0 - 6)    | 5.5<br>(3 - 9)    | 8.7      | 0.01  |
| Roland T0                                | 11.0<br>(3 - 19)  | 17.0<br>(12 - 19) | 15.5<br>(5 - 24)  | 5.7      | 0.05  |
| Roland T1                                | 5.0<br>(1 - 15)   | 7.5<br>(0 - 15)   | 11.5<br>(4 - 21)  | 5.5      | 0.06  |
| Roland T2                                | 4.5<br>(0 - 15)   | 8.5<br>(0 - 20)   | 10.0<br>(2 - 24)  | 2.1      | 0.4   |
| Roland T3                                | 7.5<br>(1 - 14)   | 7.0<br>(0 - 19)   | 10.5<br>(1 - 20)  | 2.0      | 0.4   |
| Roland T4                                | 7.5<br>(1 - 17)   | 6.5<br>(1 - 19)   | 12.0<br>(4 - 22)  | 4.8      | 0.09  |
| Oswestry T0                              | 37.0<br>(16 - 48) | 45.0<br>(24 - 60) | 43.0<br>(28 - 88) | 2.7      | 0.3   |
| Oswestry T1                              | 24.0<br>(0 - 44)  | 28.0<br>(0 - 44)  | 35.0<br>(10 - 82) | 2.7      | 0.3   |
| Oswestry T2                              | 24.0<br>(0 - 38)  | 23.5<br>(0 - 46)  | 35.0<br>(2 - 88)  | 2.2      | 0.3   |
| Oswestry T3                              | 22.0<br>(2 - 50)  | 24.5<br>(0 - 35)  | 34.5<br>(2 - 78)  | 2.7      | 0.3   |
| Oswestry T4                              | 24.0<br>(4 - 44)  | 20.0<br>(0 - 35)  | 38.0<br>(14 - 78) | 8.4      | 0.01  |
| Variation Vas T0-T1                      | -3<br>(-7 - 0)    | -4<br>(-1 - -2)   | -4<br>(-8 - 0)    | 1.6      | 0.6   |
| Variation Roland T0-T1                   | -5<br>(-9 - 0)    | -9<br>(-19 - -4)  | -3<br>(-9 - 1)    | 10.6     | <0.01 |

remission at medium-long term (comparison of VAS score at T4). These results are coherent with 810 nm effect in order to recover nerve fiber, acting on the first cause of LBP and ensuring a functional recovery. The tendency towards a minor improvement with treatments with wavelengths of 650 nm or TRIAX

could be justified as a more superficial action in the first case and submaximal action in the second case. In fact, in the administration TRIAX each wavelength corresponds to a third of the value of watts and joules of the programmed protocol.

However, the biological effects of each wave



**Table III.** Recorded scores for the three evaluation scales (VAS score for pain; Roland and Oswestry scores for disability) in all study population for all four FUs.

| Times | Scores   | Average | DS   | Range      |
|-------|----------|---------|------|------------|
| T0    | Vas      | 7.5     | 1.5  | 4.0 – 10.0 |
|       | Roland   | 14.6    | 5.0  | 3.0 – 24.0 |
|       | Oswestry | 43.2    | 16.4 | 16.0 - 88  |
| T1    | Vas      | 3.5     | 2.6  | 0.0 – 10.0 |
|       | Roland   | 8.9     | 6.1  | 0.0 – 21.0 |
|       | Oswestry | 28.4    | 17.9 | 0.0 – 82.0 |
| T2    | Vas      | 3.4     | 2.6  | 0.0 – 10.0 |
|       | Roland   | 9.0     | 7.0  | 0.0 – 24.0 |
|       | Oswestry | 26.9    | 19.7 | 0.0 – 88.0 |
| T3    | Vas      | 3.8     | 2.4  | 0.0 – 8.0  |
|       | Roland   | 9.0     | 6.6  | 0.0 – 20.0 |
|       | Oswestry | 25.8    | 19.4 | 0.0 – 78.0 |
| T4    | Vas      | 4.1     | 2.5  | 0.0 – 9.0  |
|       | Roland   | 8.8     | 6.4  | 0.0 – 22.0 |
|       | Oswestry | 26.3    | 18.0 | 0.0 - 78.0 |

(T0: recruitment, T1: at the end of treatment, T2, T3 e T4: after 1, 3 and 4 months, respectively). The data are expressed as average, standard deviation and range.

length showed to be efficacious for management of LBP. The affinity between laser 650 nm and collagen is responsible for anti-inflammatory effects on connective and muscle tissue (12). The reduction of penetration capacity due to chromophore interference, in particular of melanin and hemoglobin, justifies a local action on the surface (13). 810 nm laser therapy does not have a good capacity to modulate nociceptive pathways (14). At this wave length, the laser is able to reach the tissues in depth decreasing in chromophore resorption (melanin and Hb) (14). 980 nm laser therapy has a direct effect on microcirculation, thermal and mechanical receptor (14). The interference with chromophores is minimized (14), the main factors are melanin and Hb which allow larger action surface (15). The laser 1064 nm is able to develop an anti-inflammatory effect on muscle tissue. It is able to avoid the scattering effect

caused by tissue chromophors (melanin e la ossi-Hb) (14), acting more deeply.

In conclusion, high energy laser is a promising method that is effective in the treatment of LBP with and without leg pain. Our preliminary results demonstrate that HELT at 810 nm wave length does show greater specificity in LBP.

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## LETTER TO THE EDITOR

## ASSESSING THE ASSOCIATION BETWEEN 25-OH VITAMIN D LEVELS AND ROMA SCORE IN A POPULATION OF OBESE WOMEN

E. ANASTASI<sup>1</sup>, D. CAPOCCIA<sup>2</sup>, T. GRANATO<sup>3</sup>, V. VIGGIANI<sup>1</sup>,  
S. TARTAGLIONE<sup>1</sup>, L. MANGANARO<sup>4</sup>, A. ANGELONI<sup>1</sup> and F. LEONETTI<sup>2</sup>

<sup>1</sup>Department of Molecular Medicine, "Sapienza" University of Rome, Rome, Italy;

<sup>2</sup>Department of Experimental Medicine, "Sapienza" University of Rome – Policlinico Umberto I, Rome, Italy; <sup>3</sup>CNR-IBPM, National Research Council, Rome, Italy; <sup>4</sup>Department of Radiology, "Sapienza" University, Rome, Italy

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The "Risk Of Malignancy Algorithm" (ROMA) combines the diagnostic power of the CA125 and HE4 markers with menopausal status to predict the risk for developing epithelial ovarian cancer (EOC). The aim of this study was to evaluate the association between 25-OH vitamin D levels and ROMA score in obese women. One hundred and eighteen patients with a Body Mass Index (BMI) > 30 kg/m<sup>2</sup> (Group 1) and 80 women with a BMI <25 kg / m<sup>2</sup> (Group 2) were studied. The 25-OH vitamin D was quantified with LUMIPULSE® G 1200. As a threshold value, identified by ROC curve analysis, 20.2 ng/mL (sensitivity 73.3%, specificity 84%) was chosen corresponding to the limit between sufficient and insufficient 25-OH vitamin D according to the World Health Organization (WHO). Low 25-OH vitamin D levels were observed in 64% of obese women and in 11% of normal-weight women (p<0.001). ROMA score above 13% was detected only in obese women (19%). An association between low levels of 25-OH vitamin D and ROMA score was observed. Indeed, 64% of obese women with ROMA score >13% had concomitant insufficient levels of 25-OH vitamin D, while only 36% of obese women with ROMA score >13% had sufficient 25-OH vitamin D levels (p< 0.0001). This study suggests that the deficiency of 25-OH vitamin D in obese women has a possible correlation with high ROMA score.

To the Editor,

Vitamin D, although classified as a nutrient, is more fittingly described as a steroid-like prohormone/hormone which exists in two forms with similar biological activity: vitamin D<sub>2</sub> and vitamin D<sub>3</sub>. The primary source of vitamin D is from skin synthesis as there are few naturally occurring dietary sources. Both vitamin D<sub>2</sub> and vitamin D<sub>3</sub> are capable of binding to their vitamin D receptor (VDR) which plays a central role in the biological actions of vitamin D as

it regulates the expression of numerous genes in a largely ligand-dependent manner (1). Recently, VDRs have been identified and proven to have also anti-neoplastic effects, confirming the establishment of a broad scientific bibliography on the anti-carcinogenic properties of 25-OH vitamin D (2). In addition to the organs responsible for calcium/phosphate homeostasis, VDRs are expressed in a variety of tissues and cells, such as hepatocytes, adipocytes, and pancreatic  $\beta$ -cells, all of which are associated with

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*Mailing address:*

Dr Emanuela Anastasi,  
Laboratory of Tumor Markers,  
Department of Molecular Medicine,  
University "Sapienza",  
Viale Regina Elena 324, 00161 Rome, Italy  
Tel.: +39 064472347 - Fax: +39 064478381  
e-mail: emanuela.anastasi@uniroma1.it

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obesity and its metabolic complications (3). Obesity is a recognized epidemic worldwide and it has been demonstrated that it intensifies tumor improvement and reduces response to treatment: obese cancer patients often have poorer prognoses, and are more likely to develop metastatic disease than normal-weight patients. Various epidemiologic studies suggested that obesity is a risk factor for several types of cancer: in particular it is an established risk and progression factor for many hormone-related cancers, such as endometrial, breast, prostate, hepatocellular and ovarian cancer (4). The correlation between epithelial ovarian cancer (EOC) and obesity is still under discussion; a recent study found that high BMI was associated with increased risk of EOC in 15 case-control studies (5). Strong preclinical and clinical evidence supported an association between obesity and ovarian cancer incidence, progression, metastasis and poor survival (6). Due to a typically asymptomatic early stage and lack of early diagnostic tools, EOC is usually diagnosed at a late stage; at present, a multimodal approach including the utilization of tumor biomarkers associated with ovarian cancer, besides imaging techniques seems to be the best strategy for the determination of the early stages of cancer form (7).

Numerous serum biomarkers have been assessed for the diagnosis of ovarian cancer and growing evidence suggests that the biomarker CA125 should be complemented by the encouraging Human Epididymis protein 4 (HE4) for EOC early diagnosis. To assess the risk for EOC, recently an algorithm was developed named “risk of malignancy algorithm” (ROMA) for postmenopausal and premenopausal women. It is a quantitative test calculated from the predictive index (PI) which derives from HE4 and CA125 measurements; it has been studied for its prognostic ability in ovarian cancer and has been accepted by the Food and Drug Administration (FDA) for differentiating malignant from benign pelvic masses (8).

The co-occurrence of tumors or tumor markers and low vitamin D is now the subject of many scientific studies. Recently, it was observed that 25-OH vitamin D is decreased at time of diagnosis in nearly all patients affected by ovarian cancer and

that only a small percentage of patients affected by gynecological benign diseases had levels below the cutoff for 25-OH vitamin D (2).

The aim of this study was to evaluate whether 25-OH Vitamin D deficiency in obese women could represent a risk factor for the onset of EOC. Specifically, the objective was to demonstrate whether 25-OH Vitamin D deficiency could increase the ROMA predictive power in a population of obese women.

## MATERIALS AND METHODS

### *Patients*

Group 1 comprised 118 female patients (mean age: 34 years, range: 23-45) referred to the Obesity Center of the Policlinico Umberto I, Rome, Italy, with a body mass index (BMI)  $>30 \text{ kg/m}^2$ , therefore classified as obese.

Group 2 was composed of 80 healthy female blood donors (mean age: 36 years, range: 21-47) whose samples were obtained through collaboration with the Blood Bank of the Policlinico Umberto I, Rome, Italy. These women, unlike the previous ones, had a body mass index (BMI)  $<25 \text{ kg/m}^2$  and were therefore classified as normal-weight.

BMI categories were defined according to the National Institute of Health: underweight (BMI  $<18.5 \text{ kg/m}^2$ ), ideal body weight (BMI 18.5 to  $25 \text{ kg/m}^2$ ), overweight ( $25 \leq \text{BMI} \leq 30 \text{ kg/m}^2$ ), and obese ( $>30 \text{ kg/m}^2$ ).

All women underwent abdominal and transvaginal ultrasound (TVUS) with color Doppler to exclude ovarian masses as well as undergoing an ultrasound scan checking for adrenal masses, as this might also be a factor of increased ROMA score.

On enrolment, both normal subjects and obese patients completed questionnaires on lifestyle factors (including smoking history and alcohol consumption), and clinical data (including creatinine concentration) were acquired. Medical history was collected for each participant and peripheral blood samples were drawn and immediately sent to the laboratory for analysis. All patients signed written informed consent to participate in the study. The local ethics review board of the institution gave approval for the study.

### *Sample collection*

Sample sera were acquired following a standard

collection protocol. Samples were collected in a red top Vacutainer, clotted 60–90 min and centrifuged for 10 min at 1300×g. The serum fractions were aliquoted and stored at –80°C until analysis.

#### 25-OH Vitamin D, CA125 and HE4 determination

25-OH Vitamin D, CA125 and HE4 levels were measured with the Lumipulse® G1200 (Fujirebio, Japan), an automated assay system that allows quantitative measurements in specimens and is based on chemiluminescent enzyme immunoassay (CLEIA) technology. Lumipulse® G1200 uses a novel two-step sandwich method (Innogenetics-Fujirebio, Belgium). The assay utilizes a solid-phase and alkaline phosphatase (ALP)-labeled monoclonal antibodies immobilized on the particles that specifically bind to the analyte forming antigen–antibody immunocomplexes. Particles are then washed and rinsed in order to remove unbound materials. ALP-labeled monoclonal antibody specifically binds to the antigen of the immunocomplexes. After a second wash, substrate solution is added. Adamantyl-1,2-dioxetane phosphate (AMPPD) contained in the substrate solution is dephosphorylated by the catalysis of ALP indirectly conjugated to the particles. A luminescent signal is generated by the cleavage reaction of dephosphorylated AMPPD and reflects the amount of antigen in the sample. The cut off value between sufficient and insufficient 25-OH vitamin D was chosen corresponding to 20.2 ng/mL, according to the WHO [13]. The normal cut-off level for CA125 is <35 U/mL. The normal cut-off level for HE4 is <150 pmol/L.

#### ROMA score

ROMA was calculated as per the HE4 package insert. For

premenopausal women it is defined as follows: predictive index (PI) =  $-12.0 + 2.38 \times \text{LN}[\text{HE4}] + 0.0626 \times \text{LN}[\text{CA125}]$ . For postmenopausal women, it is defined as follows: PI =  $-8.09 + 1.04 \times \text{LN}[\text{HE4}] + 0.732 \times \text{LN}[\text{CA125}]$ , where LN stands for natural log. ROMA was calculated from the PI as follows:  $\text{ROMA} = \exp(\text{PI}) / [1 + \exp(\text{PI})] \times 100$ . A predicted probability (PP) greater than 13.1% ( $p < 0.0001$ ) suggests a high risk of having ovarian malignancies in premenopausal women.

#### Statistical analysis

Statistically significant differences were assessed using a *Chi-squared* test for categorical variables. All statistical analyses were carried out using MedCalc V 4.30 Software.

## RESULTS

#### 25-OH Vitamin D distribution in non-obese and obese women

As a threshold value, identified by ROC curve analysis, 20.2 ng/ml (sensitivity 73.3%, specificity 84%) was chosen corresponding to the limit between sufficient and insufficient 25-OH vitamin D according to the WHO.

25-OH Vitamin D values were evaluated in 118 obese women (Group 1) and 80 normal-weight blood donors (Group 2). The patients were divided according to their 25-OH vitamin D status into a deficient (< 20 ng/mL) and a non-deficient (>20 ng/mL) group. Insufficient levels of 25-OH vitamin D were observed in 76/118 (64%) Group 1 women, and in 9/80 (11%) Group 2 patients. A statistically significant difference ( $p$  value <0.0001) was observed between the values observed in obese patients (Group

**Table I.** ROMA index >13% in obese women.

| ROMA score >13%   | Number of obese women (n=22) |
|-------------------|------------------------------|
|                   |                              |
| He4 pmol/L        | 63-850 (range)               |
| Ca125 IU/mL range | 9-22 (range)                 |

1) vs normal weight women (Group 2) (Fig. 1).

## DISCUSSION

### ROMA score

The risk prediction score was investigated in obese women in comparison with normal-weight ones. ROMA score above the cut-off ( $>13\%$ ) was detected in 19% (22/118) of obese women (Group 1). Whereas in the normal-weight women group (Group 2) no woman showed a ROMA score above 13% (Table I).

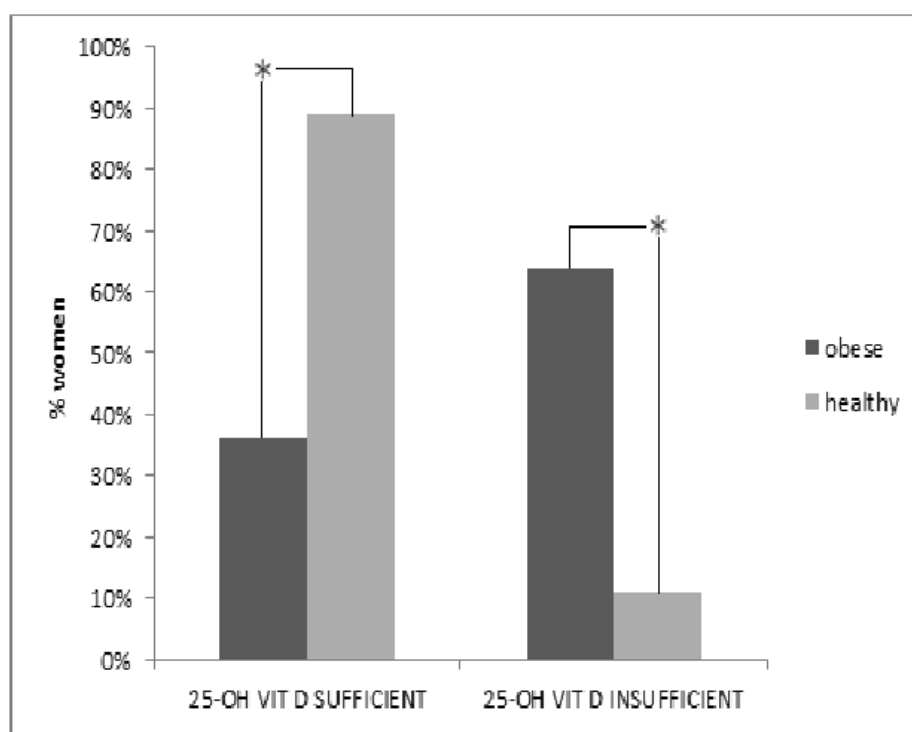
In the high risk ovarian cancer obese women group (ROMA score  $>13\%$ ) the relationship was investigated between levels 25-OH vitamin D and ROMA score. We observed that low levels of 25-OH vitamin D associate mainly with ROMA score  $>13\%$ . In fact, 64% of obese women with ROMA score  $>13\%$  had concomitant insufficient levels of 25-OH vitamin D, while only 36% of obese women with ROMA score  $>13\%$  had high levels 25-OH vitamin D. The difference was statistically significant ( $p < 0.0001$ ) (Fig. 2).

Strong preclinical and clinical evidence has established an association between obesity and EOC risk (a 30% increased risk has been described of developing EOC in obese woman compared to those with a BMI in the “healthy” range) incidence, progression, and metastasis (9).

The relationship between higher risk of EOC and obesity has great implications considering the rising prevalence of the latter. Obesity has long been associated also with poor 25-OH vitamin D status.

Many epidemiological studies, including Framingham, have shown increasing prevalence of hypovitaminosis D with increasing BMI. There is an inverse association between 25-OH vitamin D levels and anthropometric measures of body size: body weight and body fat are inversely correlated with 25-OH Vitamin D levels across the spectrum of body weight ranging from normal to obese.

The objective of this study was to evaluate whether



**Fig. 1.** Percentages of women with 25-OH vitamin D insufficiency in the group of obese women (Group 1) and normal weight women (Group 2); (\*  $p < 0.0001$ ).



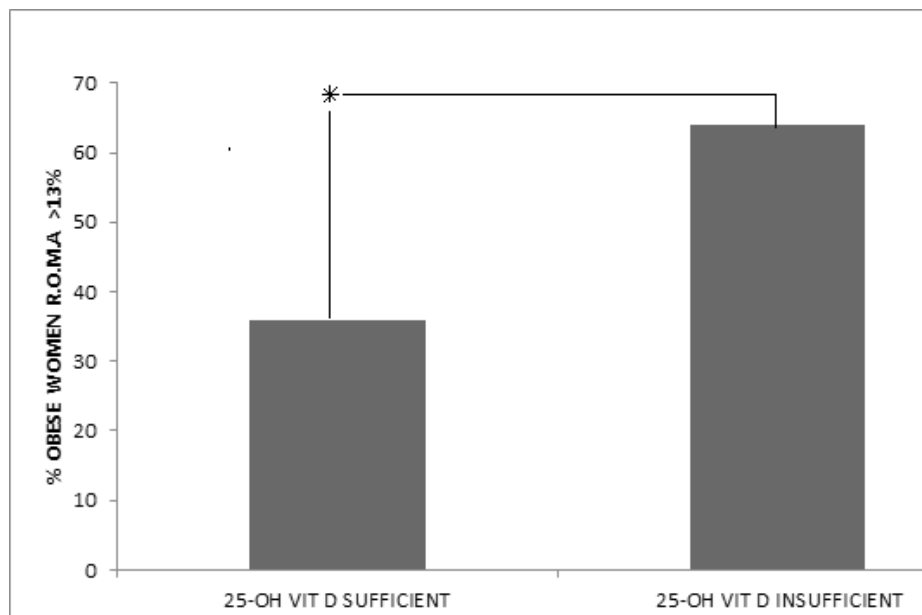
25-OH vitamin D deficiency could increase ROMA predictive power in obese women, therefore 118 obese patients with BMI higher than 30kg/m<sup>2</sup> and 80 normal-weight women with BMI below 25kg/m<sup>2</sup> as a control group were studied. There is literature supporting evidence that menopausal status is a key factor in the development of EOC, therefore in the present study only women in premenopausal age were included to better characterize the predisposition to EOC.

Firstly, a significantly higher percentage was observed of 25-OH Vitamin D deficiency in the obese patients compared to the normal weight women: this result is in agreement with previous studies showing that obesity is significantly associated with the low concentration of serum 25-OH vitamin D since it is a fat-soluble micronutrient, easier to be stored in the adipose tissue, which will reduce its bioavailability. The effect of excess body fat is theorized to: i) increase the metabolic clearance of vitamin D and its metabolites through enhanced uptake; ii) decrease the bioavailability of 25-OH vitamin D once deposited

in adipocytes; and/or iii) create a volumetric dilution of 25-OH vitamin D due to a larger body mass. 25-OH vitamin D could also prevent differentiation and maturation of preadipocytes to adipocytes by inhibiting activity and expression of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) (10). All the above findings show that the low levels of 25-OH vitamin D observed in our study are potentially related to obesity.

Several investigations have linked low serum levels of 25-OH vitamin D with increased cancer incidence. Most tissues express VDR and studies of *in vitro* cell culture and *in vivo* experimental models suggest that 25-OH vitamin D promotes cell differentiation, inhibits cancer cell proliferation, exhibits antiinflammatory, proapoptotic, and antiangiogenic properties (11). It has also recently been proposed that VDR polymorphisms may be risk factors for developing ovarian cancer (12).

To further explore the relationship between high risk of EOC and deficiency of 25-OH vitamin D in



**Fig. 2.** In obese women with ROMA score >13% a statistically significant difference was observed in the percentages of 25-OH vitamin D insufficiency.

obese women, ROMA score was evaluated in all the women included in this study: for this purpose, CA125 and HE4 were evaluated for each subject, considering that even if obesity could influence CA125 levels, conversely HE4 serum concentrations are not affected by body mass index.

ROMA score is currently used in clinical practice for identifying patients at high risk of ovarian malignancy onset, and many publications have confirmed its usefulness as a regression model to predict the risk of EOC in female patients with a pelvic mass. A ROMA score  $>13\%$  was identified in 19% of obese women, therefore classifying these patients at high risk of developing an EOC (serous and endometrioid cancer). Interestingly, a high percentage of obese women with ROMA score  $>13\%$  had concomitant insufficient levels of 25-OH vitamin D.

These results could be interpreted according to the 25-OH vitamin D antiproliferative effects and its ability to block cell mitosis in the G1 phase. Many investigations have also assessed the genomic actions of 25-OH vitamin D via VDR regulation of several genes through repression of different key molecules involved in cell cycle regulation. Evidence from *in vitro* studies supports the role of 25-OH vitamin D in stimulating cell cycle arrest and promoting apoptosis in malignant transformed cells. Such mechanisms range from preventing cell proliferation (cell cycle arrest) to inducing or suppressing cell adhesion molecules and growth factors that promote cellular homing and metastasis.

With regard to EOC, it has been shown that treating EOC cell lines with 25-OH vitamin D results in p27(Kip1) stabilization and EOC cell arrest in G1 (13). There is environmental evidence attributing high incidence of EOC to lack of sunlight exposure and it has also been demonstrated that VDR mRNA is highly expressed in EOC (14).

Additionally it has been recently hypothesized that 25-OH vitamin D could inhibit EOC by modulating cytokines, suggesting a role of 25-OH vitamin D deficiency as a possible risk factor for developing EOC (15).

In summary, this study reports that there is an association between low 25-OH vitamin D levels

and ROMA score in obese women: the results show that ROMA  $>13\%$  and deficiency of vitamin D are more frequent in obese patients than in normal weight controls. These findings are worth investigating in future prospective studies with a bigger sample size because, if the correlation between 25-OH vitamin D levels and ROMA score in obese women is real, it can be hypothesized that the detection of 25-OH vitamin D levels could be eventually reflected as a potential improvement for the predictive power of ROMA score for ovarian malignancy in obese women.

## ACKNOWLEDGEMENTS

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## LETTER TO THE EDITOR

ANTI-PLAQUE AND ANTIMICROBIAL EFFICIENCY OF DIFFERENT ORAL RINSES  
IN A 3-DAY PLAQUE ACCUMULATION MODELS. CANTORE<sup>1#</sup>, A. BALLINI<sup>1#</sup>, G. MORI<sup>2#</sup>, V. DIBELLO<sup>1</sup>, M. MARRELLI<sup>3</sup>, R. MIRGALDI<sup>1</sup>,  
D. DE VITO<sup>1#</sup> and M. TATULLO<sup>4#</sup><sup>1</sup>Department of Basic Medical Sciences, Neurosciences and Sense Organs, University of Bari Aldo Moro, Bari, Italy; <sup>2</sup>Department of Clinical and Experimental Medicine, Medical School, University of Foggia, Italy; <sup>3</sup>Calabrodental SRL, Experimental Medicine Section, Crotone, Italy; <sup>4</sup>Tecnologica Research Institute, Biomedical Section, St. E. Fermi, Crotone, Italy

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#These authors contributed equally to this study

The idea of incorporating a mouthrinse with normal tooth brushing could be a useful adjunct to oral hygiene. Despite the principle nature of the toothpaste vehicle, most alcohol-based chemical plaque-control agents have been evaluated and later formulated in the mouthrinse vehicle. The current study was aimed to investigate the persistence of antimicrobial action and plaque inhibitory properties of a new alcohol-free mouthrinse when compared with positive control, chlorhexidine 0.12%, and placebo control, physiologic saline solution mouthrinses. The evaluation of the antimicrobial activity was performed by saliva samples collected during the 3 days of usage. The results of this study indicate that this new oral rinse has an equivalent plaque inhibitory action to chlorhexidine, and the plaque inhibitory action of the rinse appears to be derived from a persistence of antimicrobial action in the mouth. Furthermore, no side effects were reported during the study, and the additional benefit of no alcohol presence in the rinse solution.

To the Editor,

Dental plaque (DP) is a biofilm that slowly accumulates on the surface of teeth. If not removed regularly, it can lead to dental cavities (caries) or periodontal problems (such as gingivitis) (1, 2). Colonization of enamel surfaces by the cariogenic bacterium *Streptococcus mutans* (*S. mutans*) is thought to be initiated by attachment to a saliva-derived conditioning film, the acquired enamel pellicle (2-5).

Mechanical control of dental biofilm has had a somewhat limited success in part because it is regarded as time-consuming and technically difficult

by most patients. This fact, coupled with an increase in the information available on the microbiology of periodontal diseases, has stimulated a great interest in developing topical antimicrobial agents to control dental biofilm (1).

In fact, periodontitis is initiated by oral biofilm formation which, if untreated, progresses to gingivitis further leading to periodontal disease. (6) According to the Evidence Based Dentistry, mouthwashes have been particularly well accepted by patients due to their ease of use, considering also their adverse effect if containing ethanol (7-9).

*Key words: chlorhexidine, gingivitis, gingival index, plaque index*

## Mailing address:

Dr. Andrea Ballini,  
Department of Basic Medical Sciences,  
Neurosciences and Sense Organs,  
School of Medicine, University of Bari Aldo Moro,  
P.zza Giulio Cesare, 11, 70124, Bari, Italy  
Tel./Fax: +39 0803430657  
e-mail: andrea.ballini@me.com

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On the one hand, chlorhexidine (CHX) is considered to be the gold standard for oral antiseptics (10), on the other hand, according to some reporters, organisms may develop resistance against long-term effects of 0.2% CHX (up to two years) after continuous use (11).

Tetrasodium pyrophosphate, disodium dihydrogen pyrophosphate, and sodium tripolyphosphate are all tartar control ingredients (7). Studies have shown that these not only serve to disrupt or retard the formation of calcium phosphate crystals, but also inhibit some bacterial growth (12).

The purpose of the present study was to evaluate the antimicrobial and anti-plaque efficiency of two different mouthwashes and a placebo through a standard 3-day plaque accumulation model in patients affected by peri-implantitis or gingivitis and periodontally healthy subjects.

## MATERIALS AND METHODS

Twenty-two healthy volunteers participated in the study. All subjects were screened for suitability by the research team. Selection criteria were a dentition with  $\geq 20$  evaluable teeth (minimum of five teeth per quadrant), no oral lesions, no severe periodontal problems (no probing depth  $\geq 5$  mm), and no removable prostheses or orthodontic bands or appliances. Persons allergic to several mouthwash components were excluded from the study. All the selected subjects were informed about the products and the purpose of the study and informed consent was given. The study was conducted in accordance with the ethical principles originating in the Declaration of Helsinki and consistent with good clinical practices.

Prior to the study period, the volunteers received a soft and hard tissue examination of the oral cavity, followed by a professional scaling and polishing to remove all calculus, plaque and extrinsic tooth stain. Professional scaling and polishing was performed using hand instruments, mechanical scalers, rotating brushes with polishing paste, and dental floss in the interproximal areas. Randomization of subjects into a control or a test group was performed using computer-generated random numbers. A person not directly involved in the research project carried out the allocation of tests or control products.

The subjects in the test group received a bottle of

non-alcoholic calculus dissolution-based oral rinse containing tetrapotassium pyrophosphate (TKPP), sodium tripolyphosphate (STPP), sodium bicarbonate, sodium fluoride and citric acid (Periogen<sup>®</sup>, USA). The positive control was a 0.12% CHX solution, while the negative control was a physiologic saline solution (PSS) (placebo - no mouthwash dilution added).

All bottles with mouthwash provided to subjects were pre-weighed. All participants were instructed to refrain from using any other means of oral hygiene during the experimental period. All subjects were instructed to rinse twice a day, in the morning and in the evening, with an applicator spoonful dissolved in one cup of warm water for 60 seconds, or 20 ml of solution for CHX and PSS. Subsequent rinsing with water was not allowed and the subjects were also asked not to brush their teeth during the study period. For plaque scores, the participants rinsed for 30 seconds using a disclosing solution (1% erythrosine), according to Quigley and Hein index, Turesky modification (13).

All measurements were carried out under similar clinical settings and by the same blinded investigator. The plaque and bleeding scoring were recorded prior to saliva collection. All returned mouthwash bottles were weighed to calculate the amount of mouthrinse used and to check for compliance.

In order to have microbial samples representative of health- and disease-associated conditions, the 22 volunteers selected included 11 periodontally healthy individuals (less than 10% of the sites with bleeding of gingival margin, plus a maximum of 2 sites with probing depth  $> 6.0$  mm or attachment level  $> 5.0$  mm); 11 individuals presenting gingivitis (bleeding of gingival margin in at least 10% of the sites), but also no more than 2 sites with probing depth  $> 6.0$  mm or attachment level  $> 5.0$  mm, and 2 with peri-implantitis.

The volunteers were asked to collect 2–3 mL of unstimulated saliva in the morning as a baseline sample and at 15 minutes after swishing the solution around inside the mouth. The saliva collection was repeated on the first, second and third day.

### *Preparation of bacterial samples*

Samples were put in a sterile vial and then stored at 4°C until they were processed on the same day. Brain-heart infusion agar was used as selective medium for *S.*



*mutans*. Purity of growth was checked by examining them under an optical microscope (Carl Zeiss ICS/KF2, Oberkachen, Germany).

For the assessment of the *in-vivo* antimicrobial activity of the solutions, a sensitivity test was carried out according to the agar dilution method described by the National Committee for Clinical Laboratory Standards

(14). The plates were incubated at 37°C in an incubation chamber for 24 h and then colony forming units (CFUs) were counted (Fig. 1, A-C). The results were presented as colony-forming units (CFU)/mL, in qualitative form only, following the study design.

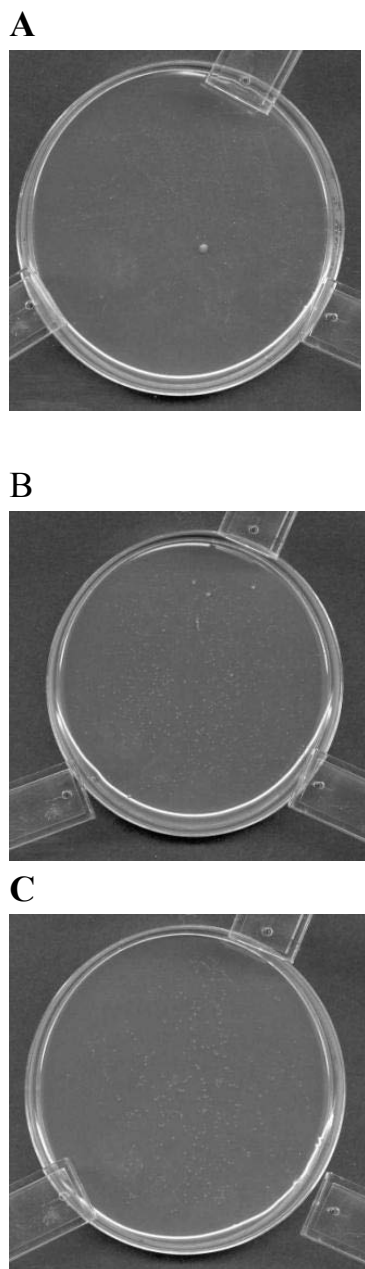
#### Statistical analysis

The statistical analyses were performed by using Statistical Package for Social Sciences (SPSS for Windows, Version 11.5, Chicago, Ill, USA) software. The values of repeated bacterial count (CFU/mL) after PSS, CHX and Periogen® mouthrinsing were expressed as the minimum (min), maximum (max), and median. The recorded values were transferred to log values before statistical analyses. The bacterial count differences in median ranks for repeated measures among time intervals before mouth rinsing (T0), 15 minute effect (T1), second day effect (T2), and third day effect (T3), were compared by the non-parametric Friedman test, and significance levels were set at 0.05. Wilcoxon signed rank test was used to compare two dependent nonparametric values, and significance level was chosen as 0.01 according to Bonferroni adjustment. The Friedman test was used to determine the bacterial count difference at each time interval for different mouth-rinsing solutions, and the Wilcoxon signed rank test was used for two dependent non-parametric values. Significance level was chosen as 0.01 according to Bonferroni adjustment. Briefly, the general level of significance was 0.05, and the local *P* values were adjusted according to Bonferroni due to multiple testing.

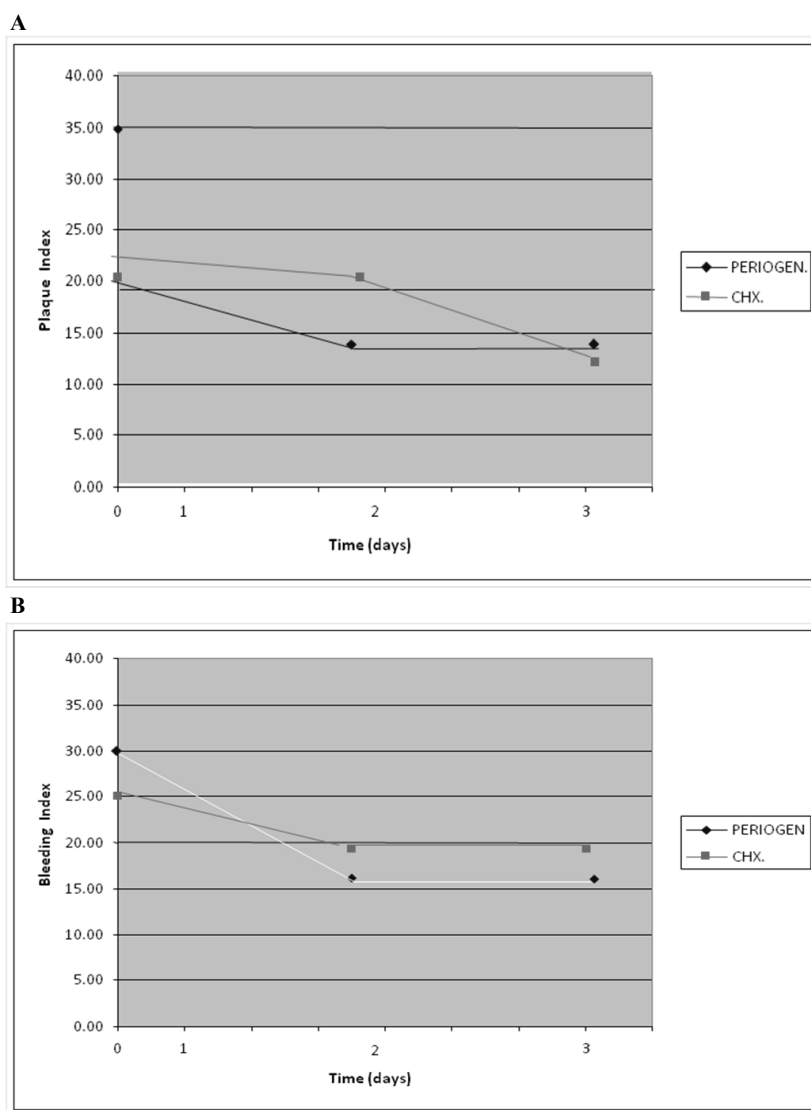
## RESULTS

All subjects (N = 22) completed the trial, and there were no missing values. The amounts of mouthwashes used indicated good compliance with the instructions. No adverse events or side effects were reported or observed. The plaque and bleeding scores for the test and control groups at the end of the experimental period are shown in Fig. 2 A-B.

The minimum, maximum, and median CFU of total *S. mutans* bacteria, in the saliva of subjects before mouth rinsing (T0), at 15 minutes after swishing the solution around inside the mouth, and on the second, and third days during treatment with antiseptics are



**Fig. 1.** CFUs on agar plates of physiologic saline solution group (A), of Periogen® group (B), and of chlorhexidine group (C).



**Fig. 2.** Plaque (**A**) and bleeding (**B**) scores for the test and control groups at the end of the study period.

presented in Table I. The antiseptic efficacy of CHX 0.12% on *S. mutans*, was very similar to the efficacy observed with Periogen® mouth solution throughout the entire study period.

There was no significant difference between antibacterial effects of CHX and Periogen® during the study period, advocating the additional benefit of an alcohol-free solution.

## DISCUSSION

In the present study, the efficacy of a new mouthwash (Periogen®) was compared with 0.12%

chlorhexidine which was considered as a gold standard. Many studies use the 3-day *de novo* plaque accumulation and non-brushing model to assess the effect of various mouthwashes (15). The results from such studies indicate that the assessment was carried out under experimental conditions. On the basis of the results obtained in the present clinical study, it can be stated that Periogen® mouthwash had a promising plaque inhibitory potential. These findings are also consistent with those reported in a six-month clinical study by Saini R. (16), who found that Periogen® solution provides a clinically relevant reduction in calculus formation as a result

**Table I.** Minimum, maximum, and median CFU ( $\log^{10}$ ) of *Streptococcus mutans* in the saliva of subjects during treatment with different antiseptics.

| Solution <sup>a</sup> | <i>Streptococcus mutans</i> |      |        |
|-----------------------|-----------------------------|------|--------|
| Time <sup>b</sup>     | Min                         | Max  | Median |
| PERIOGEN -T0          | 1.00                        | 2.45 | 1.72   |
| PERIOGEN -T1          | NG                          | 2.10 | NG     |
| PERIOGEN -T2          | 0.40                        | 1.05 | 0.72   |
| PERIOGEN -T3          | 0.50                        | 1.10 | 0.80   |
| * <i>P</i> value      |                             |      | <0.001 |
| CHX-T0                | 1.30                        | 2.40 | 1.35   |
| CHX-T1                | NG                          | 1.70 | NG     |
| CHX-T2                | 0.30                        | 1.66 | 0.98   |
| CHX-T3                | 0.50                        | 1.72 | 1.11   |
| * <i>P</i> value      |                             |      | <0.001 |
| PSS-T0                | 1.00                        | 2.00 | 1.50   |
| PSS-T1                | 1.00                        | 2.85 | 1.93   |
| PSS-T2                | 1.00                        | 2.80 | 1.90   |
| PSS-T3                | 1.00                        | 2.75 | 1.87   |
| * <i>P</i> value      |                             |      | <0.001 |

*a* PSS: physiologic saline solution; Periogen: sodium tripolyphosphate, tetrapotassium pyrophosphate, baking soda citric acid and fluoride 0.04%; CHX: chlorhexidine gluconate;

*b* T0: before mouth rinsing; T1: 15 min after swishing solution around inside the mouth; T2: second day effect; T3: third day effect.

*c* NG indicates no growth.

\* Friedman test.

of a reduced calcification of DP. However, studies of longer duration with cross-over study design and wash-out period would have been more authenticating as it eliminates the bias of viable host.

Further studies where safety and microbiological parameters on other bacteria species would be evaluated are essential to establish the true effectiveness of the mouthwash and its position among the other rinses that are used adjunctively to mechanical oral hygiene procedures (5).

Though CHX mouthwash is considered as a gold standard, it has been reported as having a number of local side effects including brown extrinsic tooth and tongue staining, taste disturbance, dryness of mouth, burning sensation (9). These side effects limit its acceptability to users and its long-term use, whereas

Periogen® mouthwash had no reported side effects.

From the public health point of view, Periogen® mouthwash can serve as a good alternative to avoid alcohol-based products or those with any artificial preservatives. There is need to investigate other alternative mouthwashes (such as Periogen®) to prove their efficacy as equivalent to alcohol-based ones in reducing the plaque scores and gingival inflammation. As this is a short-term study, the results can be used as a baseline data for future studies with similar study design.

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## LETTER TO THE EDITOR

MOLECULAR CHARACTERIZATION AND ANTIBIOTIC RESISTANCE OF  
SALMONELLA SEROVARS ISOLATED IN THE APULIA REGION OF ITALY

R. MIRGALDI<sup>1#</sup>, A. BALLINI<sup>2#</sup>, A.M. DIONISI<sup>3</sup>, I. LUZZI<sup>3</sup>, G. DIPALMA<sup>4</sup>,  
F. INCHINGOLO<sup>4</sup> and D. DE VITO<sup>2#</sup>

<sup>1</sup>Department of Soil, Plant and Food Science, University of Bari Aldo Moro, Bari, Italy;

<sup>2</sup>Department of Basic Medical Science, Neurosciences and Sense Organs, University of Bari Aldo Moro, Bari, Italy; <sup>3</sup>Department of Infectious, Parasitic and Immuno-Mediated Diseases, Istituto Superiore di Sanità, Rome, Italy; <sup>4</sup>Department of Interdisciplinary Medicine, University of Bari Aldo Moro, Bari, Italy

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#These Authors equally contributed to the work

During the period January 2013-December 2015, 175 cases of human salmonellosis were reported in the Apulia Region of Italy. The aim of this study was to characterize salmonella strains from the standpoints of serovars prevalence, antimicrobial resistance and clonal origin. The serological typing was performed by agglutination against antisera followed by a multiplex polymerase chain reaction (m-PCR). The obtained results were analyzed following the Kauffmann-White scheme. Susceptibility to antimicrobial agents was tested using the disk diffusion method on Muller-Hinton agar plates. All strains were tested by pulsed-field gel electrophoresis (PFGE) according to the PulseNet protocol, and cluster analysis was performed using BioNumerics software. It was found that the most prevalent isolated serovars were in order: i) *S. Enteritidis*, ii) *S. Typhimurium* and iii) *S. 4,[5],12:i:-*. The most common resistances were: i) Ampicillin (A) (38%), ii) Amoxicillin/Clavulanic Acid (AmC) (11%), iii) Streptomycin (S) (19%), iv) Sulphonamides (Su) (19%), v) Tetracycline (T) (30%), and vi) Piperacillin (Pip) (25 %). Ten multidrug-resistant (MDR) patterns were identified among the isolates, and the two most diffused ones were ASSuT and ASSuTPip, respectively. MDR patterns were predominantly expressed by *Salmonella Typhimurium* and *Salmonella 4,[5],12:i:-*. Molecular typing by PFGE yielded 60 different macrorestriction profiles among 33 serotypes.

To the Editor,

*Salmonella* spp. are important food borne pathogens and increasing occurrence of antimicrobial resistance in both typhoidal and non-typhoidal *Salmonellae* is a major public health problem. Human salmonellosis is caused by various

*salmonella* serovars and it occurs in a wide variety of forms presenting a broad clinical spectrum. It may occur as an acute, self-limiting gastroenteritis or as systemic infection characterized by septicemia and ultimate localization in extra-intestinal sites (1).

The emergence of antimicrobial resistance among

*Key words: antibiotic resistance, pulsed field gel electrophoresis, salmonella, water borne disease, multiplex polymerase chain reaction*

Mailing address:

Dr. Andrea Ballini,  
Department of Basic Medical Science,  
Neurosciences and Sense Organs,  
University of Bari Aldo Moro, Bari, Italy  
Tel./Fax: +39 0805448583  
e-mail: andrea.ballini@uniba.it

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salmonella spp. makes it more difficult to choose the proper antimicrobial therapy, when required. Since 1990 there has been a dramatic increase in the occurrence of multidrug-resistant (MDR) strains of *Salmonella* spp. in many developed countries (2). This is of great concern as the majority of infections with MDR *Salmonella* are acquired through the consumption of contaminated foods of animal origin such as swine and chicken eggs. Currently, there is an increasing global concern for the transmission of extended spectrum  $\beta$ -lactamase- (ESBL) producing salmonella and *Enterobacteriaceae*, from primary production to humans (3). This could potentially compromise therapeutic treatment with third-generation cephalosporins in humans, that are classified as critically important antimicrobials for human health, and are the drugs of choice for treating invasive infections. Since they are the first choice for treating children, there may be serious public health implications and consequences (2,3).

Furthermore, there is a need for continuous surveillance and sharing of antimicrobial susceptibility data for salmonella among countries worldwide (3).

The aim of this work was the surveillance of human salmonellosis in the Apulia Region during a two-year period in relation to the framework "Enter-Net Italia" and to characterize salmonella strains from the standpoints of serovars prevalence, antimicrobial resistance and clonal origin. *Salmonella* strains were characterized by genotypic typing methods in order to define clonal relationships between the isolates, obtaining in this way information on their distribution.

## MATERIALS AND METHODS

### *Bacterial strains*

A total of 175 salmonella spp. strains were isolated from different samples (fecal specimens, rectal swabs and blood), collected in Apulian Hospitals from patients with gastrointestinal symptoms typical of salmonellosis, and sent to the Public Health Laboratory, Department of Basic Medical Sciences, Neurosciences and Sense Organs, University of Bari "Aldo Moro" during the period January 2013 - December 2015.

The confirmation of the salmonella genus identification was performed using automated biochemical tests (MicroScan analysis).

### *Serotyping of salmonella*

Serotyping of salmonella spp. isolates was performed by slide agglutination according to the Kauffmann-White scheme (4). All the strains were tested against polyvalent and monovalent antisera (Antiserum *Salmonella*, Bio-rad) and saline control (negative control test) to identify the antigenic formula.

*Salmonella* phase inversion was performed following the Sven-Gard Method (5). In order to discriminate *Salmonella* 4,[5],12:i:- from *S. Typhimurium* strains, a multiplex polymerase chain reaction (m-PCR) was carried out to identify the *invA* housekeeping gene and the *fljB* gene typical of *S. Typhimurium*, respectively. The test was performed using colonies isolated on solid media. DNA was extracted from 2-3 bacterial cells grown on a Nutrient, suspended in 100  $\mu$ L of phosphate-buffered saline (Oxoid), boiled for 5 min and centrifuged at 8000 g for 5 min. The supernatant was used for PCR and stored at -20°C. The PCR assay was performed in a total volume of 25  $\mu$ L containing 1.5 mM  $MgCl_2$ , 0.2 mM of dNTPs (Invitrogen, Carlsbad, CA, USA), 1X Buffer-Taq, 1U of Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), 0.5 mM of each were primers specific for *InvA* (*invA* F 5'-GTGAAATTATCGCCACGTTCCGGGCA-3'; *invA* R 5'-TCATCGCACCGTCAAAGGAACC-3') and *fljB* (*fljB-s* 5'-ACGAATGGTACGGCTTCTGTAAACC-3'; *fljB-as* 5'-TACCGTCGATAGTAACGACTTCCG-3'), and 2.5  $\mu$ L of template DNA.

Amplification was carried out in MyCycler® Thermal Cycler (Bio-rad). The amplification profile was: (i) denaturation: 95°C for 3' (ii) amplification 30 cycles: 95°C 60'' - 65°C 60'' - 72°C 60'', (iii) final extension: 72°C 7'. A 17  $\mu$ L aliquot of PCR product was loaded onto a 1.1% agarose gel, and electrophoresis was performed in TAE Buffer 1x (2 M TRIS, 0.05 M EDTA, pH 8) at 100 V/cm for 45 min. The expected amplicons were: *InvA* 526 bp, *fljB* 284 bp.

### *Antimicrobial susceptibility analysis*

The susceptibility to antimicrobial agents was tested using the disk diffusion method on Muller-Hinton agar plates according to the European Committee on Antimicrobial Susceptibility Testing (www.eucast.org).

To ensure the accuracy and reproducibility of



antimicrobial susceptibility test results, internal quality control testing was regularly performed using *Escherichia coli* ATCC 25922 as strain for quality control.

The tested antibiotics were: Ampicillin (A), Amoxicillin + Clavulanic acid (AmC), Gentamicin (G), Cefotaxime (Ctx), Ceftazidime (Caz), Ciprofloxacin (Cip), Chloramphenicol (C), Nalidixic Acid (NA), Tetracycline (T), Kanamycin (K), Piperacillin (Pip), Streptomycin (S), Sulfonamide (Su), Trimethoprim (Tr), Trimethoprim-Sulfametossiazole (SXT).

#### *Detection of extended-spectrum-beta-lactamase-producing salmonella*

Automated MicroScan analysis (Dade Behring) was used to detect ESBL-producing salmonella. All procedures were performed according to the manufacturer's instructions (6).

#### *Pulsed field gel electrophoresis and cluster analyses*

Pulsed field gel electrophoresis (PFGE) analysis was performed according to the PulseNet standardized protocol (7) using *XbaI* as the restriction enzyme (Sigma-Aldrich). *Salmonella enterica* serotype Braenderup H9812 strain was used as the molecular size marker. Agarose gels were stained with ethidium bromide, in order to visualize the DNA fragments and capture them digitally for analysis. Dendrogram and cluster analysis were performed using algorithms available within the BioNumerics software package (v. 7.5, Applied Maths, Sint-Martens-Latem, Belgium). The similarity percentage among different chromosomal fingerprints was scored by the Dice coefficient. The unweighted pair group method with arithmetic means (UPGMA), with a 1% tolerance limit and 1% optimization, was used to obtain the dendrogram. DNA profiles which differentiate by one or more DNA fragments were considered as distinct patterns. Strains with a coefficient of similarity  $\geq 85\%$  were considered to be genetically closely related.

## RESULTS

During the investigated period a total of 175 salmonella strains were isolated from human samples confirming the salmonellosis diagnosis. Overall, reported episodes of salmonellosis have declined steadily for years; in fact the annual number of confirmed cases were: i) 74 in 2013, ii) 66 in 2014,

iii) 35 in 2015.

Salmonella isolates belonged to 33 serotypes, with *Enteritidis* being the most frequent one (46/175, 27%), followed by *Typhimurium* (42/175, 24%), its monophasic variant 4,[5],12:i:- (32/175, 18%).

The change in serovars prevalence during the years 2013-2015 is reported in Fig. 1

In 2015 the number of cases with *S. Enteritidis* (4/35, 11%) decreased, compared to 2014 (21/66, 32%) and to 2013 (21/74, 28%); while cases with *S. Typhimurium* (7/35, 20%) decreased compared to 2014 (14/66, 32%) and to 2013 (21/74, 28%). Conversely, the number of *S. 1,4,[5],12:i:-* cases in 2015 (8/35, 23%) increased, compared to 2014 (8/66, 12%) and to 2013 (12/74, 16%).

All 175 salmonella isolates were susceptible to Gentamicin, and nearly half of the examined strains (86/175, 49%) were fully susceptible to the tested antimicrobials, although the most common resistances were: i) Ampicillin (38%), ii) Amoxicillin/Clavulanic Acid (11%), iii) Streptomycin (19%), iv) Sulphonamides (19%), v) Tetracycline (30%), and vi) Piperacillin (25%). Interestingly, ten MDR patterns were identified among the isolates, and the two most diffused ones were ASSuT and ASSuTPip, respectively (Tab. 1). Also, three ESBL-producing strains were identified in 2013 and 2014: *Salmonella* Muenchen, *Salmonella* II and *Salmonella* Infantis. Thus, over the years the percentage of MDR strains increased, 16% in 2013, 22% in 2014, 57% in 2015.

The percentage of the resistances to each antimicrobial tested by years is shown in Figure 2. The histograms highlight that percentages of strains resistances to antimicrobials were increasing by year: i) Ampicillin, ii) Amoxicillin/Clavulanic Acid, iii) Streptomycin, iv) Sulphonamides, v) Tetracycline, vi) Trimethoprim - Sulfamethoxazole, vii) Amikacina, viii) Piperacillin.

PFGE, performed on the 175 salmonella strains using the *XbaI* enzyme, yielded 60 different macrorestriction profiles (PFGE.0001-PFGE.0060). The observed banding pattern comparison for different salmonella serovars is illustrated in Fig. 3.

Five PFGE patterns (PFGE.0052, PFGE.0053, PFGE.0054, PFGE.0055 and PFGE.0056) were obtained from 46 strains of *S. Enteritidis*. All

**Table I.** Prevalence of MDR-type among salmonella strains by year.

| R-TYPE       | 2013  | 2014  | 2015  | Total  |
|--------------|-------|-------|-------|--------|
| ASSuT        | 04/74 | -     | 05/35 | 09/175 |
| AAkSPip      | -     | -     | 02/35 | 02/175 |
| ASSuTSXT     | -     | 01/66 | 03/35 | 04/175 |
| ASSuTPip     | 02/74 | 09/66 | 03/35 | 14/175 |
| AAmcSSuPip   | -     | 01/66 | 02/35 | 03/175 |
| AAmcSSuTPip  | 02/74 | 03/66 | 01/35 | 05/175 |
| ACSSuTSXT    | 03/74 | 01/66 | 02/35 | 06/175 |
| AAmcCSSuTPip | -     | -     | 01/35 | 01/175 |
| AAmcSSuTSXT  | -     | -     | 02/35 | 02/175 |
| ESBL         | 01/74 | 02/66 | -     | 03/175 |
| <b>Total</b> | 12/74 | 15/66 | 20/35 | 48/175 |

PFGE.0052 (19/46 42.2%) PFGE.0056 (1/46, 2.2%) and PFGE.0053 (24/46, 52.2%) patterns were susceptible, instead PFGE.0054 (1/46, 2.2%) and PFGE.0055 (1/46, 2.2%) showed R-type T and ASSuT, respectively. PFGE.0052, PFGE.0053, PFGE.0054, PFGE.0056 were considered as closely related genetically with the coefficient of similarity >85%.

*S. Typhimurium* showed high genetic variability: 16 pulsotypes were presented by 42 strains. Among the patterns, two clusters were identified: “cluster a” grouped PFGE.0013, PFGE.0014, PFGE.0060, PFGE.0012, PFGE.0011, PFGE.0010 with similarity >80%, “cluster b” grouped PFGE.0025, PFGE.0059 and PFGE.0057 with similarity >88%, suggesting a close common ancestor. In particular, PFGE.0025 and PFGE.0026, were detected in all three investigated years and were MDR.

*Salmonella* 4,[5],12:i:- showed less genetic

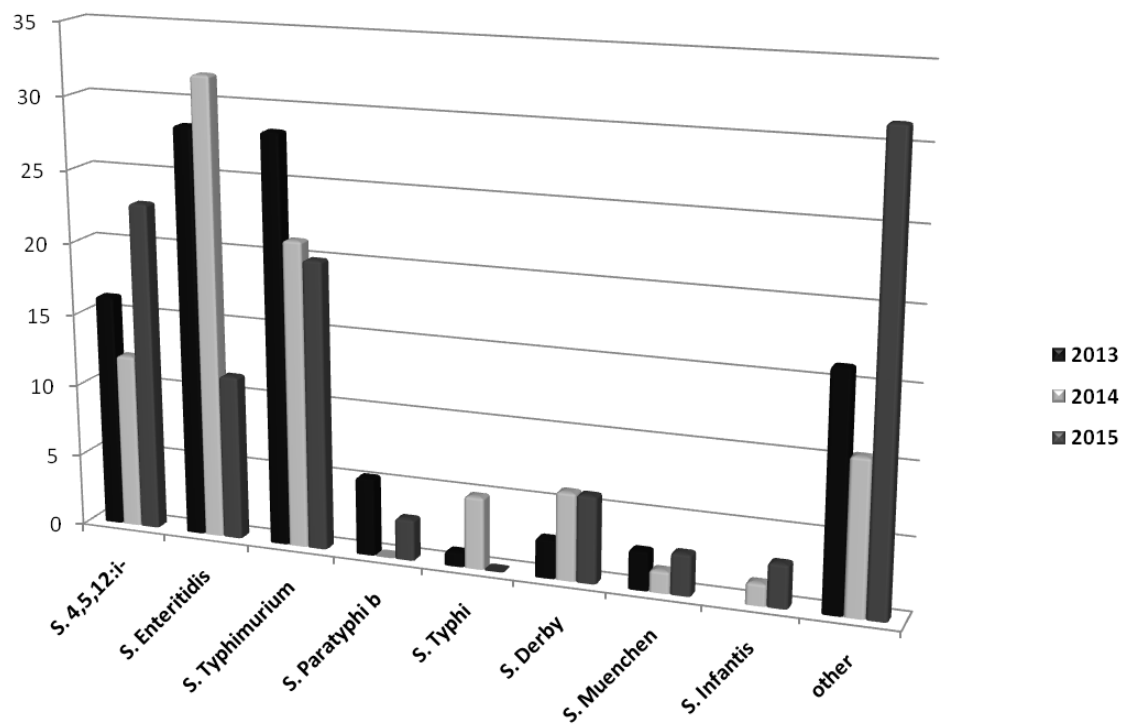
variability than *Typhimurium*: 6 pulsotypes were presented by 28 strains. Among the patterns, one cluster was identified and it grouped PFGE.0019, PFGE.0020, PFGE.0021 and PFGE.0022 with similarity >88%.

Particularly for *Salmonella* 4,[5],12:i:- PFGE.0020 R-types detected were ASSuT (6/7) and ASSuTPip (1/7), and all were genetically closely related.

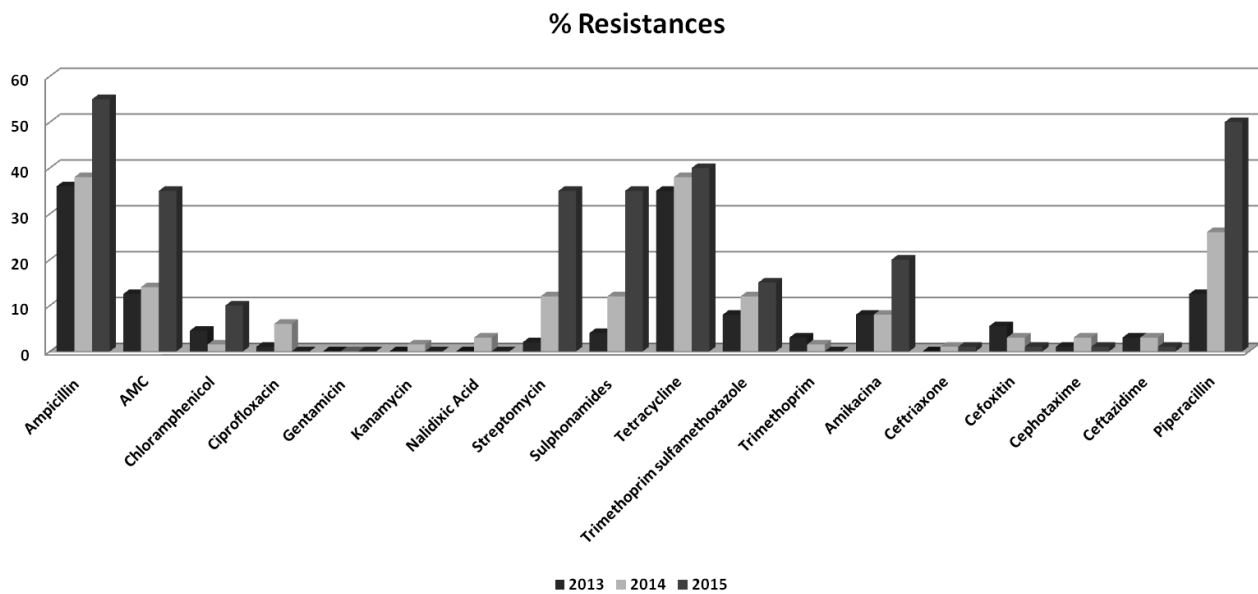
All isolates were included in the database of the Enter-net Surveillance Network at the Section of Hygiene (Dept. of Basic Medical Science, Neurosciences and Sense Organs) of the University of Bari Aldo Moro (Italy) coordinated by the Istituto Superiore di Sanità, Rome (Italy).

## DISCUSSION

The aim of this work was to characterize *Salmonella* strains, responsible for human cases in



**Fig. 1.** Change in serovars distribution in the Apulia region during the period 2013-2015.



**Fig. 2.** Percentage of antimicrobial resistance to different antimicrobial agents by study year.

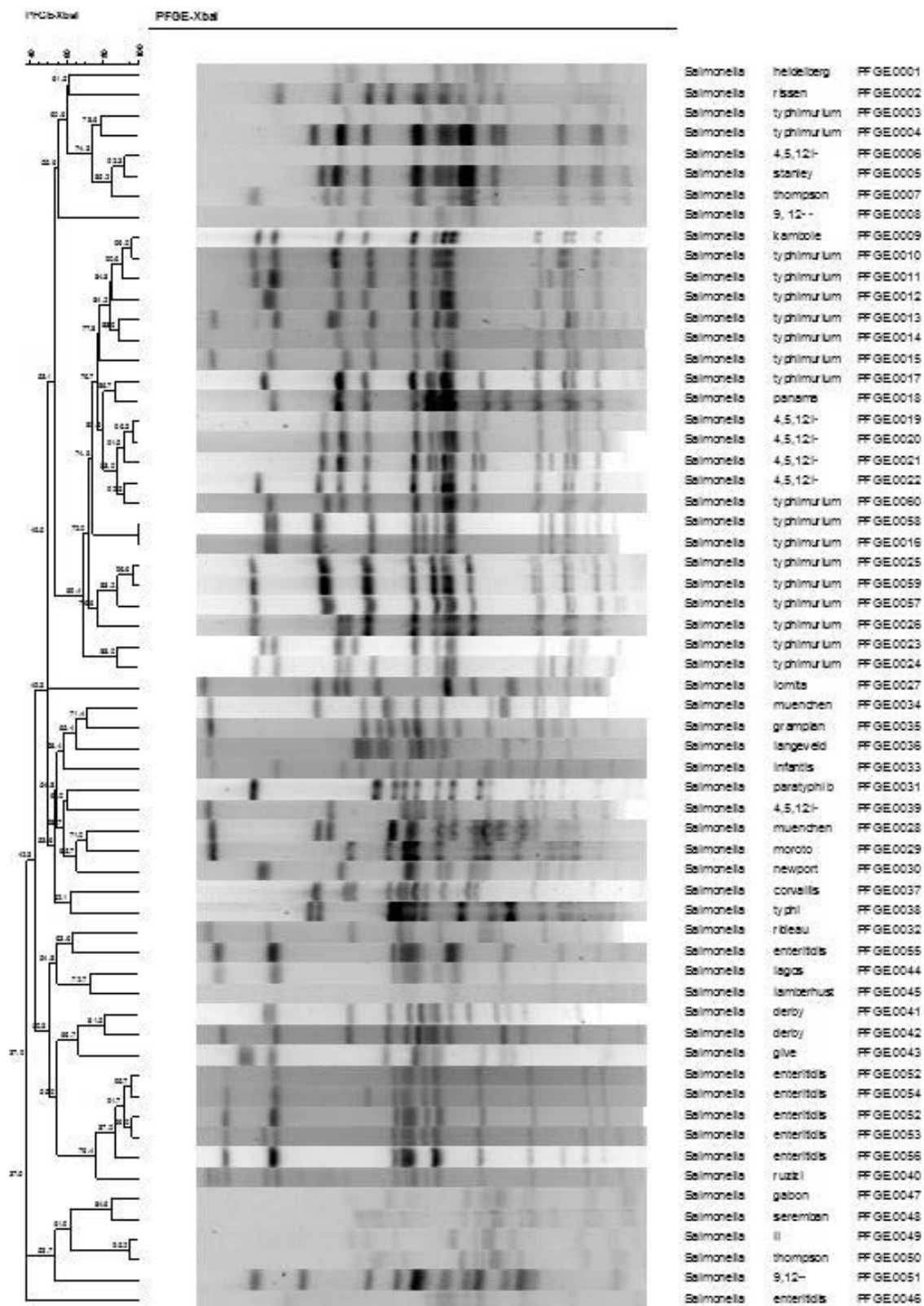


Fig. 3. Dendrograms of all obtained PFGE profiles.

Apulia Region during the years 2013-2015, from the standpoints of serovars prevalence, antimicrobial resistance and clonal origin. It was found that the number of human salmonellosis cases notified in Apulia Region showed a constant lowering in the investigated period. This finding is in agreement with the data collected by the European Centre for Disease Prevention and Control for the European Union (EU) countries, which reported that since 2006 salmonellosis rates have continued to decrease with a significant lowering trend in 17 EU countries (8). This decrement is mainly attributed to the implementation of successful veterinary control programs, particularly in poultry (9).

As in other countries (10, 11), the strains of salmonella isolated in Italy are frequently MDR (14). For this reason there is an ever growing perception of the need and importance for surveillance systems in order to control salmonella infections, the emergence of resistant strains and to monitor the source of infections to reduce the transfer risk of MDR salmonella to humans. (12).

In the present research, the antimicrobial susceptibility analyses showed that resistance to antimicrobials occurred in 51% (89/175) of all isolates and PFGE, performed on the 175 strains of salmonella using the *XbaI* enzyme, yielded 60 different macrorestriction profiles among 33 serotypes (Fig. 3). Among the prevalent serovars, *S. Enteritidis* had less genotypic variability; in fact, 5 pulsotypes were obtained among 46 isolates and 4 of these were closely related genetically except for PFGE.0055 that identified the only MDR *S. Enteritidis* with ASSuT type. On the contrary, *S. Typhimurium* showed high genetic variability: 16 pulsotypes were presented by 42 strains and two clusters (a and b) grouped respectively 6 and 3 pulsotypes. *Salmonella* 4, [5], 12: i:—showed less genetic variability than *Typhimurium*: 6 pulsotypes were presented by 28 strains. In conclusion, in accordance to literature data (13-15), resistance to antimicrobials appears to increase salmonella spp. Analysis of these results may aid in the development of practices that will prevent resistance or slow salmonella spp. spread and thus reduce its impact on human and animal health. In these settings, the surveillance in Apulia

Region may play an important role as ‘sentinel’ for emerging pathogens and resistance patterns.

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## LETTER TO THE EDITOR

**FINE NEEDLE ASPIRATION CYTOLOGY OF 650 THYROID NODULES OPERATED FOR MULTINODULAR GOITER: A CYTO-HISTOLOGICAL CORRELATION BASED ON THE NEW ITALIAN CYTOLOGICAL CLASSIFICATION (SIAPEC 2014)**

F. TARTAGLIA<sup>1</sup>, A. GIULIANI<sup>2</sup>, L. TROMBA<sup>1</sup>, S. CARBOTTA<sup>1</sup>, M. KARPATHIOTAKIS<sup>1</sup>, G. TORTORELLI<sup>1</sup>, F. PELLE<sup>1</sup>, R. MEROLA<sup>1</sup>, C. DONELLO<sup>1</sup>, G. CARBOTTA<sup>3</sup>, L. DE ANNA<sup>1</sup>, G. CONZO<sup>4</sup>, S. SORRENTI<sup>1</sup> and S. ULISSE<sup>1</sup>

<sup>1</sup>Department of Surgical Sciences, "Sapienza" University of Rome, Rome, Italy; <sup>2</sup>Department of Environment and Health, Istituto Superiore di Sanità, Rome, Italy; <sup>3</sup>Department of Experimental Medicine, "Sapienza" University of Rome, Rome, Italy; <sup>4</sup>Department of Clinical and Experimental Medicine and Surgery, Second University of Naples, Naples, Italy

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The new Italian cytological classification (SIAPEC 2014) of thyroid nodules, in line with those of Bethesda and BTA-RCPATH, replaces the previous TIR3 class with two new classes (TIR3A and TIR3B), which correspond to different risks of malignancy and clinical actions required. The present study was conducted to evaluate the diagnostic accuracy of the new SIAPEC classification as opposed to its previous version (SIAPEC 2007). Preoperative cytology was compared with the final histology obtained from 650 consecutive patients who underwent total thyroidectomy for multinodular goiter. Of this total, 434 patients (group A) had their cytological diagnosis based on the old SIAPEC 2007 classification and 216 patients (group B) had their cytological diagnosis based on the SIAPEC 2014 classification. In group A 111 patients (25.6%) had a TIR3 diagnosis, while in group B 52 patients (24.1%) received a TIR3 diagnosis, of whom 30 had TIR3A and 22 had TIR3B. In group A, 46 (41.4%) out of the 111 patients with TIR3 diagnosis had, based on histology, a thyroid carcinoma. In group B, only 2 (6.7%) out of 30 patients with TIR3A diagnosis had a thyroid carcinoma. This rate of malignancy was significantly lower ( $p < 0.001$ ) than that observed in patients with TIR3B diagnosis, in which 12 (54.5%) out of 22 patients had a carcinoma. The observations here reported show that, in respect to the previous version, the new Italian cytological classification provides greater diagnostic accuracy for detecting thyroid nodule malignancy.

To the Editor,

Fine needle aspiration cytology (FNAC) is considered the gold standard diagnostic test in the evaluation of a thyroid nodule. It is a simple, cost-effective, readily repeatable test that is quick to perform and well accepted by patients (1). The management of patients with indeterminate or

suspicious FNAC specimens remains problematical, the main challenge being the differentiation of nodules requiring surgical treatment from benign ones that can simply be monitored clinically (2, 3). Surgical excision, required to obtain a definitive diagnosis, shows that about 80% of these patients harbor a benign lesion. In order to

*Key words: thyroid nodules, thyroid cancer, fine needle biopsy, cytology, thyroidectomy, goiter*

## Mailing address:

Prof. Salvatore Ulisse,  
Department of Surgical Sciences,  
"Sapienza" University of Rome,  
Viale Regina Elena 324, 00161-Rome, Italy  
Tel.: +39 06 49972607  
e-mail: salvatore.ulisse@uniroma1.it

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reduce unnecessary thyroidectomy, a number of instrumental and molecular diagnostic approaches have been proposed (4-8). Over the years, also the cytological classifications have been developed in a bid to further improve the management of patients with indeterminate or suspicious thyroid cytology (9-11). According to the Bethesda System for Reporting Thyroid Cytopathology (BSRTC), indeterminate nodules can be classed as: class III, "atypia of undetermined significance (AUS)/follicular lesions of undetermined significance (FLUS)"; class IV, "follicular neoplasm (FN)/suspicious for a follicular neoplasm (SFN) (9). In 2009, the classification of the British Thyroid Association-Royal College of Pathologists (BTA-RCPATH) was modified along the lines of the BSRTC (10). In 2014, the Italian Society for Anatomic Pathology and Cytology together with the Italian Thyroid Association, the Italian Society of Endocrinology, and the Italian Association of Clinical Endocrinologists (SIAPEC 2014) altered the previous Italian Society of Anatomical Pathology and Cytology and the Italian Division of the International Academy of Pathology (SIAPEC 2007) classification, replacing its TIR3 class with two new classes (TIR3A and TIR3B), which differ in the risk of malignancy and in the clinical actions required (11). TIR3A and TIR3B are comparable both to the BSRTC classes III and IV and to the BTA-RCPATH classes Thy 3a and Thy 3f.

Multinodular goiter (MNG) often present local compressive symptoms that require surgical treatment, including dysphagia, neck tightness, and airway obstruction (12-15). The present study was conducted to evaluate the diagnostic accuracy of the SIAPEC 2014 classification as opposed to the SIAPEC 2007 version, by comparing preoperative cytology with final histology in a series of 650 patients who underwent total thyroidectomy for MNG.

## MATERIALS AND METHODS

### *Patients*

In this retrospective study, we analysed data relating to a series of 650 patients who underwent total thyroidectomy for MNG between January 2008

and June 2016 in the Department of Surgical Sciences of the "Sapienza" University of Rome. Preoperative cytological reports and histological diagnosis were available for all patients. All patients provided their written informed consent.

The patients were divided into two groups: group A included 434 patients (79 male and 355 female, median age 55 yr, range 16-84 yr) operated between January 2008 and August 2013, while group B included 216 patients (37 male and 179 female, median age 55, range 17-84 yr) operated between September 2013 and June 2016. The cytological classification system used for group A was the SIAPEC 2007 classification (five classes: TIR1 to TIR5), whereas patients' cytology for group B was reported using the new SIAPEC 2014 classification, in which the old class TIR3 is divided into two subclasses (TIR3A and TIR3B). Cytological and histological diagnoses were made by expert pathologists of the Pathology Department of the Policlinico Umberto I Hospital of Rome, Italy. Being affected by MNG, all patients underwent total thyroidectomy.

We retrospectively analyzed the final histology reports on the surgical specimens taken from all the patients, in order to establish whether the new classification has an improved diagnostic accuracy.

### *Statistical analysis*

The statistical significance of the malignancy rate between group A and group B patients was performed using either the *Chi*-squared and/or the Fisher exact test. Values of  $p < 0.05$  were considered significant.

## RESULTS

We retrospectively evaluated 650 patients with preoperative cytology who were underwent total thyroidectomy for MNG. Of these, 434 were cytologically classified based on the old SIAPEC criteria (group A), and 216 (group B) had their cytological classification based on the new SIAPEC criteria. Their distribution by cytological class and their final histological outcome is shown, respectively, in Tables I and II.

Examining each group as a whole, a statistically significant difference ( $p < 0.0001$ ) in the rate of malignancy between the two groups was observed. In fact, histology disclosed malignancy in 255 (58.8%) out of 434 patients of group A, and in 58 (26.8%) out of 156 patients of group B. The overall performance of the new SIAPEC 2014 classification of thyroid nodules, as reported in Table III and Fig. 1, is significantly ( $p < 0.0001$ ) improved, in respect to the old SIAPEC classification.

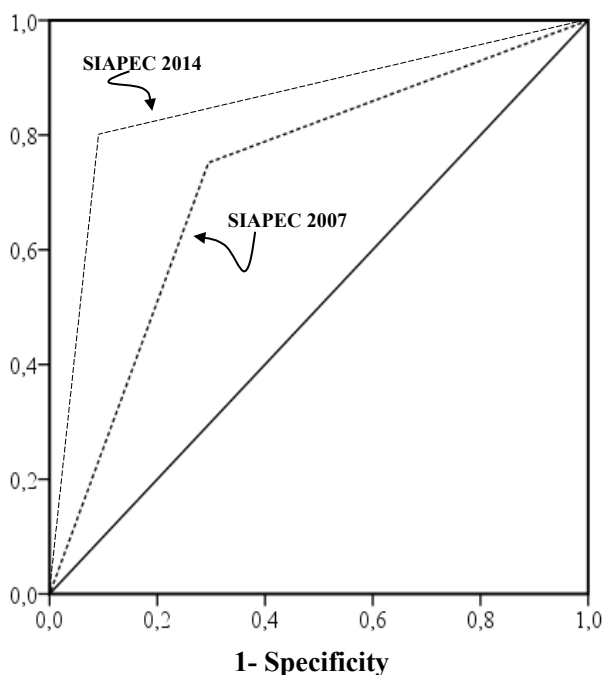
In fact, although sensitivity and positive predictive value remain unchanged, specificity, negative predictive values and accuracy are sensibly improved following the adoption of the SIAPEC 2014 criteria (see Table III). This improved accuracy appears to be due to a better classification of the old TIR3 category into TIR3A and TIR3B categories, showing a statistically significant ( $p < 0.001$ ) difference in the rate of malignancy. In particular, 163 patients presented with a cytological diagnosis of “thyroid follicular proliferation”, 111 of whom were of group A (92 women and 19 men), and 52 were of group B, of whom 30 (23 women and 7 men) were diagnosed

as TIR3A, and 22 (19 women and 3 men) as TIR3B (Tables I and II). Among the patients of group A with TIR3 diagnosis, 41.4% had a histological diagnosis of malignancy, whereas patients of group B with TIR3 diagnosis had a positive cancer diagnosis at histology in 6.7% of cases in TIR3A patients, and in 54.5% of cases in TIR3B patients.

To the improved accuracy above reported may also contribute the significantly lower rate of malignancy observed in the TIR2 ( $p < 0.001$ ) category in the new SIAPEC classification, compared with the old classification. A lower rate of malignancy in the SIAPEC 2014 classification was also observed in the TIR1 category, in respect to the old classification, but it did not reach a statistical significance ( $p = 0.084$ ). The rate of malignancy in the TIR4 and TIR5 categories remains unchanged between the two classification systems.

## DISCUSSION

FNAC has been previously found to have a malignancy predictive value of about 60%, a



**Fig. 1.** ROC (Receiver Operating Characteristic) curves for the cytological diagnosis of thyroid nodules based on the old SIAPEC 2007 or the new SIAPEC 2014 classifications.

**Table I.** Distribution by cytological diagnosis of 434 patients in Group A with correspondent histology and percentage of malignancy.

| <b>GROUP A: patients with cytological diagnosis based on the 2007 SIAPEC criteria</b> |                               |                                                                                                                                                |                               |
|---------------------------------------------------------------------------------------|-------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------|
| <b>Cytological diagnosis</b>                                                          | <b>Number of patients (%)</b> | <b>Histological diagnosis</b>                                                                                                                  | <b>Rate of malignancy (%)</b> |
| <b>TIR1</b>                                                                           | 21 (4.8)                      | <b>8 malignant:</b> 6 PTC and 2 FTC.<br><b>13 benign:</b> 1 CLT and 12 MNH.                                                                    | 8/21 (38.1)                   |
| <b>TIR2</b>                                                                           | 135 (31.1)                    | <b>38 malignant:</b> 33 PTC and 5 FTC.<br><b>97 benign:</b> 1 teratoma, 2 FA, 5 CLT, 1 De Quervain thyroiditis and 88 MNH.                     | 38/135 (28.1)                 |
| <b>TIR3</b>                                                                           | 111 (25.6)                    | <b>46 malignant:</b> 35 PTC, 9 FTC and 2 MTC.<br><b>65 benign:</b> 10 FA, 4 CLT, 51 MNH.                                                       | 46/111 (41.4)                 |
| <b>TIR4</b>                                                                           | 131 (30.2)                    | <b>127 malignant:</b> 121 PTC, 3 FTC and 3 MTC.<br><b>4 benign:</b> 4 MNH.                                                                     | 127/131 (96.9)                |
| <b>TIR5</b>                                                                           | 36 (8,3)                      | <b>36 malignant:</b> 35 PTC and 1 ATC                                                                                                          | 36/36 (100)                   |
| <b>Total</b>                                                                          | 434 (100)                     | <b>255 malignant:</b> 230 PTC, 19 FTC, 5 MTC and 1 ATC.<br><b>179 benign:</b> 10 CLT, 155 MNH, 12 FA, 1 teratoma and 1 De Quervain thyroiditis | 255/434 (58.8)                |

*CLT: chronic lymphocytic thyroiditis; MNH: multiple nodular hyperplasia; FA: follicular adenomas; PTC: papillary thyroid carcinoma; FTC: follicular thyroid carcinoma; MTC: medullary thyroid carcinoma; ATC: anaplastic thyroid carcinoma.*

low value in part determined by diagnoses of indeterminate nodules (4). The Bethesda System recommends repeating fine needle aspiration (rFNA) for the management of nodules with non-diagnostic and AUS/FLUS readings due to their low risk of malignancy (1–4% and 5–15%, respectively) (3, 9). However, rFNA does not seem to be a satisfactory course of action in such cases, given that a non-diagnostic reading is again obtained in 17–47% of nodules that previously gave non-diagnostic readings, while non-diagnostic and AUS/FLUS readings occur in 1–7% and 19–31% of nodules with previous AUS/FLUS FNA readings (3, 16). The core needle biopsy (CNB) is more useful than rFNA for reducing the frequency of inconclusive diagnoses

after initial AUS/FLUS, but it is less tolerated by patients and is associated with a higher rate of complications. In 2014, the SIAPEC proposed a five-tiered classification system in which the old TIR3 diagnostic class was divided in two subclasses, TIR3A and TIR3B, which differ in the risk of malignancy and the clinical actions required (11). TIR3A and TIR3B are comparable to the BSRTC diagnostic classes III (AUS/FLUS) and IV (FN/SFN) and the BTA-RCPATH classes Thy 3 “a” and “f”. However, unlike the BSRTC and BTA-RCPATH, the SIAPEC extends TIR3B to include those cases with “mild/focal nuclear atypia” at expected higher risk of malignancy (11).

In our study, focusing only on the TIR3 class,

**Table II.** Distribution by cytological diagnosis of 156 patients in Group B with correspondent histology and rate of malignancy.

| <b>GROUP B:</b> patients with cytological diagnosis based on the SIAPEC 2014 criteria |                               |                                                                                       |                               |
|---------------------------------------------------------------------------------------|-------------------------------|---------------------------------------------------------------------------------------|-------------------------------|
| <b>Cytological diagnosis</b>                                                          | <b>Number of patients (%)</b> | <b>Histological diagnosis</b>                                                         | <b>Rate of malignancy (%)</b> |
| <b>TIR1</b>                                                                           | 23 (10.6)                     | <b>3 malignant:</b> 3 PTC.<br><b>21 benign:</b> 5 CLT and 16 MNH.                     | 3/23 (13.1)                   |
| <b>TIR2</b>                                                                           | 108 (50.0)                    | <b>9 malignant:</b> 7 PTC and 2 FTC.<br><b>99 benign:</b> 20 FA, 14 CLT and 65 MNH.   | 9/108 (8.3)                   |
| <b>TIR3A</b>                                                                          | 30 (13.9)                     | <b>2 malignant:</b> 2 PTC.<br><b>28 benign:</b> 17 FA and 11 MNH.                     | 2/30 (6.6)                    |
| <b>TIR3B</b>                                                                          | 22 (10.2)                     | <b>12 malignant:</b> 11 PTC and 1 FTC.<br><b>10 benign:</b> 6 FA and 4 MNH.           | 12/22 (54.5)                  |
| <b>TIR4</b>                                                                           | 18 (8.3)                      | <b>17 malignant:</b> 16 PTC and 1 FTC.<br><b>1 benign:</b> 1 MNH.                     | 17/18 (94.4)                  |
| <b>TIR5</b>                                                                           | 15 (7.0)                      | <b>15 malignant:</b> 13 PTC and 2 FTC                                                 | 15/15 (100)                   |
| <b>Total</b>                                                                          | 216 (100)                     | <b>58 malignant:</b> 52 PTC and 6 FTC.<br><b>159 benign:</b> 19 CLT, 97 MNH and 43 FA | 58/216 (26.8)                 |

CLT: chronic lymphocytic thyroiditis; MNH: multiple nodular hyperplasia; FA: follicular adenomas; PTC: papillary thyroid carcinoma; FTC: follicular thyroid carcinoma.

**Table III.** Diagnostic performance of the SIAPEC 2007 and SIAPEC 2014 criteria for reporting cytology of thyroid nodules.

|                        | <b>SIAPEC 2007</b>  | <b>SIAPEC 2014</b>  |
|------------------------|---------------------|---------------------|
| <b>Sensitivity (%)</b> | 82.0<br>[76.7-86.5] | 75.9<br>[62.8-86.1] |
| <b>Specificity (%)</b> | 61.5<br>[53.9-68.6] | 93.0<br>[87.9-96.5] |
| <b>PPV (%)</b>         | 75.2<br>[69.7-80.1] | 80.0<br>[67.0-89.6] |
| <b>NPV (%)</b>         | 70.5<br>[69.7-80.1] | 91.3<br>[85.8-95.2] |
| <b>Accuracy (%)</b>    | 72.8<br>[67.8-77.9] | 84.5<br>[78.5-90.4] |

NPV: negative predictive value; PPV: positive predictive value. In square brackets the 95% confidence interval is reported.



which is divided into two subclasses in the new classification, in Group B, this subdivision resulted in a 6.7% cancer detection rate in patients classed as TIR3A *versus* 54.5% in the TIR3B patients, and 41.4% in Group A. It is worth mentioning that if the Group B had been classified using the old cytological criteria, the rate of malignant disease would have been 26.9%, which is not statistically different ( $p=0.083$ ) from that observed in Group A. Therefore, the new SIAPEC 2014 classification distinguishes more accurately among TIR3 patients those requiring surgery from those who can be referred for clinical monitoring. In particular, the rate of malignancy observed in the TIR3A patients did not differ significantly from those with TIR2 ( $p=1.000$ ) or TIR1 ( $p=0.642$ ) diagnosis. This accounts, at least in part, to the increased diagnostic accuracy of the new SIAPEC system. It has to be noted, however, that this improved accuracy in the diagnosis of indeterminate lesions has not been detected in recent studies using either the PTA-RCPath or the BSRTC classification systems (17, 18). In particular, in a very recent study, which analyzed a series 109 Thy-3a and 99 Thy-3f nodules using the BTA-RCPath classes, did not find any significant difference in the rate of malignancy between thyroid nodules diagnosed as Thy-3a, in respect to those classified as Thy-3f (17). Similar findings emerged from a recent meta-analysis of 51 studies, using the BSRTC classification system, including 4475 AUS/FLUS and 3202 FN/SFN nodules (18). The reported rate of malignancy for the AUF/FLUS FNAC was 27%, and that observed for the FN/SFN FNACs was 31% (18). These observations have prompted a number of studies to evaluate the role of ultrasonography and TIRADS (thyroid imaging reporting and data system) score to ameliorate the diagnostic accuracy of these indeterminate lesions (5, 6, 19, 20). It is finally worthy of mention that in the present study only patients with multinodular goiter were considered. Whether this represents a bias in our study remains to be determined.

In conclusion, our data reveal that the SIAPEC 2014 classification offers a better diagnostic accuracy in respect to the old classification system. However, these observations should be confirmed in a larger

series of patients, and until then clinical decisions for patients with TIR3A cytology should be carefully evaluated in order to not miss cancers.

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## LETTER TO THE EDITOR

# TENDONITIS OF THE ROTATOR CUFF TREATED WITH EXTRACORPOREAL SHOCK WAVE THERAPY: RADIOGRAPHIC MONITORING TO IDENTIFY PROGNOSTIC FACTORS FOR DISINTEGRATION

A. NOTARNICOLA<sup>1,2</sup>, L. MORETTI<sup>2</sup>, G. MACCAGNANO<sup>2</sup>, S. TAFURI<sup>3</sup> and B. MORETTI<sup>1,2</sup>

<sup>1</sup>*Department of Medical Sciences of Basis, Neurosciences and Organs of Sense, Course of Motor and Sports Sciences, Faculty of Medicine and Surgery, University of Study of Bari, Bari, Italy;*

<sup>2</sup>*Department of Medical Sciences of Basis, Neurosciences and Organs of Sense, Orthopedics Section, Faculty of Medicine and Surgery, University of Study of Bari, General Hospital, Bari, Italy;* <sup>3</sup>*Department of Biomedical Sciences and Human Oncology, Faculty of Medicine and Surgery, University of Study of Bari, Bari, Italy*

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Extracorporeal shock wave therapy (ESWT) is widely used for calcific tendonitis of the shoulder. The initial rationale for this therapy was to break the calcification, but this effect does not always occur. To date, we do not know how calcifications evolve or why they may be less responsive to the action of the shock waves. One hundred and seventy-four shoulders with calcific tendinitis were prospectively evaluated before and after ESWT, using the radiographic classifications according to Gartner and Heyer, to Bosworth and to Molè. Three months after ESWT therapy, we observed the disappearance of calcification in 36.8% of the shoulders, a reduction in size in 21.8% and no change in 41.4%. The calcifications that disappeared were large according to Bosworth ( $p=0.004$ ). The probability of disappearance of calcification increased with increasing age ( $p=0.011$ ), for medium calcifications according to Bosworth ( $p=0.001$ ), and calcifications of type A according to Molè ( $p=0.043$ ). The results of our study suggest that the radiographic aspects of calcific tendonitis of the rotator cuff could influence the disruptive effects after ESWT. With this knowledge we could define the timing of treatment and therapeutic choice for each patient.

To the Editor,

Described as early as 1872, calcific tendonitis of the rotator cuff is a common disorder caused by the deposition of calcium hydroxyapatite (hydroxyapatite, HAP) (carbonate apatite crystal) within an area of tendon degeneration or hypovascularity. The treatment of choice is primarily conservative. Extracorporeal shock wave therapy (ESWT) is a promising non-invasive treatment that

has been reported to be comparable to surgery in the long term (1, 2). In 1997 an international “Consensus Conference” defined the SW physical characteristics for clinical applications: high peak pressure ( $> 500$  bar), short duration ( $<10$  microseconds) and rapid rise in pressure ( $<10$  ns) (2). With regard to the mechanisms responsible for the disintegrating effect of the shockwave therapy there are some hypotheses. The first hypothesis is a mechanical effect. Different

*Key words: shoulder, tendinopathy, radiology, high-energy shock waves, calcific tendonitis, classification*

*Mailing address:*

Angela Notarnicola, MD, Ph.D.,  
Orthopedics Section, Department of Medical Sciences of Basis,  
Neurosciences and Organs of Sense,  
Faculty of Medicine and Surgery, University of Study of Bari,  
General Hospital, Piazza Giulio Cesare 11, 70124 Bari, Italy  
e-mail: angelanotarnicola@yahoo.it

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*in vivo* studies on calcific tendinopathy treatment demonstrated that the disintegration effect occurs with low energy level (0.04-0.32 mJ/mm<sup>2</sup>) and with longer times than that of lithotripsy for renal stone (3). In the treatment of tendinopathy, ESWT promotes pro-inflammatory and catabolic processes that are associated with removing damaged matrix constituents (4). Disappearance of the deposits may be due to breakthrough into the adjacent subacromial bursa and local resorptive reactions in the surrounding soft tissues (1).

Knowing the therapeutic effect of shock wave (SW) therapy on painful entesopathies by physiological mechanisms, and justifying the clinical improvement even in those patients in whom calcification may remain unchanged, we conducted a study in order to examine the shock wave therapy on tendon calcific deposit. The purpose of our study was to report the radiologic changes of calcific deposits after ESWT in tendinitis of the shoulder. Although SW treatment is the good option for calcific tendinitis, only a few studies have documented the radiologic changes over time or the extent of resolution of a calcific deposit (5). Knowing whether the radiographic features at the beginning could influence the calcific deposit and reabsorption after the treatment, may lead to improved insight for orthopedic surgeons, physiatrists and physiotherapists to predict the extent of recovery and adjust rehabilitation and patient expectations accordingly.

## MATERIALS AND METHODS

Between July 2012 and January 2015 we performed a prospective observational study to monitor the evaluation of calcific deposits after SW therapy. Institutional review board approval was obtained for this study protocol, and the patients gave informed consent to be involved in the study. To be eligible, the participants had to have a history of at least 6 months of pain or tenderness from idiopathic calcific tendonitis of the shoulder that was resistant to conservative treatment (rehabilitation, with strengthening and stretching exercises for the muscles and tendons of shoulder and instrumental therapy, with laser therapy and ultrasound therapy). We carried out an objective examination of the shoulder and ruled out tendon tear by sonographic

examination, and when in doubt, with magnetic resonance imaging prior to treatment. The recruited patients were evaluated at 3 months after the end of SW therapy.

### ESWT

ESWT was administered with an electromagnetic (EM) generator equipped with in-line ultrasound guidance (Minilith SL1, Storz, Switzerland), during three sessions at 7 day intervals. No local anesthesia was needed. We used an energy level of between 0.05-0.017 mJ/mm<sup>2</sup>, which was consistent with patients' pain tolerance levels, during the treatment, according to our clinical experience (2, 6-8). The energy was not increased during the treatment. The patient was placed in a supine position. The shock wave head was coupled to the shoulder and coupling gel was used between the shock wave head and shoulder. Under ultrasound guidance, the shock wave head was rotated until the calcific deposit was identified in a free position. We administered 2,400 impulses on supraspinatus calcific tendinopathy (2, 6-8).

### Radiographic assessment

We assessed the presence of calcified deposits by conventional radiography. The presence of calcifications was determined by anteroposterior radiographs of the shoulder obtained in 45° external and 45° internal rotation. The technique was standardized in terms of position of the shoulder and arm, distance from the radiographic film, and exposure. The X-ray image was obtained in 1:1 scale. These radiographs were obtained within 14 days before intervention to exclude spontaneous healing before treatment and again at 3 months after treatment and analyzed by an independent observer according to the radiographic classification systems, as follows:

- i. the staging of calcifying tendinitis according to Gartner and Heyer (9) identifies 3 types (I-III), where type I is dense with well-defined borders, formative phase, type II is dense with indistinct borders or transparent with well-defined borders, and type III is transparent with indistinct borders, resorptive phase;
- ii. The classification of morphology of calcifying tendinitis described by the French Society of Arthroscopy (SFA) (Molè) (10) classified into 4 types (A-D), where A is dense, well-defined, and circumscribed, type B is dense, well-defined, and segmented, C is transparent and non-homogenous, and D is dystrophic deposit at the origin

of the tendon;

- iii. the classification of longitudinal size of calcific deposit according to Bosworth (11) categorizes into 3 types according to dimensions (large, medium and small), where large is  $> 1.5$  cm, medium is 1.5-0.5 cm and small is  $< 0.5$  cm.
- iv. The localization of calcification is valued in relation to a perpendicular line drawn at the lateral border of the acromion, as medial or lateral orientation of the deposit. This system was analogous to quadrant technique previously described in literature (12);
- v. The shape of calcific deposit has been cataloged as arc or oval (13).

At 3 months after the end of treatment, we evaluated new radiographic data. The changes in radiologic aspects of the calcification, including staging, morphology, size and shape, were determined using the previous standardized methods. The evolution of calcification could be three possibilities: complete resolution, reduction, or no change.

#### *Clinical evaluation*

The Roles and Maudsley score was adopted for the evaluation of the therapeutic outcome (14). A score of 1, meaning excellent, was given to an absence of pain and a full functional recovery; a score of 2, meaning good, was given to patients who experienced occasional pain, but had made a satisfactory functional recovery; a score of 3, meaning fair, was awarded when patients experienced discomfort after prolonged activity; a score of 4, meaning poor, was used when pain limited functionality. Scores 1 and 2 were considered as a clinical improvement and a successful outcome to the treatment, while 3 and 4 with persistent discomfort meant that the therapy was unsuccessful.

#### *Demographic and clinical assessments*

For each subject enrolled we completed a file in which the demographic variables (age, gender), evaluation of calcification, side (right, left, bilateral), shock wave repetition, radiographic classification according to Gartner, Molè, Bosworth, shape and localization of calcification were reported. At follow-up the radiological features of calcific deposit were reassessed. The clinical improvement was measured by Roles and Maudsely Score.

#### *Statistical analysis*

The forms were entered into a database using FileMaker

Pro software. STATA MP11 software was used to analyse the data. Quantitative variables were expressed as mean and comparison of averages into three groups of calcification development (disappearance, reduced or unchanged) by ANOVA test. Fisher test was used to compare means in the different groups, while Chi-squared test was used to compare proportions in 3 groups. The percentages were compared according to the distribution in the three groups by gender and radiographic classification.

Furthermore, multivariable regression models were carried out in order to evaluate the determinants for the disappearance of calcification at follow-up. The following confounder factors were analyzed: age, gender, side of pathology, shock wave repetition, Gartner, Bosworth and Mole classifications, calcification site and shape. Odds ratio (OR) value was measured in the confidence range at 95%. For each test a value of  $p < 0.05$  was considered to be statistically significant.

## RESULTS

During the study period 192 subjects affected by shoulder calcific tendinopathy were enrolled. Twenty-eight subjects were excluded. The enrolled subjects were treated with SW therapy in our ESWT Service, national reference center for this treatment. At the 3 month follow-up (FU) 34 patients had dropped out due to various causes: family reasons (16 patients), lack of time to carry out the FU (14 patients) or because they underwent surgery for increased pain and functional limitation (4 patients).

At the FU, 158 patients, 48 of whom were males (30.4%) and the other 110 were females (69.6%) were assessed. The average of age was  $54.1 \pm 10.7$  years. In the study 174 shoulders affected by calcific tendinopathy were analyzed: 58.6% ( $n=102$ ) of calcifications was in the right side and 41.4% ( $n=72$ ) in the left side, 18.4% of patients presented bilateral calcifications; 73.6% of calcifications affected females ( $n=128$ ) and the other 26.4% males ( $n=46$ ) (Table I). The disease had begun within the previous  $2.8 \pm 3.5$  years.

At 3-month radiographic FU, the following conditions were revealed:

- in 64 shoulder calcific tendinopathies (36.8%) the deposits had disappeared (groups

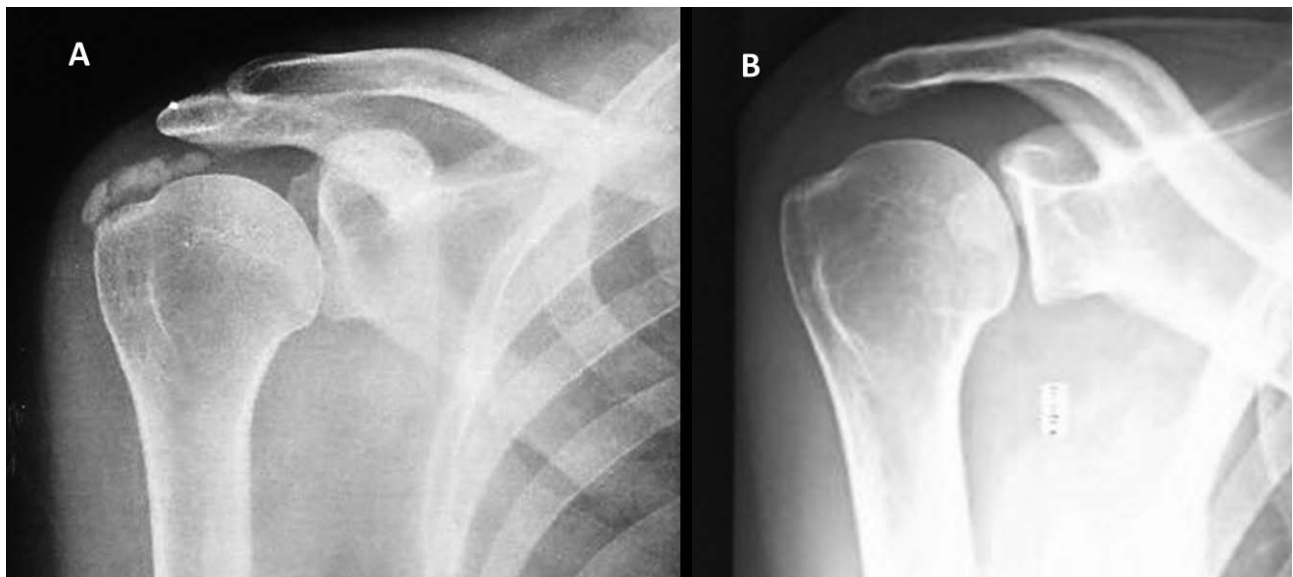
**Table I.** Influence of epidemiological characteristics of patients and radiographic classification of calcifications at enrollment for the disintegration of calcification after extracorporeal shock wave therapy.

| Groups in response to treatment of shock waves | Group absent | Group unchanged | Group decreased |                              |
|------------------------------------------------|--------------|-----------------|-----------------|------------------------------|
| At recruitment                                 |              |                 |                 |                              |
| Epidemiological features                       |              |                 |                 |                              |
| Side                                           |              |                 |                 |                              |
| Right                                          | 46           | 34              | 22              | Chi-squared= 4.25;<br>p=0.12 |
| Left                                           | 18           | 38              | 16              |                              |
| <i>Of which were bilateral</i>                 | 6            | 20              | 6               |                              |
| Age (years)                                    | 57.9±9.8     | 51.5±11.8       | 52.6±8.3        | 52.6±8.3; F=3.44;<br>p=0.04  |
| Male                                           | 12           | 24              | 10              | Chi-squared=1.85;<br>p=0.396 |
| Famale                                         | 52           | 48              | 28              |                              |
| onset of pain (years)                          | 3.1±4.4      | 2.8±3.4         | 2.3±1.5         | F=0.32; p=0.73               |
| number of cycles of treatment                  | 1.3±0.5      | 1.6±0.8         | 1.5±0.6         | F=2.33; p=0.10               |
| Radiograph classification                      |              |                 |                 |                              |
| Gartner                                        |              |                 |                 |                              |
| I                                              | 20           | 40              | 18              | Chi-squared=5.96;<br>p=0.20  |
| II                                             | 38           | 26              | 20              |                              |
| III                                            | 6            | 6               | 0               |                              |
| Bosworth                                       |              |                 |                 |                              |
| Tiny                                           | 16           | 20              | 4               | Chi-squared=15.2;<br>p=0.004 |
| Medium                                         | 14           | 38              | 26              |                              |
| Large                                          | 34           | 14              | 8               |                              |
| Molè                                           |              |                 |                 | Chi-squared=5.19;<br>p=0.52  |
| A                                              | 12           | 24              | 10              |                              |
| B                                              | 30           | 32              | 20              |                              |
| C                                              | 12           | 10              | 8               |                              |
| D                                              | 10           | 6               | 0               |                              |
| Side (orientation of the deposit)              |              |                 |                 |                              |
| medial                                         | 22           | 38              | 18              | Chi-squared=2.38;<br>p=0.304 |
| lateral                                        | 42           | 34              | 20              |                              |
| Shape                                          |              |                 |                 |                              |
| Arc shape                                      | 24           | 24              | 18              | Chi-squared=1.04;<br>p=0.593 |
| Oval shape                                     | 40           | 48              | 20              |                              |
|                                                |              |                 |                 |                              |
| At the follow up                               |              |                 |                 |                              |
| Radiograph classification                      |              |                 |                 |                              |
| Gartner                                        |              |                 |                 |                              |
| I                                              | -            | 40              | 2               | Chi-squared=13.6;            |
| II                                             | -            | 24              | 24              | p=0.001                      |



|                                          |    |    |    |                              |
|------------------------------------------|----|----|----|------------------------------|
| III                                      | -  | 8  | 12 |                              |
| <b>Bosworth</b>                          |    |    |    |                              |
| Tiny                                     | -  | 20 | 16 | $\chi^2=1.73$ ;              |
| Medium                                   | -  | 36 | 18 | $p=0.42$                     |
| Large                                    | -  | 16 | 4  |                              |
| <b>Molè</b>                              |    |    |    |                              |
| A                                        | -  | 22 | 2  | $\chi^2=13.6$ ;              |
| B                                        | -  | 36 | 12 | $p=0.001$                    |
| C                                        | -  | 6  | 16 |                              |
| D                                        | -  | 8  | 8  |                              |
| <b>Side (orientation of the deposit)</b> |    |    |    |                              |
| medial                                   | -  | 38 | 18 | $\chi^2=0.14$ ;              |
| lateral                                  | -  | 34 | 20 | $p=0.703$                    |
| <b>Roles and Maudsely Score</b>          |    |    |    |                              |
| 1                                        | 28 | 4  | 6  | $\chi^2=33.07$<br>$P<0.0001$ |
| 2                                        | 26 | 10 | 16 |                              |
| 3                                        | 4  | 18 | 8  |                              |
| 4                                        | 6  | 40 | 8  |                              |

Quantitative variables are expressed as mean and standard deviation.  $p < 0.05$ .



**Fig. 1.** A 53-year-old female affected by right shoulder calcific tendinopathy which had been present for 1 year. **A)** Before the treatment: the calcification was classified as type II according to Gartner; as large according to Bosworth, and as type B according to Molè, with oval and arc shape. The calcification was in lateral side of acromion margin. **B)** At follow-up, 3 months after the end of treatment. The calcification had disappeared in both X-rays. The patient expressed an excellent clinical recovery according to Roles and Maudsley Score.

“absent”) (Fig. 1),

- in 72 shoulder calcific tendinopathies (41.4%) the deposits were unchanged (groups “unchanged”),
- in 38 shoulder calcific tendinopathies (21.8%) the deposits had decreased in size (groups “decreased”).

The three groups did not present any statistically significant differences related to gender distribution (*Chi-squared*=1.85; *p*=0.396). The average age was higher in the “absent” group ( $57.9 \pm 9.8$ ) compared to the “unchanged” ( $51.5 \pm 11.8$ ) and the “decreased” group ( $52.6 \pm 8.3$ ; *F*=3.44; *p*=0.04). With regard to the calcific deposit localization site, no statistically significant differences were revealed between the 3 groups (*Chi-squared*=4.25; *p*=0.12). The average of disease onset was indifferent in the 3 groups (*F*=0.32; *p*=0.73). The therapy protocol for each patient was the same without any statistical differences (*F*=2.33; *p*=0.10).

The statistical evaluation of radiographic classification according to Bosworth before treatment, revealed a higher frequency of disappearance of calcification for the large type and a higher frequency of calcifications unchanged for the medium type (*p*=0.004).

The statistical evaluation of radiographic classification demonstrated higher frequency of unchanged calcification when the calcific deposit was type I according Gartner (*p*=0.001) or type B according to Molè at the end of treatment (*p*=0.001) (Table I).

The clinical evaluation by Roles and Maudsley Score allowed to define the level of clinical improvement: scores 1 and 2 when resorption of calcification was observed and scores 3 and 4, in cases of unchanged calcification (*P*<0.0001), when there is the persistent discomfort.

The multivariable regression model revealed that the probability of disappearance of calcification increases with age (*OR*=1.10; 95% *CI*=1.02-1.18; *z*=2.54; *p*=0.011) is related to the medium size according to Bosworth (*OR*=0.03; 95% *CI*=0.004-0.23; *z*=3.36; *p*=0.001) and to type A according to Mole before treatment (*OR*=0.05; 95% *CI*=0.003-0.91; *z*=2.02; *p*=0.043).

## DISCUSSION

In the present study, we investigated the influence of the characteristics of calcification on the resolution of calcific tendinitis after SW. Three months after ESWT we found a reduction of calcification in 21.8% and disintegration in 36.8% of the treated calcifications.

The first aspect that we analyzed was the radiologic density according to Gartner and Molè classification (9, 10). In our study, the multiple regression analysis showed that when calcification in pre-treatment was dense (type A, according Molè), there was a higher chance of complete disintegration and disappearance (*p*=0.043). We also demonstrated that the calcifications of medium and large size according to Bosworth classification have a tendency to disappear (respectively, *p*=0.001; *p*=0.004).

One of the indications of ESWT is to induce “destruction of calcific deposits” (15). In clinical practice we can observe many patients with functional clinical improvements that do not match the disappearance of calcification or the total reabsorption. The latter occurs only at a distance after the conclusion of treatment and early clinical improvement (8). These results can be explained, considering that when an SW bombards a tissue, the kinetic energy generated induces different trophic-metabolic effects, such as antalgic, anti-inflammatory, anti-edema, angiogenetic and proliferative (16). Consequently, it is possible that the effects of resorption may follow a temporal progression parallel to a “washout mechanism” of chemical inflammatory mediators and a nociceptive inhibition (gate control theory) on tendons in close proximity to calcifications (17, 18).

A weakness of our study is that, although we used a standardized method for radiologic analysis, the shape of the deposit was usually irregular. The best way is to measure the area by digitizing the radiographs. For this reason we decided not to calculate the area of calcification and we used the classification of Bosworth. For ethical reasons patients did not undergo CT examination before and after therapy. Also, we did not perform pain assessment with VAS and functional evaluation

with Constant score. We had only a single evaluation time point after ESWT. We did not have a control group. Another limitation of the study is the absence of a prior determined study power and the sample size to test the hypothesis. Comparative studies with other methods and with appropriate control groups are needed to evaluate its actual effectiveness. The strong points of this study are that it is one of the first clinical studies to analyze the effects of SW on the calcific deposit in relationship to radiological features. Another strong point is the high number of enrolled patients.

In conclusion, ESWT is effective in calcific tendinitis of the shoulder for the biological effects induced on the tendinopathy. In more than a third of the shoulders treated with SW, the disintegration of calcification occurred after three months. Orthopedic surgeons and physiatrists can recommend with more confidence medium-large and dense calcifications to treatment with shock waves, due to the good disruptive response. Understanding the different disruptive responses among different calcifications, scientific research will be able to define which calcific tendonitis of the shoulder may derive more benefit from shock wave treatment. Future studies may also verify which shoulders may benefit most as regards pain and functional recovery, even in the absence of removal of calcific deposit.

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## LETTER TO THE EDITOR

## CIRCULATING sRAGE IN THE DIAGNOSIS OF OSTEOLYTIC BONE METASTASIS

E. GALLIERA<sup>1,2</sup>, M.G. MARAZZI<sup>3</sup>, E. VIANELLO<sup>3</sup>, L. DRAGO<sup>2,3</sup>, A. LUZZATI<sup>2</sup>,  
P. BENDINELLI<sup>3</sup>, P. MARONI<sup>2</sup>, L. TACCHINI<sup>3</sup>, M.A. DESIDERIO<sup>3</sup>  
and M.M. CORSI ROMANELLI<sup>3,4</sup>

<sup>1</sup>Department of Biomedical, Surgical and Oral Science, Università degli Studi di Milano, Milan, Italy; <sup>2</sup>IRCCS Galeazzi Orthopedic Institute, Milan, Italy; <sup>3</sup>Department of Biomedical Sciences for Health, Università degli Studi di Milano, Milan, Italy; <sup>4</sup>U.O.C SMEL-1 Patologia Clinica IRCCS Policlinico San Donato, San Donato, Milan, Italy

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Despite the clinical importance of metastasis to the skeleton, the diagnostic tools for early detection and monitoring of bone metastasis lack sensitivity and specificity. We evaluated a promising new serum biomarker, the soluble form of the Receptor of Advanced Glycosylated End-products (sRAGE). sRAGE is involved in the Wnt-signaling pathway, and has been reported to reduce the risk of cancer. We investigated the diagnostic potential of sRAGE to improve the detection and monitoring of bone metastasis. We measured sRAGE in the serum of control healthy subjects, patients with primary tumors and patients with bone metastasis. sRAGE was also correlated with the Wnt inhibitors DKK-1 and sclerostin, the bone resorption markers MMP-2, MMP-9 and TRAP5, and the metastatic marker survivin. sRAGE was significantly lower in primary tumor and metastatic patients than in healthy subjects. sRAGE also showed a strong negative correlation with DKK-1, sclerostin, MMP-2, MMP-9, TRAP5b and survivin. These results indicated that sRAGE might play a protective role in bone metastasis progression, and it may diagnostic significance for detecting and monitoring osteolytic metastases.

To the Editor,

Bone metastases are a major problem in the management of neoplastic disease, because they cause various adverse skeletal events (1-3). Understanding the interaction between cancer and bone has led to the identification of innovative diagnostic tools (4), and a pivotal role in bone metastasis is played by the Wnt signaling pathway. Wnt function is balanced by several secreted endogenous inhibitors, such as the Dickkopf (DKK) family, secreted FRZ-related proteins and sclerostin (5). DKK-1 and sclerostin

have been described as playing an important role in cancer progression and bone metastasis colonization (6-8). These molecules have been described as potential new therapeutic targets (9), and have also been suggested as a diagnostic tool for aggressive tumors that metastasize to the skeleton (6). The Receptor of Advanced Glycosylated End-products (sRAGE) is an emerging molecule involved in the Wnt pathway. After AGE-RAGE interaction, osteoblast proliferation is inhibited and osteoclast activity increases because of suppression of the Wnt

*Key words: bone biomarkers, osteolytic metastases, soluble RAGE*

*Mailing address:*

Dr Emanuela Galliera,  
Department of Biomedical, Surgical and Oral Science,  
Università degli Studi di Milano,  
Via Mangiagalli 31, 20133, Milano, Italy  
Tel.: +39 02 5031 5354 - Fax: +39 02 5031 5338  
e-mail: emanuela.galliera@unimi.it,

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pathway (10). In contrast, the soluble form of the RAGE receptor, sRAGE, resulting from alternative splicing or proteolytic cleavage of the membrane-bound RAGE, acts as a decoy receptor for different AGE ligands (11). sRAGE therefore has a protective role in several inflammatory diseases (12).

Serum sRAGE levels can be used as a marker of disease status in different pathological conditions, but in neoplastic disease its potential diagnostic role as a marker of metastasis has not been investigated. We assessed the diagnostic potential of sRAGE, which could be important to improve the detection and monitoring of osteolytic bone metastasis, by evaluating its correlation with bone metastasis markers, Wnt inhibitors, DKK-1 and sclerostin, the bone resorption markers TRAP5b and MMP-2 and MMP-9, and the canonical metastatic marker survivin. Our experimental evidence supports the role of sRAGE as a bone metastasis marker (13).

## MATERIALS AND METHODS

### *Patients*

Thirty-eight selected patients were enrolled from January 2012 at the Oncological Orthopedic Surgical Unit, at the IRCCS Galeazzi Orthopedic Institute (Milan, Italy), who were classified as having metastatic or non-metastatic tumor. One group comprised 18 patients with bone metastases from different primary non-osseous tumors: 4 thyroid carcinomas, 3 renal carcinomas, 4 lung carcinomas, 4 breast carcinomas, 1 teratoma, 1 pheochromocytoma, 1 larynx carcinoma. Bone metastases, evidenced with CT, were confirmed by histological analysis of the biopsies. The second group of 20 patients had primary tumors without metastatic spread (8 myeloma, 5 chondrosarcoma, 7 cordoma); the tumor was staged according to the Enneking classification (14). To examine the serum concentrations of the bone markers in a healthy population, we enrolled a control pool of 33 healthy humans without skeletal disease, matched for age and sex. We checked the medical records of all participants to make sure they were not taking any medication known to interfere with bone turnover, such as calcium supplementation or bisphosphonates. All procedures followed were in accordance with the 1975 Declaration of Helsinki, as revised in 2000 and 2008.

Informed consent was obtained from all participants. The study was in line with the guidelines for experimental studies on humans applicable in the Galeazzi Orthopedic Institute, and approved by the local ethics committee.

### *Serum sRAGE, Wnt inhibitors (sclerostin, DKK-1), survivin, MMP-2, MMP-9 and TRAP5b*

Serum was obtained from all participants from routine pre-surgery blood. After centrifugation at 1500 g for 15 min, samples were stored at -80°C until analysis. sRAGE, sclerostin, DKK-1, survivin, MMP-2, MMP-9 and TRAP5b in serum were determined by commercial assays, according to the manufacturers' instructions (Quantikine Human RAGE ELISA, human DKK-1, human SOST (sclerostin), human survivin, human MMP-2 and human MMP-9 immunoassays were from R&D Systems, Minneapolis, Minnesota, USA; TRAP5b EIA Kit was from METRA, Hannover, Germany). For sRAGE, the sensitivity of the assays was 16.14 pg/mL, with intra- and inter-assay coefficients of variation respectively 5.7% and 7.7%. For DKK-1, the sensitivity was 15.6 pg/mL, and intra- and inter-assay coefficients of variation were respectively 3.8% and 6.1%. For sclerostin, the sensitivity was 3.8 pg/mL, and intra- and inter-assay coefficients of variation were respectively 2% and 9.5%. For survivin, the sensitivity was 4.4 pg/mL, with intra- and inter-assay coefficients of variation respectively 5.5% and 5.6%. For MMP-2, the sensitivity was 0.082 ng/mL, and intra- and inter-assay coefficients of variation were respectively 4.8% and 6.7%. For MMP-9, the sensitivity was 0.156 ng/mL, and intra- and inter-assay coefficients of variation were respectively 2.3% and 7.5%. For TRAP5b, the sensitivity was 0.2 U/L, and intra- and inter-assay coefficients of variation were respectively 1.9% and 2%.

### *Statistical analysis*

For all parameters, the normality of distribution of the three groups was verified by Kolmogorov-Smirnov's test. Statistical analysis was carried out using one-way analysis of variance (ANOVA), with  $p < 0.05$  considered significant and  $p < 0.001$  highly significant. Correlation was verified using Graph Pad Prism v5.0 software (Graph Pad Software Inc., La Jolla, CA, USA), by linear regression analysis between the different groups of data, calculating the 95% confidence interval of the regression line.



## RESULTS

*sRAGE levels and correlations with serum Wnt inhibitors in primary tumors and in bone metastases*

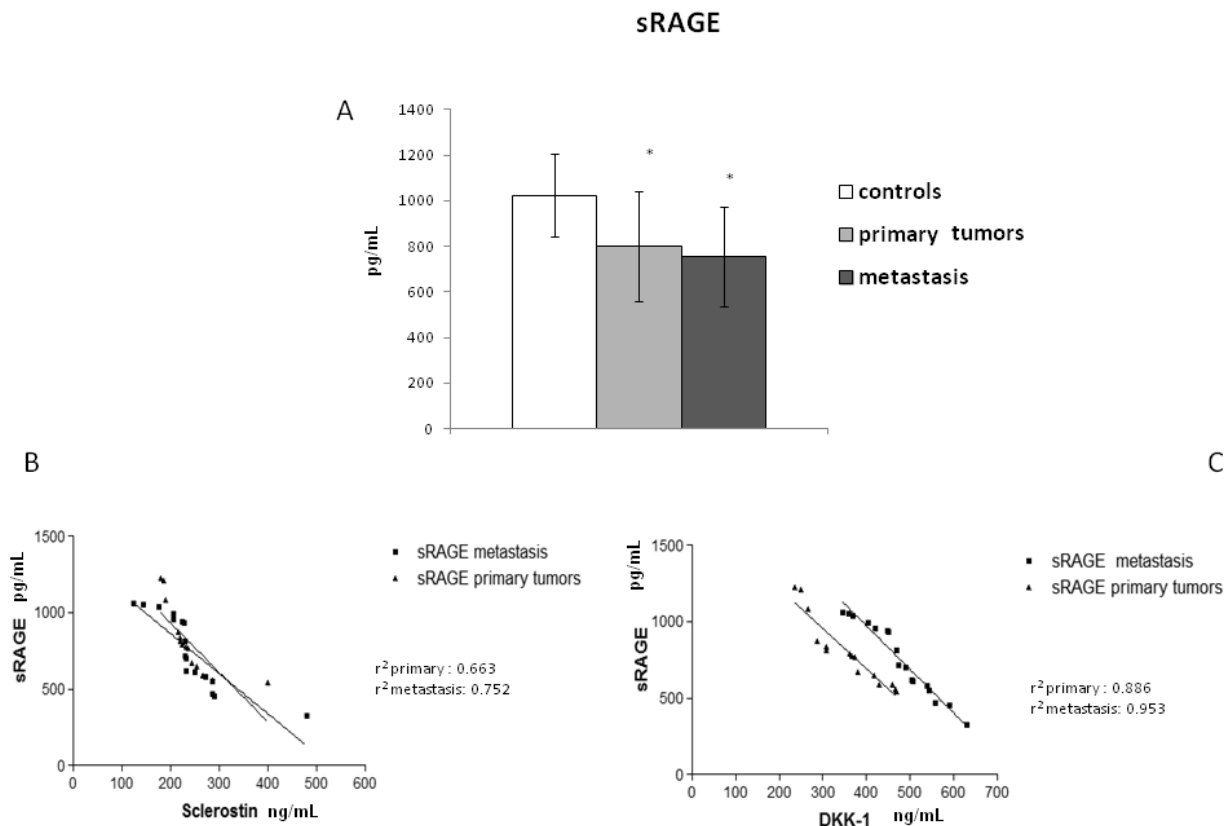
sRAGE was measured in the serum of three groups of individuals: control healthy humans, patients with primary tumors, patients bearing bone metastasis (Fig. 1). sRAGE was significantly higher ( $p<0.05$ ) in the control group ( $1022.27\pm374.49$  pg/mL) than in the groups with primary tumors ( $801.28\pm222.92$  pg/mL) and bone metastases ( $756.08\pm237.99$  pg/mL) (Panel A). sRAGE was 6% lower in metastatic patients than in primary tumor patients though the difference was not statistically significant.

Panels B and C show the correlations for sRAGE with Wnt inhibitors: there was a weak negative correlation ( $r^2=0.663$ ,  $p<0.05$ ) with sclerostin in primary tumors but a good one in metastases

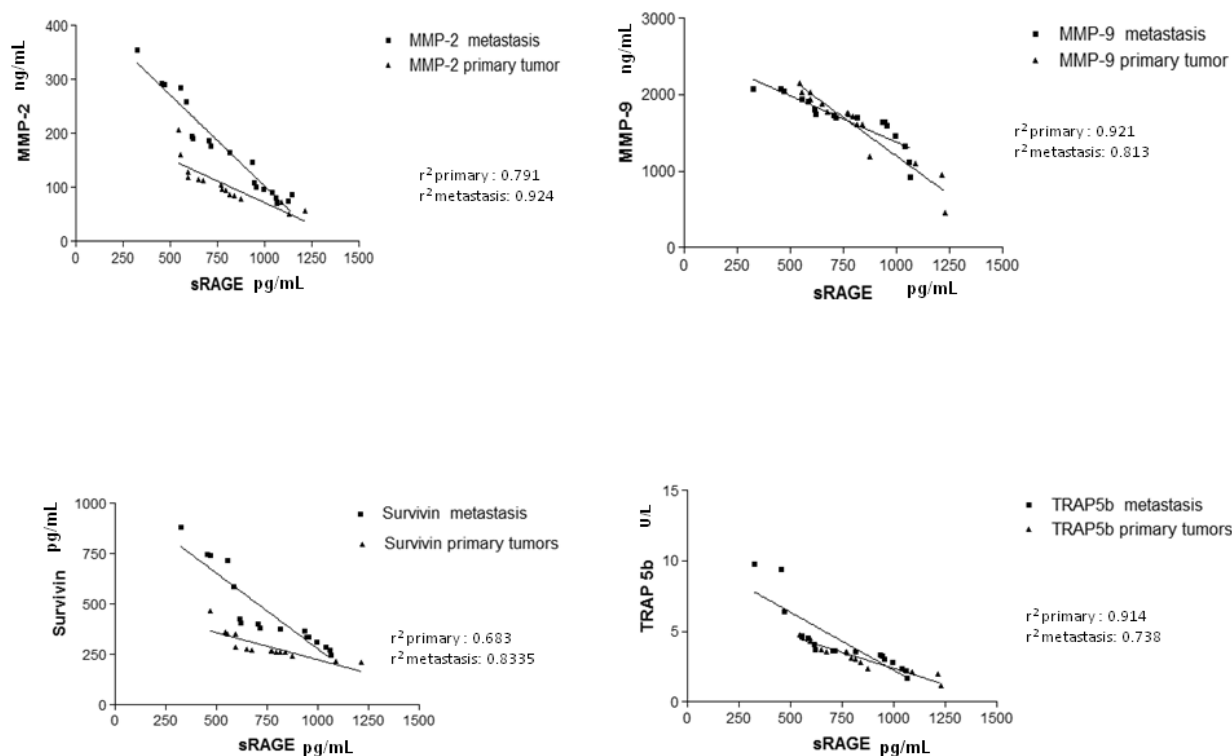
( $r^2=0.752$ ,  $p<0.05$ , panel B). Similarly, there was a good negative correlation between sRAGE and DKK-1 in primary tumors ( $r^2=0.886$ ,  $p<0.05$ ), and the correlation was even more significant in metastatic patients ( $r^2=0.953$ ,  $p<0.05$ , panel C).

*Correlation between sRAGE and bone metabolic and metastatic markers*

To assess the clinical importance of sRAGE in the diagnosis of bone osteolytic metastasis, we carried out a linear correlation analysis of sRAGE and a panel of bone metabolic and metastatic markers, considering the two distinct groups of patients bearing primary tumors and bone metastases. MMP-2 and MMP-9 were measured as markers of bone matrix remodeling, TRAP5b as a canonical marker of bone resorption and survivin as a metastatic marker. Fig. 2 shows the correlation analysis for sRAGE



**Fig. 1.** sRAGE and Wnt inhibitors in primary tumors and in bone metastases. **A)** sRAGE serum levels in controls (white bar), patients with primary tumors (gray bar), and bone metastatic patients (dark gray bar). \* $p<0.05$ . **B)** linear correlation of serum levels of sRAGE and sclerostin in metastatic patients (black square) and in patients with primary tumors (black triangle). **C)** linear correlation of serum levels of sRAGE and DKK-1 in metastatic patients (black square) and in patients with primary tumors (black triangle)



**Fig. 2.** Correlation between sRAGE and bone metabolism and metastatic markers. Linear correlation between sRAGE and MMP-2 (A), MMP-9 (B), survivin (C) and TRAP5b (D); serum levels of the markers in metastatic patients (black square) and in patients with primary tumors (black triangle).

(panel A) with MMP-2: there was a good negative correlation ( $r^2=0.791$   $p<0.05$ ) in primary tumors; the significance in bone metastases ( $r^2=0.924$   $p<0.01$ ) was higher than in the primary tumor group. In contrast, the negative correlation between sRAGE and bone matrix remodeling marker MMP-9 was significant in primary tumors ( $r^2=0.921$ ,  $p<0.01$ ) but weak in metastases ( $r^2=0.813$ ,  $p<0.05$ , panel B). Similarly, the correlation analysis between sRAGE and TRAP5b was also negative and more significant in primary tumors than in metastases (panel D,  $r^2=0.914$ ,  $p<0.01$  and  $r^2=0.738$ ,  $p<0.05$ , respectively).

The negative correlation between sRAGE and survivin in primary tumors and bone metastases was similar (panel C,  $r^2=0.683$   $p<0.01$  and  $r^2=0.833$ ,  $p<0.01$ , respectively).

## DISCUSSION

This study investigated the diagnostic potential of sRAGE to improve the detection and monitoring of bone metastasis. We measured sRAGE in the serum of control healthy persons, patients with primary tumors and patients with bone metastasis. sRAGE might act as a negative regulator of RAGE, thus exerting a protective effect against metastatic disease. This explains why it is usually higher in healthy states than in pathological conditions (15). Our findings confirmed this. The lower levels of circulating sRAGE in the patients with primary tumors - and even more so in patients with bone metastases - resulted in a defect of the protective function of sRAGE, allowing the development of bone metastatic lesions. Since the metastases

analyzed were mainly osteolytic, in order to evaluate bone resorption we assayed serum TRAP5b (16) and the two main bone-related metalloproteinases MMP-2 (17) and MMP-9 (18). In addition, since sRAGE is involved in the Wnt pathway (19), serum sRAGE was compared to two Wnt pathway inhibitors, DKK-1 and sclerostin, recently reported to be involved in the pathogenesis of bone metastasis (5, 20). sRAGE was significantly lower in primary tumor and metastatic patients than in healthy controls. sRAGE also showed strong negative correlations with DKK-1, sclerostin, MMP-2, MMP-9, TRAP5b and survivin. These results indicate that sRAGE might play a protective role in bone metastasis progression, and might also have diagnostic significance, helping detect and monitor osteolytic metastases.

In conclusion, sRAGE has been described as an emerging diagnostic biomarker for several diseases. The present study suggests that the measure of serum sRAGE might also find a potential new application in the clinical evaluation of bone metastases, helping to improve early diagnosis and in monitoring the bone metastatic process.

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## LETTER TO THE EDITOR

## COMPARATIVE MOLECULAR ANALYSIS OF BACTERIAL SPECIES ASSOCIATED WITH PERIODONTAL DISEASE

V. DE IULIIS<sup>1</sup>, S. URSI<sup>2</sup>, L.M. DI TOMMASO<sup>2</sup>, M. CARUSO<sup>1</sup>, A. MARINO<sup>1</sup>,  
S. D'ERCOLE<sup>1</sup>, S. CAPUTI<sup>1</sup>, B. SINJARI<sup>1</sup>, F. FESTA<sup>1</sup>, M. MACRI<sup>1</sup>,  
S. MARTINOTTI<sup>1,2</sup>, G. VITULLO<sup>2</sup> and E. TONIATO<sup>1</sup>

<sup>1</sup>Department of Medical, Oral and Biotechnological Sciences, University of Chieti, Chieti, Italy;

<sup>2</sup>SS Annunziata University Hospital, Unit of Clinical Molecular Biology and Predictive Medicine, University of Chieti, ASL Lanciano-Vasto-Chieti, Chieti, Italy

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Periodontal disease is an inflammatory disorder affecting the supporting teeth structures, including gingiva, periodontal ligament and alveolar bone, causing loss of connective tissue, reabsorption of alveolar bone and formation of periodontal pockets. The aim of this study is to find a correlation between bacterial growth and periodontal disease. Fifty-seven patients aged between 21 and 65 years, median age 46 years, were enrolled. According to gingival pocket depth, ranging from 3 to 7 mm, patients were divided into two groups: the first (30 patients, 53%) with deep pockets  $\geq 5$  mm and the second (27 patients, 47%)  $< 5$  mm. The samples taken were processed for microbiological analysis by absolute quantitative real-time Taq-Man technique. Patients affected by periodontal disease were 32 (56%) and patients with gingival bleeding were 35 (61%). This data showed that the presence, the type and the bacterial load in gingival pockets were strongly correlated with gingival depth, periodontal disease and gingival bleeding. Quantitative microbiological analysis is a key point to improve patient compliance, allowing to choose the specific antibiotic treatment, avoiding antibiotic resistance and ensuring the successful outcome of therapy for periodontal disease.

To the Editor,

Periodontal disease is an inflammatory disorder affecting the supporting teeth structure, including gingiva, periodontal ligament and alveolar bone. It is a multifactorial disease and many different factors are responsible for its onset and progress, such as the existence of certain bacteria and different exogenous risk factors such as smoking, bad oral hygiene, systemic diseases and stress (1). Periodontal pathology is customarily classified as disease that can affect only the gingival substrate or it is associated

with the destruction of the underlying structures of the periodontium. Gingival inflammation can extend into the periodontal ligament and alveolar bone triggering a periodontitis process causing loss of connective tissue, reabsorption of alveolar bone and formation of periodontal pockets (2). The most common symptoms of gingivitis are: swollen gums, bright red or purple gums that are tender or painful to the touch, bleeding gums or bleeding after brushing and/or flossing and bad breath (halitosis) (3).

Early studies (4) demonstrated a direct relationship

*Key words: antibiotic, bacterial genetics, biofilm, RT-PCR analysis*

Mailing address:

Dr Elena Toniato,  
Department of Medical, Oral and Biotechnological Sciences,  
University of Chieti, Via dei Vestini 31, 66013 Chieti, Italy  
Tel.: +39 0871 3554161 - Fax: +39 0871 357340  
e-mail: e.toniato@unich.it

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between bacterial plaque accumulation and gingival disease. In these studies, tissues without bacterial plaque showed little level of inflammation, while those with plaque exhibited a strong inflammatory reaction. Thus, periodontal disease is a source of recognizable pathogenic species, where virulence factors and inflammatory mediators can create and sustain a chronic inflammatory burden. It has been estimated that in this area we can pick up more than 700 species (5). A study by Socransky et al. examined more than 13,000 samples of subgingival plaque, recognizing the presence of five groups of closely-related bacterial species using DNA probes (6). Where the bacterial species within the complexes were closely associated, the complexes had specific interrelationships (7). This indicates not only specific relationships between subgingival species, but also suggests a possible pattern or sequence of colonization (8). The main difference between health and disease is represented by the increase in the count of the complexes of bacterial species (9).

Retrospective studies have suggested that microbiological analysis on critical levels of *A. Actinomicetemcomitans* and *P. intermedia gingivalis* in subgingival sites may have diagnostic value (10). In addition, recent studies have supported an association between periodontal and systemic diseases such as coronary heart disease (11), preterm infants with low birth weight (12), diabetes (13) and respiratory infections (14). For this reason, it is important to know the microbiological composition of the oral cavity to prescribe correct antibiotic treatments (15).

The aim of this study is to find a correlation between bacterial growth and periodontal disease. It is possible to underline the state of the periodontal disease through microbiological analysis to choose a correct personalized therapy based on the specific strains found.

## MATERIALS AND METHODS

### *Study participants*

In this study, 57 patients aged between 21 and 65 years, median 46 years, were enrolled. All subjects were systemically healthy, possessed at least 20 teeth and had not taken systemic

antibiotics, anti-inflammatory or immunosuppressive drugs during the month preceding study enrollment. Patients were selected and classified according to the criteria outlined by the International Classification of periodontal disease (16). Patients with pockets < 3 mm and attachment loss < 1 mm were considered in good periodontal health, while those who had more than 30% of sites with attachment loss > 4 mm were considered having periodontal disease.

All patients were examined by the same periodontist. Exclusion criteria included diabetes, pregnancy, HIV infection and use of immunosuppressant, bisphosphonates, steroids and antibiotics. Patients with aggressive periodontitis or necrotizing periodontal disease were excluded from this study.

Informed consent was obtained from patients whose data were used for this study. In addition, the study was conducted according to the guidelines of the declaration of Helsinki and we notified our University Department and its local Ethics Committee, even though internal procedures do not require the Ethics Committee's formal approval.

### *Clinical assessment*

At each visit, the patients were treated only to remove supragingival plaque. For each patient, the following clinical parameters were recorded in all quadrants of six sites per tooth (mesiobuccal, buccal, distobuccal, mesiolingual and distolingual) using a PCP-UNC 15 probe: probing depths (PD), clinical attachment levels (CAL) and bleeding on probing (BOP+). The third molars were not considered.

### *Sample collection and analysis*

The samples were collected using a kit consisting of paper cones and tubes (Meridol, Perio-Diagnostics, GABA International, Switzerland). The sterile paper cones were inserted for 10 s into pockets and gingival sulcus after the identification and removal of the supragingival plaque, and then transferred to a sterile tube. In subjects with chronic periodontitis, the subgingival plaque samples were collected from 4 proximal nonadjacent sites, presenting a pocket depth of at least 5 mm and a loss of attachment of at least 6 mm. In subjects with periodontal health, samples were taken from mesial sulcus of 15 teeth chosen by a random method.

### *Quantification of periodontal bacteria*

The samples included in the appropriate tubes were processed for microbiological analysis by absolute quantitative real-time Taq-Man technique (kit CEIVD - Meridol-Perio-



Diagnostics, GABA International, Switzerland). Initially the solution was warmed to room temperature and centrifuged at 10,000 g for 20 min. The supernatant was removed and the DNA was extracted by using a High Pure PCR Template Preparation Kit (Qiagen, Hilden, Germany). Briefly, 200 µl of tissue lysis buffer and 40 µl of proteinase K solution were added to the sediment and the mixture was incubated with shaking at 72°C for 10 min. After adding 200 µl of binding buffer and only after mixing, the sample was incubated at 95°C for 5 min. PCR amplification was carried out in a reaction volume of 50 µl consisting of 5 µl of template DNA and 45 µl of reaction mixture containing 35 µl of primer–nucleotide mix, 5 µl of 10 X PCR buffer, 5 µl of 25-mM MgCl<sub>2</sub> and 1 U Taq polymerase and specific gene probe Taqman. PCR cycling was carried out in a TRIO-Thermoblock thermal cycler (Biometra, Gottingen, Germany). The cycling conditions comprised an initial denaturation step at 95°C for 5 min, 10 cycles at 95°C for 30 s and at 60°C for 2 min, 20 cycles at 95°C for 10 s, at 55°C for 30 s and at 72°C for 30s, and a final extension step at 72°C for 10 min. Negative and positive controls were included in each batch of samples.

#### Statistical analysis

A descriptive analysis of the samples was carried out by means of median, interquartile range (IQR) and range for quantitative variables and absolute and relative frequencies for qualitative ones. For evaluating differences in bacterial load among different gingival pocket depth class populations, between healthy subjects and patients with periodontal disease and between patients with and without gingival bleeding, a Mann-Whitney test was used. Non-parametric tests were chosen because data were not normally distributed as demonstrated by Shapiro-Wilk test. The analysis was performed using SPSS software version 12.0 for Windows and statistical significance level was set at  $p < 0.05$ .

## RESULTS

#### Main demographic and clinical characteristics of the analyzed population

As shown in Table I, the baseline clinical parameters of the study population according to a gingival pocket depth were the following: from 3 to 7 mm (median: 5; IQR: 2), 27 patients (47%) with a pocket depth  $< 5$  mm and 30 (53%) 5 mm ( $p > 0.05$ ); 32 patients (56%) affected by periodontal disease and

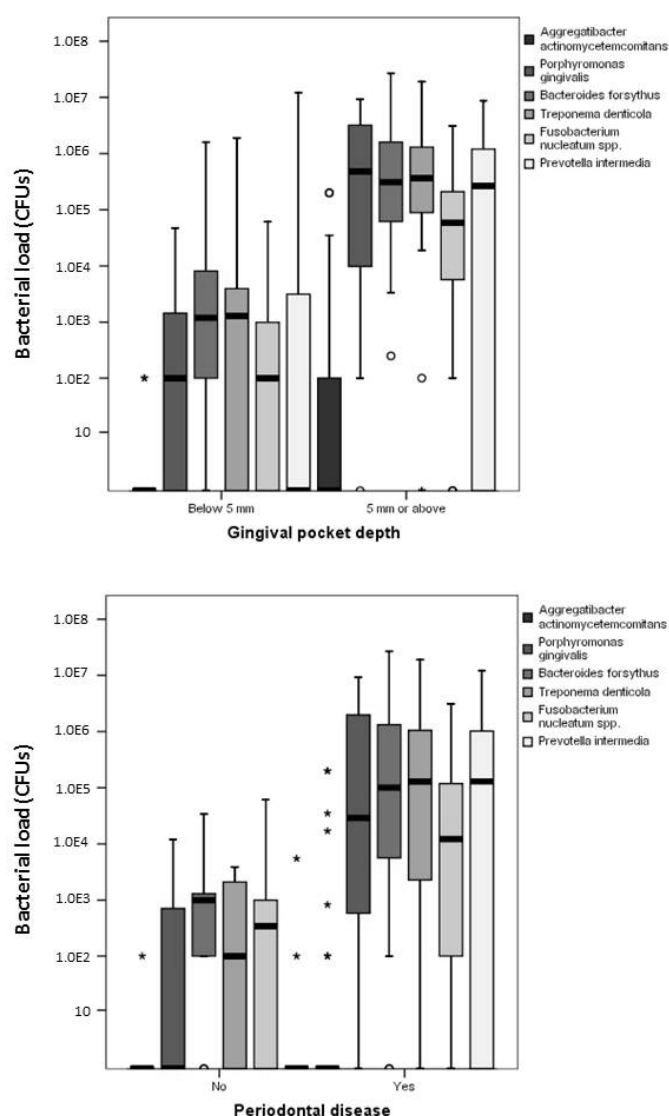
25 (44%) without periodontal disease ( $p > 0.05$ ); 35 patients (61%) with gingival bleeding and 22 (59%) without gingival bleeding ( $p > 0.05$ ). Table II shows the clinical characteristics and bacterial load of the analyzed population. Specifically, *P. Gingivalis* resulted the most representative with an average of  $5.4E+03$  CFU, while *A. actinomycetemcomitans* was the least representative with a maximum range of  $2.0E+05$  CFU.

#### Bacterial load in different gingival pocket depth population classes

This data showed that the type of bacteria strains in gingival pockets was strongly correlated with gingival depth. In fact, *T. denticola* and *B. forsythus* ( $1.3E+03$  and  $1.2E+03$ , respectively) were principally found in patients with gingival pocket depth under 5 mm, while *P. gingivalis* and *T. denticola* ( $4.8E+05$  CFU and  $3.8E+05$  CFU, respectively) in patients with gingival pocket depth over 5 mm. *A. actinomycetemcomitans* was non detectable. In addition, the results evidenced a statistically significant difference between gingival pockets depth 5 mm compared to those  $< 5$  mm, regarding the bacterial load for *P. gingivalis*, *B. forsythus*, *T. denticola* and *F. nucleatum spp.* ( $p < 0.001$ ) and for *P. intermedia* ( $p = 0.023$ ) (Table III and Fig. 1).

#### Bacterial load in patients with or without periodontal disease

Bacterial species mostly detected in patients affected by periodontal disease were: *P. gingivalis* ( $3.3E+05$  CFU), *P. intermedia* ( $1.4E+05$  CFU), *T. denticola* ( $1.3E+05$ CFU), *B. forsythus* ( $1.0E+05$  CFU) and *F. nucleatum spp.* ( $1.6E+04$  CFU). Instead, in subjects without periodontal disease they were: *F. nucleatum spp.* ( $3.4E+02$  CFU), *T. denticola* ( $1.0E+02$  CFU) and *B. forsythus* ( $1.0E+03$  CFU). Other species, such as *P. intermedia* and *A. actinomycetemcomitans*, were not detectable. These results showed a significantly higher bacterial load in patients with periodontal disease compared to patients without periodontal disease, in particular regarding *P. gingivalis* ( $p = 0.003$ ), *B. forsythus* ( $p = 0.001$ ), *T. denticola* ( $p = 0.002$ ) and *P. intermedia* ( $p = 0.004$ ) (Table III and Fig. 1).



**Fig. 1.** Box plots representing bacterial load distribution in the study population. Subjects are divided based on the gingival pocket depth (at the top in the figure) or on the presence of periodontal disease. The vertical axis is on a logarithmic scale.

#### *Bacterial load in patients with or without gingival bleeding*

*B. forsythus* ( $7.3\text{E}+02$  CFU) was the most representative bacterial strain among people without gingival bleeding, while *F. nucleatum* spp. ( $5.7\text{E}+04$  CFU), *T. denticola* ( $4.7\text{E}+05$  CFU) and *P. gingivalis* ( $4.7\text{E}+05$  CFU) were commonly present in patients with gingival bleeding. In order to compare patients with or without gingival bleeding, a significantly greater

bacterial load in patients with this symptom was found for the following bacteria strains: *P. gingivalis* and *F. nucleatum* spp. ( $p=0.001$ ), *B. forsythus*, *T. denticola* and *P. intermedia* ( $p<0.001$ ) (Table III).

#### DISCUSSION

Infectious disease of many organs is caused by relatively defined pathogens, which can act individually or in small groups. Each of

**Table I.** *Clinical features of the analyzed patients.*

| Clinical parameter    | Variable | N° of cases/Total cases |         |
|-----------------------|----------|-------------------------|---------|
|                       |          | Frequency (%)           | p-value |
| Periodontal disease   | +        | 32/57 (56%)             | > 0.05  |
|                       | –        | 25/57 (44%)             |         |
| Gingival pocket depth | < 5 mm   | 27/57 (47%)             | > 0.05  |
|                       | ≥ 5 mm   | 30/57 (53%)             |         |
| Gingival bleeding     | +        | 35/57 (61%)             | > 0.05  |
|                       | –        | 22/57 (59%)             |         |

**Table II.** *Clinical characteristics of the study population and bacterial load of gingival plaque.*

| Clinical parameter    | Variable | N° of cases/Total cases |         |
|-----------------------|----------|-------------------------|---------|
|                       |          | Frequency (%)           | p-value |
| Periodontal disease   | +        | 32/57 (56%)             | > 0.05  |
|                       | –        | 25/57 (44%)             |         |
| Gingival pocket depth | < 5 mm   | 27/57 (47%)             | > 0.05  |
|                       | ≥ 5 mm   | 30/57 (53%)             |         |
| Gingival bleeding     | +        | 35/57 (61%)             | > 0.05  |
|                       | –        | 22/57 (59%)             |         |

*N.D.: Not Detectable.*

**Table III.** *Statistical analysis of bacterial load in different gingival pocket depth population classes, in patients with or without periodontal disease and gingival bleeding.*

| Clinical parameter    | Variable | N° of cases/Total cases |         |
|-----------------------|----------|-------------------------|---------|
|                       |          | Frequency (%)           | p-value |
| Periodontal disease   | +        | 32/57 (56%)             | > 0.05  |
|                       | –        | 25/57 (44%)             |         |
| Gingival pocket depth | < 5 mm   | 27/57 (47%)             | > 0.05  |
|                       | ≥ 5 mm   | 30/57 (53%)             |         |
| Gingival bleeding     | +        | 35/57 (61%)             | > 0.05  |
|                       | –        | 22/57 (59%)             |         |

*Median bacterial load, CFUs (IQR). N.S.: Not significant; N.D.: Not detectable bacterial load; \*Mann-Whitney test.*

these infections requires the use of a different chemotherapeutic agent and the choice of each agent is based on the results of the diagnostic tests. It has been shown that periodontal disease has a multifactorial nature, in which the presence of certain bacteria strains can initiate and sustain the inflammatory process (17-19). In the present study, we identified associations among the amount and type of bacteria in the subgingival plaque and the presence of periodontal disease, gingival bleeding and the depth of the gingival pocket. In particular, the study indicates a statistical relationship between periodontal disease and a great bacterial load consisting of *P. gingivalis* ( $p = 0.003$ ), *B. forsythus* ( $p = 0.001$ ), *T. denticola* ( $p = 0.002$ ) and *P. intermedia* ( $p = 0.004$ ) in the subgingival plaque. Meanwhile, subgingival flora of healthy individuals was characterized by the presence of *F. nucleatum spp.*, *T. denticola* and *B. forsythus*. Furthermore, a gingival pocket depth  $\geq 5$  mm was correlated with a higher amount of *P. gingivalis*, *B. forsythus*, *T. denticola* and *F. nucleatum spp.* ( $p < 0.001$ ); as long as *T. denticola* and *B. forsythus* were found in gingival pocket depth  $< 5$  mm. Moreover, gingival bleeding, a hallmark sign of periodontal disease, seems to be related to a high amount of *P. gingivalis* and *F. nucleatum spp.* ( $p = 0.001$ ), *B. forsythus*, *T. denticola* and *P. intermedia* ( $p < 0.001$ ). *A. actinomycetemcomitans* was not reliably detected in the subgingival plaque, perhaps due to the small number of cases and to the appropriateness of the specimen used.

These data highlight how physiologic gingival flora is constituted by a small amount of *F. nucleatum spp.*, *T. denticola* and *B. forsythus*. We also show that periodontal disease is characterized by an increased bacterial load of the same pathogens and that the bacteria more frequent in shallow pockets (*T. denticola* and *B. forsythus*) are probably associated with active initial periodontal lesions. Additionally, the presence and the increased amount of *P. gingivalis* in patients with periodontal disease, with gingival pocket depth  $\geq 5$  mm and gingival bleeding, seems to be necessary for the onset and the progression of periodontal pathology. For these reasons, a single treatment cannot control periodontal diseases and the therapeutic choice should be based on the nature of

the infectious agents. Appropriate therapy, however, should stop the progression of the disease or would significantly increase the potential for long-term periodontal stability, but such therapy can only be effective in the presence of certain bacterial species. The results obtained show that the involvement of these pathogens can influence the status and progression of periodontal disease and that a correct therapy could improve prognosis. In fact, a correct antibiotic treatment could improve the regeneration of the gingival bacterial flora homeostasis and could avoid residual disease and antibiotic resistance. Several studies show that it is very important to choose the right implant surface as it plays a crucial role in osseointegration (20-21).

In conclusion, this study points out the correlation between bacterial load and the presence of periodontal disease, gingival pocket depth and gingival bleeding in order to establish a target antibiotic therapy for patients undergoing dental treatments. A correct microbiological analysis is a key point in order to improve patient compliance, avoid the loss of implants, restore the bacterial flora homeostasis and minimize the presence of residual disease and the increasing incidence of antibiotic resistance.

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## LETTER TO THE EDITOR

**CONGENITAL MUSCULAR DYSTROPHY AND EPILEPSY:  
A PROSPECTIVE CASE SERIES OF PEDIATRIC PATIENTS**

G. VITALITI<sup>1</sup>, P. PAVONE<sup>1</sup>, C. ROMANO<sup>1</sup>, M. BARBAGALLO<sup>2</sup>, M. VECCHIO<sup>3</sup>, C. LEDDA<sup>4</sup>,  
R. LUBRANO<sup>5</sup> and R. FALSAPERLA<sup>1</sup>

<sup>1</sup>General Paediatrics O.U., Policlinico-Vittorio Emanuele University Hospital, University of Catania, Italy; <sup>2</sup>General Paediatrics O.U., Azienda Ospedaliera di Rilievo Nazionale e di Alta Specializzazione Garibaldi, Catania, Italy; <sup>3</sup>Centre of Physical Medicine and Rehabilitation, Policlinico-Vittorio Emanuele Hospital, University of Catania, Italy; <sup>4</sup>Clinical Pathology Unit, Azienda Ospedaliera di Rilievo Nazionale e di Alta Specializzazione Garibaldi, Catania, Italy; <sup>5</sup>Pediatric Department, Pediatric Nephrology Operative Unit of Sapienza University of Rome, Rome, Italy

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**Congenital Muscular Dystrophies (CMDs) can be considered as a heterogeneous group of diseases characterized by marked weakness, generalized hypotonia and joint contractures. They are divided into pure and classical forms, without ocular and cerebral involvement, and complex forms, which are associated with cerebral abnormalities. Seizures have rarely been described in the pure forms while they seem to occur more frequently in complex forms. The aim of our study was to evaluate the incidence of seizure in CMD. Herein, the authors describe 16 cases of congenital muscular dystrophy (CMD) associated with different kinds of epileptic events, in order to study the pathogenic connection between the two clinical manifestations. In all described patients we reviewed the clinical, neurophysiologic, and neuroimaging data to determine any associations with epilepsy. The patients were divided into two groups: 14 cases with merosin positive CMD in one group and 2 patients with Walker Warburg syndrome (WWS) in the second group. In our study we found that in the first group, one benign myoclonic epilepsy (BME), one benign febrile convulsions had occurred. Also in one patient, the EEG revealed a moderately high voltage slow background with diffuse sharp waves reaching 300mV in amplitude with no clinical signs. In the merosin positive CMD patients, the presence of two different epileptic diseases, benign myoclonic epilepsy (BME) in one and febrile convulsion with tonic clinic seizures, may represent a new expression of merosine-positive congenital muscular disease (PCMD) in which the deficiency of an undiscovered muscular protein with a cerebral isoform may be the cause of epileptic events in this group of patients.**

To the Editor,

Congenital muscular dystrophies (CMDs) are a heterogeneous group of diseases, characterized by onset within the first 6 months of life of marked

weakness, generalized hypotonia, joint contractures and a muscle biopsy suggestive of dystrophic changes (1). Most CMDs are inherited as autosomal recessive disorders characterized by the above mentioned

*Key words: seizures, incidence and types, congenital muscular dystrophies, paediatrics*

Mailing address:

Dr Giovanna Vitaliti,  
AOU Policlinico-OVE,  
Via Plebiscito n. 628, 95100 Catania, Italy  
Tel.: +39 0957435254  
e-mail: giovitaliti@yahoo.it

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symptoms, with no mental retardation. In contrast to the common form of muscular dystrophy, classical forms of CMDs are not usually progressive and ambulation may be achieved.

Initially, CMDs were clinically subdivided into two groups based on the presence or absence of the central nervous system (CNS) involvement. The term "pure" or "classical" CMD indicates the absence of CNS abnormalities. The second group, with CNS involvement, includes Fukuyama muscular dystrophy (FCMD), an autosomal recessive disorder, common in the Japanese population, mapped on chromosome 9q31 (2), Muscle Eye Brain (MEB), common in Finland, mapped on chromosome 1q32 (3), and Walker Warburg syndrome (WWS), prevalent in other white populations (3). Recently, we described a novel congenital muscular dystrophy syndrome, which is phenotypically distinct from the recognized forms of CMD with brain involvement and in which the linkage analysis excluded the candidate loci on chromosome 6q2, 9q31, 1q32, and 1q42 (4) (5). Following discovery of merosin, a protein located on the surface of muscle fibers, pure CMD was divided into merosin positive (presence of merosin) and merosin negative (partial or total merosin deficiency) forms (6). Merosin negative CMD is linked to a mutation of a gene localized on chromosome six. The differences between merosin negative and merosin positive CMD is also based on the different clinical and neuroimaging pictures. Merosin negative CMD is usually more severe and has a slight CNS involvement (7).

The aim of the present study was to review the clinical, electrophysiological and neuroimaging data of 16 patients with CMD, whose clinical presentation was characterized by the association of epileptic events, in order to evaluate the pathophysiology connection between CMD and epilepsy.

## MATERIALS AND METHODS

This study is an observational prospective study on 16 patients with CMD, who were enrolled between 2002 and 2012 in the Pediatric Operative Unit, Policlinico-Vittorio Emanuele Hospital, University of Catania, Sicily, Italy.

The diagnosis of CMD was based on the follow-

ing criteria: clinical picture, plasma creatine-kinase (CK), electrophysiologic evaluation (EMG), brain neuroimaging study and muscle biopsy including immunocytochemical investigations for the following muscle proteins: dystrophin (I, II and III domains), sarcoglycan complex and merosin.

Patients over 16 years of age and those affected by any other chronic disease (such as diabetes, renal failure, congenital heart diseases, genetic and metabolic diseases, neurological diseases other than CMDs) were excluded. Patients undergoing any therapy that may have affected their convulsive history during the two months prior to their inclusion in this study were also excluded.

All patients were evaluated under a neurological profile to study their seizure activity, according to their personal history focused on the onset and the semiology of seizures, standard ictal and interictal EEG, and neuroimaging with magnetic resonance (MRI). Included patients were classified into two groups: Group A: (14/16) with merosin positive pure CMD and Group B: (2/16) with WWS. The study protocol was approved by the ethics committee of our institution and informed consent was signed by the parents of all the patients.

## RESULTS

Group A patients were similarly analyzed to Group B, and Table I reports their clinical history of seizures and EEG study. Group A patients included 6 girls and 8 boys aged between 4 and 24 months. In this group of patients, three were affected by convulsive events (Table I); in one case a benign myoclonic epilepsy (BME) was diagnosed, characterized by brief bilateral jerky movements, which were more prominent in the upper part of body, causing a head drop. These jerky movements were very brief and lasted several seconds. The onset of these epileptic events was at age three and their course was benign.

The second patient had had several febrile seizures (FS) from the age of 2 years. He had generalized tonic-clonic seizures (GTCS) with a benign course.

The EEG of the patient affected by BME showed a typical pattern of generalized spike-waves (S-W) or polyspike-wave (PS-W) discharges, compatible with myoclonus. The EEG of the FS patient did not show any abnormality.

**Table I.** *Clinical and electrophysiologic findings of the studied patients.*

| Clinical history of seizers of 3 out 14 patients with pure CMD |                |                                                                                  |                        |                 |                                                                              |                    |
|----------------------------------------------------------------|----------------|----------------------------------------------------------------------------------|------------------------|-----------------|------------------------------------------------------------------------------|--------------------|
| Patients Numbers                                               | Age (yrs)      | Onset of seizures (yrs)                                                          | Semeiology of seizers  | Frequency       | Clinical Course                                                              | Epileptic syndrome |
| 1                                                              | 7              | 3                                                                                | Sudden head drop       | Daily           | Benign                                                                       | BME                |
| 2                                                              | 5              | 2                                                                                | GTCS                   | =               | Benign                                                                       | FS                 |
| 3                                                              | Died           | ?                                                                                | =                      | =               | Fatal                                                                        | ?                  |
| EEG findings of patients with pure CMD and EEG abnormalities   |                |                                                                                  |                        |                 |                                                                              |                    |
| Patients Numbers                                               |                | Age performed                                                                    | Ictal                  |                 | Interictal                                                                   |                    |
| 1                                                              |                | 3 yrs                                                                            | S-W or PS-W discharges |                 | Normal                                                                       |                    |
| 2                                                              |                | 2 yrs                                                                            | -                      |                 | -                                                                            |                    |
| 2                                                              |                | 9 days<br>5 months                                                               | Diffuse sharp waves    |                 | -                                                                            |                    |
| Clinical history of seizers of two patients with WWS           |                |                                                                                  |                        |                 |                                                                              |                    |
| Patients Numbers                                               | Onset (months) | Semeiology of seizures                                                           | Frequency              | Clinical Course | Epileptic Syndrome                                                           |                    |
| 1                                                              |                | Lateral deviation head and eyes-unilateral clonie becoming secondary generalized | Daily                  | Progressive     | PSGE                                                                         |                    |
| 2                                                              | 1              | Lateral deviation head and eyes-unilateral clonie becoming secondary generalized | Daily                  | Progressive     | PSGE                                                                         |                    |
| EEG findings of patients with WWS                              |                |                                                                                  |                        |                 |                                                                              |                    |
| Patients Numbers                                               |                | Age performed (months)                                                           | Ictal                  |                 | Interictal                                                                   |                    |
| 1                                                              |                | 10                                                                               | -                      |                 | Paroxysmal s-w discharges diffused on central region of the right hemisphere |                    |
| 2                                                              |                | 11                                                                               | -                      |                 | S-W complex on the centro-temporal region of right hemisphere                |                    |

The third patient of group A, who showed epileptic events, had undergone an EEG at nine days of age, which was characterized by a low-voltage ground activity in all states, while at 5 months of age a second EEG revealed moderately high voltage slow background waves with diffuse sharp waves reaching 300 mV in amplitude. These were not associated with clinical signs (Table I).

Group B patients included one male and one female who had been observed since birth. Both of them died before the age of one year. Both patients were affected by WWS associated with partial-secondary generalized epilepsy (PSGE), with onset before the second month of life, characterized by arrest of activity, lateral deviation of the head and eyes, loss of consciousness, diffuse hypertonia with unilateral clonic jerks with secondary generalization (Table I). EEG studies on one of the patients showed paroxysmal spike-wave complexes on the central region of the right hemisphere, while in the other patient spike-wave complex figures on the centro-temporal region of the right hemisphere were detected (Table I).

We found that the neuroimaging pattern of the first group of patients was normal, while the brain MRI in both Group B patients showed a similar pattern, characterized by lissencephaly, cerebellar hypoplasia, hydrocephalous and cortical heterotopia.

## DISCUSSION

The classification of CMDs is based on a combination of clinical criteria, neuroimaging study, molecular and biochemical approaches. The European Neuromuscular Center Consortium on CMD (7) has defined four main categories of CMD up to variable involvement of the eyes and brain. First, Tomè (6) described a classical form of CMD with total or reduced expression of the laminin  $\alpha_2$  chain (LAMA2) in which there is no brain or eye involvement. Genetic studies revealed several mutations in the LAMA2 gene localized on chromosome six. This form is called merosin negative CMD and accounts for approximately 30-50% of classical CMD patients.

Brain MRI in merosin negative CMD patients usually shows an abnormal signal within the white matter on T2-weighted images with abnormal

evoked potential (8). A minority of these patients (20%) has structural brain defects, such as polymicrogyria, cortical dysplasia and cerebellar hypoplasia. Moreover, a high incidence of different types of drug-resistant epilepsies has been described (9). The association of CMD and epilepsy has been reported mainly in merosin negative CMD patients and this evidence could be explained by the different expression of the cerebral merosin isoforms. There are some descriptions of late onset epilepsy in some cases with partial merosin deficiency (10). Three patients of two Italian families with congenital merosin negative CMD and the presence of occipital epilepsy with periodic spasms and focal dysplasia were also described (11). In all these cases, seizures were diagnosed in childhood and were mainly consistent with periodic spasms, a particular type of partial seizures characterized by clusters of epileptic spasms. An interesting article reviewed thirty patients with CMD with normal mental development. Epilepsy was described in only three cases, two of whom had mild presentations, while the other was a drug-resistant case. The EEG did not reveal any common pattern, though there were various abnormalities in several children (12).

Nevertheless, we have to highlight our bias of a limited sample of patients. This is because the condition itself is rare, with rarer CNS involvement and our study was performed in a single center, considering mandatory the need for multicenter randomized controlled studies.

On the other hand, epilepsy has been associated with merosin positive CMD and mental retardation (13). In the broad clinical spectrum of pure CMD, dilated cardiomyopathy has been reported. Creatine kinase (CK) is grossly elevated and motor nerve conduction velocity is reduced, suggesting mild demyelinating peripheral neuropathy (14).

The WWS syndrome is considered as a more severe form of CMD leading to death usually before the first 6 months of life. The diagnostic criteria of WWS include cobblestone lissencephaly, retinal abnormalities, cerebellar hypoplasia and congenital muscular dystrophy or myopathy. Almost all children with WWS have enlarged ventricles and many of them have progressive hydrocephalus that often

requires treatment with shunt. In some cases, posterior encephaloceles has been reported. Most WWS patients have profound mental retardation, hypotonia and mild distal spasticity. Some authors consider WWS syndrome an allelic disorder of MEB; however, this hypothesis does not seem to be in line with genetic linkage studies. No locus for WWS has been identified, but a secondary reduction in laminin- $\alpha$ 2,  $\beta$ -dystroglycan, laminin- $\beta$ 2 and  $\alpha$ -sarcoglycan has been reported (15).

In literature there is only one case of merosin-positive CMD associated with epilepsy (15). This was the case of a Turkish girl, the clinical findings of whom were consistent with CMD, and who had been affected by epileptic events since she was 6 years of age with a marked white matter high signal on her brain MRI. In our merosin-positive CMD patients, the presence of two different epileptic diseases, MBE in one and ESE in the second, may represent a new expression of CMD in which the deficiency of an undiscovered muscular protein with a cerebral isoform leads to the development of the epileptic disease. It is important to understand the association between CMD and epilepsy, as these forms may be the consequence of merosin isoforms involving the CNS, constituting a new subgroup of merosin-positive CMD.

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## LETTER TO THE EDITOR

## VITAMIN A DECREASES AFTER A MAXIMAL INCREMENTAL STRESS TEST IN NON-PROFESSIONAL MALE RUNNERS WITH LOW AEROBIC PERFORMANCE

P. IZZICUPO<sup>1</sup>, B. GHINASSI<sup>1</sup>, M.A. D'AMICO<sup>1</sup>, T. BUCCIARELLI<sup>2</sup>, S. GALLINA<sup>3</sup>  
and A. DI BALDASSARRE<sup>1</sup>

<sup>1</sup>Department of Medicine and Aging Sciences, University "G. d'Annunzio", Chieti-Pescara, Italy;

<sup>2</sup>Department of Biomedical, Oral and Biotechnological Sciences, University "G. d'Annunzio", Chieti-Pescara, Italy; <sup>3</sup>Department of Neuroscience, Imaging and Medical Sciences, University "G. d'Annunzio", Chieti-Pescara; Italy

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The first two authors contributed equally to this paper

The data on the effects of aerobic training on plasma antioxidant vitamins are conflicting. Additionally, most studies focus on the oxidative profiles of professional athletes, but limited information is available for amateur athlete populations. The aim of this study was to evaluate the effects of high-intensity exercise on antioxidant vitamins in non-professional runners with varying levels of aerobic power. Eighty-one male runners underwent an incremental test to exhaustion. The study population was then divided into the following tertiles according to  $\text{VO}_2\text{max}$ : Group L ( $^{\text{Low}}\text{VO}_2\text{max}$ ,  $<44.2 \text{ mLkg}^{-1}\text{min}^{-1}$ ), Group M ( $^{\text{Medium}}\text{VO}_2\text{max}$ ,  $44.2\text{--}49.7 \text{ mLkg}^{-1}\text{min}^{-1}$ ) and Group H ( $^{\text{High}}\text{VO}_2\text{max}$ ,  $>49.7$ ). Comparative analyses were performed between Groups L and H. The total antioxidant capacity (TAC), Vitamin (Vit) E, Vitamin A,  $\beta$ -carotene, lycopene and thiobarbituric acid-reactive substances (TBARS) were determined before and 60 min after exercise testing. After the stress test, Vit A decreased and TBARS increased in Group L, whereas no changes in the vitamin concentrations, TAC induction and TBARS reduction were observed in group H. In individuals with low  $\text{VO}_2\text{max}$ , an incremental test determined lipid-peroxidation and Vitamin A consumption, whereas H Group increases TAC that buffer TBARS production.

To the Editor,

Reactive oxygen species (ROS) are compounds that play an important role in many signaling pathways in physiological conditions. However, these normal byproducts of the metabolism can also cause substantial damage to cellular components such as lipid peroxidation and protein oxidation; for this reason, cells possess a complex network of antioxidant molecules that include both enzymatic and non-enzymatic compounds. Physical activity

promotes the generation of ROS through several mechanisms (1). However, a growing body of evidence strongly indicates that habitual, moderate physical activity reduces the incidence of oxidative stress-based diseases (2) and that antioxidant activity differs significantly between trained and untrained individuals (3, 4). The ROS that are produced during repeated bouts of exercise stimulate the synthesis of antioxidant enzymes (5). The extent of this adaptation is influenced by both the genetic

*Key words: antioxidants, vitamin supplementation, maximal oxygen consumption, oxidative stress, physical activity for health*

*Mailing address:*

Prof. Angela Di Baldassarre,  
Dept of Medicine and Aging Sciences, University "G. d'Annunzio"  
Via dei Vestini, 31- 66100 Chieti, Italy  
Tel.: +39 0871 3554545 -Fax: +39 0871 3554568  
e-mail: a.dibaldassarre@unich.it

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profile and the training volume and  $\text{VO}_2\text{max}$  of the individual (6-8). However, the data on the effects of training on the non-enzymatic antioxidants, such as vitamins, are often contradictory. Additionally, most of the existing studies in humans have only analyzed the oxidative profiles of professional athletes (9). There is a limited amount of amateur population-based research, which could better reflect the average person who is interested in achieving and maintaining a good fitness level for health or recreational purposes.

Our hypothesis is that individuals with a lower  $\text{VO}_2\text{max}$  would also have a lower enzymatic antioxidant capacity. This reduced capacity could be reflected in the differences in plasma antioxidant vitamin concentrations after an acute bout of exercise. Therefore, the aim of this study was to measure fat-soluble vitamins after a maximal exercise test and evaluate their levels for both total antioxidant capacity and the byproducts of lipid peroxidation.

## MATERIALS AND METHODS

### *Study subjects*

One hundred twenty-seven non-professional male runners responded to a public advertisement. A short interview and a preliminary medical examination excluded 37 ineligible individuals who met the following exclusion criteria: vegetarian or vegan diet; taking antioxidant supplements for at least four months before the start of the study; cigarette use, diabetes or any other cardiovascular risk factors, or any condition related to oxidative stress. Nine of the 90 individuals enrolled in the study were excluded from the statistical analysis because they did not properly perform the incremental stress test (did not reach  $\text{VO}_2\text{max}$ ). The protocol was approved by the University of Chieti-Pescara ethics committee, and all individuals provided their written informed consent to participate in the study. Athletes were similar in age, biohumoral parameters, training history ( $9.0 \pm 2.6$  yr) and volume (Table I). All participants had been participating in long-distance running events for at least 6 years.

### *Screening*

Each subject's medical history and standard physical examination were performed by a physician. Dietary habits

and calorie intake were estimated by a dietician from 3-day dietary records (two weekdays and one weekend day) using the WinFood 2.7 software (Medimatica, Colonnella, Teramo, Italy). Physical exercise history was assessed for type of training, years of training, week frequency and duration, and estimated intensity (light, moderate, strenuous). A 12-h overnight fasting blood sample was drawn for assessing the biohumoral measurements. Body composition was assessed via electrical bioimpedance (10).

### *Incremental stress test*

The stress test was performed according to the Bruce protocol as described previously (11). Participants had performed this test annually in the past and were thus already familiar with the test. Prior to testing, participants were asked to maintain their usual dietary intake and abstain from alcohol and caffeine consumption for 24 hours. A progressive exercise stress test was performed to exhaustion on a RAM770 CE treadmill (RAM, Padova I). A breath-by-breath gas exchange analyzer (Vmax 29; Sensor Medics, Buena Vista, CA, USA) was used to measure  $\text{VO}_2$  and carbon dioxide production. Heart rate and rhythm were continuously monitored by ECG. Mean arterial pressure (MAP) was calculated using the following formula:  $[(\text{systolic blood pressure}) + (2 \times \text{diastolic blood pressure})]/3$  (12).

### *Glucose, Lipids, Vit A, Vit E, lycopene and $\beta$ -carotene determination*

Fasting blood samples were drawn before and 60 min after the stress test. Plasma was frozen at  $-80^\circ\text{C}$  for subsequent analyses. Glucose, HDL-C, TC, and TGs were assessed directly using enzymatic methods. Plasma from the L and H groups (see statistical analysis section) were analyzed by HPLC for Vit A, Vit E, lycopene and  $\beta$ -carotene content. Briefly, two Water Pumps 515 HPLC units, equipped with Waters autoinjectors (Model 717 plus autosamplers) and Waters 996 photodiode array (PDA) detectors, constituted the HPLC system (13). Data acquisition and processing were performed using the Waters chromatography software, Empower-Pro.

### *Total antioxidant status and thiobarbituric acid-reactive substances analyses*

Plasma from the L and H groups was analyzed for

total antioxidant status (TAC) using an Antioxidant Assay ELISA Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's recommendations and the resulting plates were read with a Multiscan Go Plate Reader (ThermoScientific, Waltham, MA, USA). Thiobarbituric acid-reactive substances (TBARS) levels were measured using a TBARS Assay Kit (Cayman Chemical). All samples were processed in duplicate in the same assay sections.

#### Statistical analysis

All statistical analyses were performed using SPSS 15 (SPSS, Chicago, IL, USA). The normal distribution of the data was verified by the Kolmogorov-Smirnov test. The study population was divided into tertiles based on the  $VO_{2max}$  tertiles described above: Group L (Low  $VO_{2max}$ , < 44.2 mL·kg<sup>-1</sup>·min<sup>-1</sup>), Group M (Medium  $VO_{2max}$ , 44.2-49.7 mL·kg<sup>-1</sup>·min<sup>-1</sup>) and Group H (High  $VO_{2max}$ , >49.7 mL·kg<sup>-1</sup>·min<sup>-1</sup>). Comparative analyses

were performed between Groups L and H. The correlation between lipid soluble antioxidants and plasma lipids was calculated using a Pearson (r) or Spearman's rank (rs) correlation. As there was a positive correlation between Cholesterol + triglyceride (TG) levels and Vit E ( $r = 0.611$ ;  $p < 0.001$ ), the Cholesterol + TG variable was included as a covariate in the repeated ANOVA measures. Additionally, lipid status was included in the Vit E evaluation, whereas FM was included as covariate in the analysis of retinol and carotenoids because adipose tissue represents an important storage site for these vitamins. Finally,  $\beta$ -carotene was included as a covariate in the Vit A evaluation because of its pro-vitamin activity. The groups' Vit A levels were compared using 2 (group) x 2 (time) ANOVA with repeated measures on the second factor. The group Vit E levels were compared by 2 (group) x 2 (time) ANCOVA with repeated measures on the second factor with Total Cholesterol + TG included as a covariate. When possible, post hoc *t*-tests for pair-wise comparisons

**Table I.** Basal-line parameters.

|                                 | <b>Group L</b><br><b>(n=27)</b> | <b>Group M</b><br><b>(n=27)</b> | <b>Group H</b><br><b>(n=27)</b> | <i>p1</i>        | <i>p2</i>        |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|------------------|------------------|
| <b>VO2 Max, ml/kg/min</b>       | 36.8±5.6                        | 47.0±1.5                        | 53.5±4.4                        | <b>&lt;0.001</b> | <b>&lt;0.001</b> |
| <b>Age, years</b>               | 39.6±8.4                        | 40.6±8.1                        | 42.8±6.7                        | 0.32             | 0.43             |
| <b>BMI</b>                      | 25.7±3.4                        | 24.1±1.8                        | 22.8±2.5                        | <b>&lt;0.001</b> | <b>&lt;0.001</b> |
| <b>FM %</b>                     | 21.7±6.4                        | 18.3±5.3                        | 17.1±4.9                        | <b>0.011</b>     | <b>0.011</b>     |
| <b>Volume Training, hr/week</b> | 5.3±2.9                         | 6.3±3.8                         | 6.8±3.1                         | 0.48             | 0.71             |
| <b>HR, beats per min</b>        | 60.3±10.6                       | 53.1±9.2                        | 50.7±7.1                        | <b>0.001</b>     | <b>0.001</b>     |
| <b>SBP, mmHg</b>                | 122.7±10.7                      | 121.7±8.7                       | 121.7±6.5                       | 0.89             | 1                |
| <b>DBP, mmHg</b>                | 78.5±7.7                        | 75.7±6.2                        | 75.2±6.7                        | 0.19             | 0.26             |
| <b>MAP, mmHg</b>                | 86.1±26.5                       | 91.0±6.6                        | 90.7±6.2                        | 0.46             | 0.90             |
| <b>Gly, mg/dL</b>               | 80.2±22.1                       | 81.1±6.4                        | 83.3±8.7                        | 0.71             | 1                |
| <b>TG, mg/dL</b>                | 96.9±47.6                       | 79.6±33.2                       | 72.8±27.5                       | 0.06             | 0.06             |
| <b>Cho, mg/dL</b>               | 199.6±34.8                      | 194.0±32.2                      | 194.8±27.5                      | 0.78             | 1                |
| <b>HDL, mg/dL</b>               | 57.0±13.3                       | 63.5±18.2                       | 64.4±15.0                       | 0.19             | 0.29             |
| <b>LDL, mg/dL</b>               | 123.3±30.9                      | 115.9±29.2                      | 116.0±26.7                      | 0.58             | 1                |

*VO<sub>2max</sub>*: maximal oxygen consumption; *BMI*: body mass index; *FM%*: fat mass percent; *HR*: resting heart rate; *SBP*: systolic blood pressure; *DBP*: diastolic blood pressure; *MAP*: mean arterial pressure; *Gly*: glycaemia; *TG*: triglycerides; *Cho*: cholesterol; *HDL*: high density lipoproteins; *LDL*: low density lipoproteins. *p1*: difference between all three groups. *p2*: difference between Group H and L.

**Table II.** Plasma concentrations of Vit A, Vit E,  $\beta$ -carotene and lycopene before and after a maximal incremental exercise test.

|                         | Group L<br>(n=27) |                   | Group H<br>(n=27) |                   | <i>p</i>     |
|-------------------------|-------------------|-------------------|-------------------|-------------------|--------------|
|                         | BL                | PE                | BL                | PE                |              |
| Vit A, mg/L             | 0.84 $\pm$ 0.23   | 0.825 $\pm$ 0.206 | 0.884 $\pm$ 0.172 | 0.894 $\pm$ 0.169 | <b>0.001</b> |
| Vit E, mg/L             | 17.7 $\pm$ 5.71   | 16.55 $\pm$ 5.82  | 16.53 $\pm$ 3.18  | 16.76 $\pm$ 2.74  | >0.05        |
| $\beta$ -carotene, mg/L | 0.61 $\pm$ 0.43   | 0.626 $\pm$ 0.39  | 0.74 $\pm$ 0.44   | 0.76 $\pm$ 0.40   | >0.05        |
| Lycopene, mg/L          | 0.37 $\pm$ 0.155  | 0.39 $\pm$ 0.13   | 0.35 $\pm$ 0.15   | 0.37 $\pm$ 0.15   | >0.05        |

Plasma vitamin concentrations were analyzed before (BL, Basal levels) and 60 min after (PE: Post-exercise) the maximal incremental exercise test. Significant of RM ANCOVA was assessed at  $p \leq .05$ . Data are expressed as the means  $\pm$ SD.

were performed. The statistical level of significance was set at  $p \leq 0.05$ .

## RESULTS

Sample characteristics are shown in Table I. The incremental exercise test did not induce significant plasma volume changes (hematocrit 45.1 $\pm$ 1.7 and 44.6 $\pm$ 1.8 pre- and post-exercise, respectively,  $p > .05$ ). As reported above, the study population was divided into LowVO<sub>2</sub>max (L), MediumVO<sub>2</sub>max (M) and HighVO<sub>2</sub>max (H) groups. Demographic, cardiovascular and hematologic parameters were similar between groups, except for heart rate (HR), BMI and percentage of Fat Mass (FM), which were significantly lower in Group H. Analyses were then performed comparing Groups L and H.

We first analyzed the effects of the incremental exercise test on TAC, a parameter that measures the combined activities of all plasmatic antioxidants. Although the two groups did not differ in basal TAC conditions, there was a significant increase detected in Group H after the incremental test (Fig. 1).

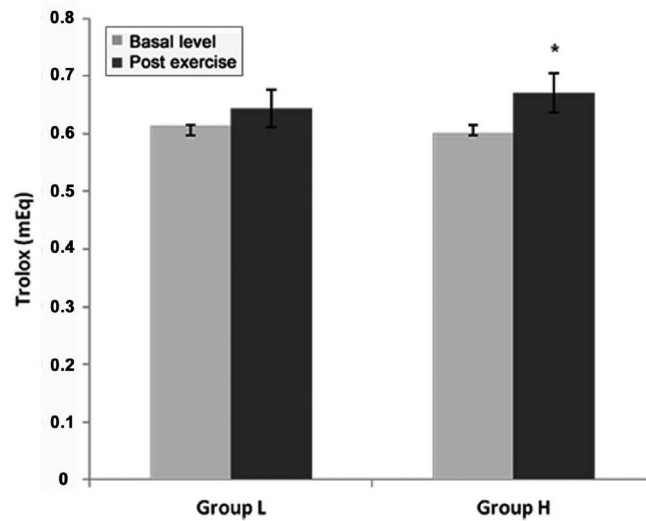
We then measured the plasma Vit A, Vit E,  $\beta$ -carotene and lycopene concentrations. In the basal condition, the concentrations of these fat-soluble vitamins were similar between the groups. After the incremental exercise test, no significant changes in vitamin concentrations were observed, except for a significant decrease in Vit A in Group L (Table II).

Finally, to measure the extent of the oxidative damage induced by the incremental test in the two

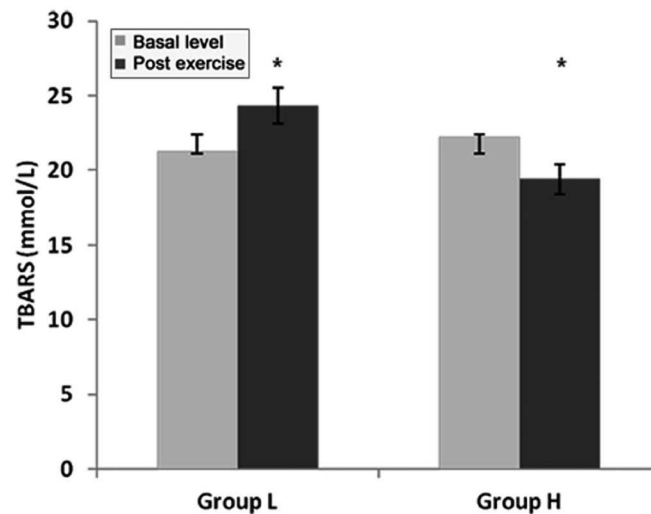
experimental groups, we measured the plasma levels of TBARS, which are formed as a byproduct of lipid peroxidation. The TBARS level was comparable in the basal condition but after the stress test TBARS levels significantly increased to 23% in Group L but decreased to 19% in Group H (Fig. 2).

## DISCUSSION

The aim of this study was to correlate the antioxidant levels to a physiological parameter, such as the VO<sub>2</sub>max. The main finding of this study is that amateur runners with diverse VO<sub>2</sub>max values respond differently to an incremental test. Group L showed decreased Vit A and increased TBARS levels, whereas Group H showed no change in the vitamin concentrations, an increase in TAC, and a reduction in TBARS concentration. When we divided participants by VO<sub>2</sub>max, there were significant differences for BMI, FM% and resting HR. This result is not surprising because individual variability exists in adaptations to prolonged endurance training (13, 14). However, one of the main findings of our study is that after the incremental test, we observed an increase in TAC in the H group, whereas no changes were observed in the L group. This finding suggests that higher VO<sub>2</sub>max values are associated with earlier and more beneficial adaptive responses to oxidative perturbations. Because we did not detect changes in fat-soluble antioxidant levels in the H group, this increase in TAC is likely due to the induction of enzymatic scavengers.



**Fig. 1.** Plasma total antioxidant capacity (TAC) in the L and H groups before and after the maximal test. Grey column: Basal Level; black column: Post Exercise. Data are expressed as the means  $\pm$ SD of molar Trolox equivalent \*indicates a significant change compared to the BL. (RM ANOVA,  $p < 0.05$ ; post hoc analysis  $p = 0.02$ ).



**Fig. 2.** Thiobarbituric acid-reactive substances (TBARS) changes after the incremental exercise test in the L and H groups. TBARS were evaluated before and 60 min after the stress test. Grey column: Basal Level; black column: Post Exercise. Data are expressed as the means  $\pm$ SD \*indicates a significant change compared to the Basal Levels. (RM ANOVA,  $p < 0.05$ ; post hoc analysis  $p = 0.02$ ).

Although Vit A, Vit E,  $\beta$ -carotene and lycopene are widely studied, fat-soluble antioxidants and their involvement in neutralizing exercise-dependent ROS production remains unclear. Interestingly, after the incremental exercise test, we did not detect significant variations of Vit E,  $\beta$ -carotene and lycopene, although a decrease in Vit A concentration was observed in the L group. Based on these findings, we hypothesize that whereas a prompt enzymatic induction may preserve Vit A in the H group, in the L group, Vit A is consumed in an attempt to buffer ROS production or regenerate other oxidized vitamins such Vit E.

Finally, after the stress test, we detected opposing changes in concentration of TBARS in the L and H groups. This observation suggests that in the H group, the reduction of the TBARS concentration may be due to the concomitant increase in TAC, while in the L group, Vit A consumption does not completely buffer ROS production and thus results in increased lipid peroxidation.

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## LETTER TO THE EDITOR

**ZENKER DIVERTICULUM IN THE RIGHT SIDE OF THE NECK RESEMBLING A THYROID MASS AT ULTRASOUND**

S. ALESSANDRINI<sup>1</sup>, I. SAMPERI<sup>1</sup>, F. DE CRISTOFARO<sup>2</sup>, E. D'ARMIENTO<sup>1</sup>,  
D. DIACINTI<sup>2</sup>, A. PERNAZZA<sup>2</sup>, D. BOSCO<sup>2</sup>, V. ASCOLI<sup>2</sup> and S. ULISSE<sup>3</sup>

<sup>1</sup>Department of Experimental Medicine, "Sapienza" University of Rome, Italy; <sup>2</sup>Department of Radiological, Oncological and Anatomic-Pathological Sciences, Sapienza University of Rome, Italy; <sup>3</sup>Department of Surgical Sciences, "Sapienza" University of Rome, Italy

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The first two authors contributed equally to this paper

Zenker's diverticulum represents the most common form of pharyngo-oesophageal diverticula usually occurring on the left side of the neck. Due to its anatomical proximity to the thyroid, it can mimic a thyroid mass. Here we describe the case of an asymptomatic 49-year-old man referred to the Thyroid Clinic of the Policlinico Umberto I Hospital-"Sapienza" University of Rome for thyroid sonography due to a family history of autoimmune thyroid disease. The patient's thyroid blood tests did not reveal any abnormalities. The sonographic examination showed a dishomogeneous and hypoechoic thyroid gland. In addition, in the third middle of the right lobe, a mass (with a diameter greater than 26 mm), with heterogeneous internal echogenicity, hypoechoic margins and internal hyperechoic spots was recorded, with no appreciable flow at the Doppler evaluation. The TI-RADS score was 4c. Hence, the patient underwent ultrasound-guided fine-needle aspiration cytology that revealed the presence of squamous cells without cytological atypia, erythrocytes, muscular and vegetable fibres, colonies of bacteria in the absence of inflammatory infiltrate. This was consistent with the diagnostic hypothesis of oesophagus diverticulum, which was confirmed by means of a barium-swallow oesophagography. This case report underlines the possibility that a suspicious thyroid mass may result from a Zenker's diverticulum, even if located on the right side, especially if the lesion has a heterogeneous echo-texture, a hypoechoic rim and internal hyperechoic spots.

To the Editor,

Pharyngo-esophageal diverticula (PED) are a saccular distension of the oesophagus internal layer through a muscular gap between the hypopharynx and the cervical oesophagus due to an anatomic weakness. Two types of PED have been described: the Zenker and the Killan-Jamieson diverticula,

with the former being the most common with an estimated prevalence between 0.01% and 0.11% and usually occurring on the left side of the neck. The Zenker diverticulum (ZD) originates in the posterior wall of the oesophagus above the *locus minoris resistentiae* called "Killan's triangle" and located between the oblique fibres of the inferior constrictor

*Key words: Zenker diverticulum, ultrasound, thyroid nodule, fine needle aspiration cytology*

*Mailing address:*

Prof. Salvatore Ulisse,  
Department of Surgical Sciences, "Sapienza" University of Roma,  
Viale Regina Elena, 324, 00161 - Roma, Italy  
Tel.: +39 06 49972607 - Fax: +39 06 49972606  
e-mail: salvatore.ulisse@uniroma1.it

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of the pharynx and the transverse muscle fibres of the cricopharyngeus muscle (1).

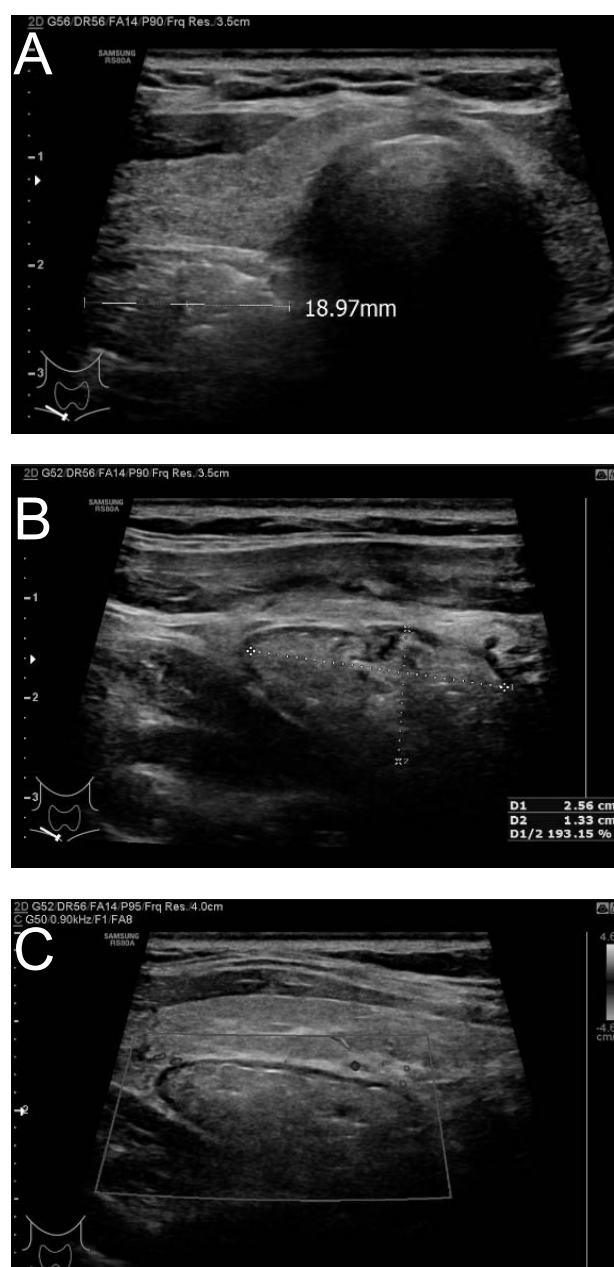
During sonography, Lixin and colleagues described four different types of ZD based on sonogram features: i) the lesion is isoechoic with a hypoechoic rim boundary and, small, internal punctuate echogenic foci; ii) the lesion contains a central hyperechoic area associated with a comet-tail artefact; iii) the mass is hyperechoic and heterogeneous, with stronger echoes anteriorly, but with no acoustic shadowing or reverberation artefacts; iv) the mass is an oval-shaped mixed nodule, with an unclear border and relatively multiple strong punctuate echoes (2). The most frequent clinical manifestations of ZD include dysphagia occurring in 80-90% of the patients, chronic cough and repeated episodes of aspiration in 30-40% of the cases. More rarely regurgitation of undigested foods, halitosis, hoarseness, weight loss and malnutrition have been observed (3). Sudden worsening of dysphagia, regurgitation and the development of symptoms such as local pain, haemoptysis or hematemesis may signal the presence of squamous cell carcinoma (0.4-1.5% of cases) (3).

Since ZD are present at thyroid level and protrude toward the thyroid, their identification during thyroid US scanning may be challenging and erroneously diagnosed as thyroid nodules. We report the case of ZD mimicking a thyroid nodule occurring on the right side of the neck of an asymptomatic patient, along with a review of the literature.

### Clinical case

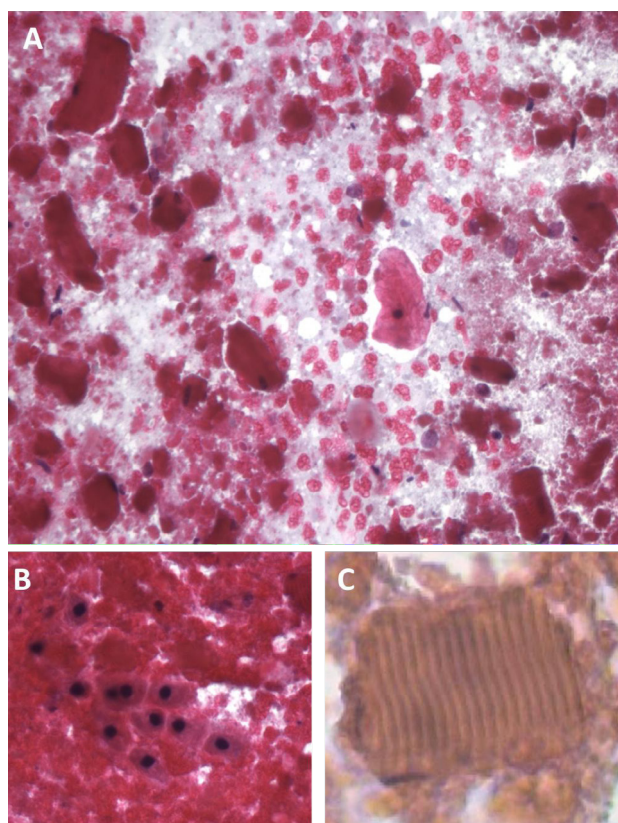
A 49-year-old man was referred to the Thyroid Clinic of the Policlinico Umberto I Hospital-“Sapienza” University of Rome for thyroid ultrasonography (US) due to a family history of autoimmune thyroid disease. He was asymptomatic for local pain, regurgitation or dysphagia. On physical examination, the thyroid gland was not palpable.

Thyroid blood tests did not show any abnormalities: TSH 1.5 mUI/l (normal range 0.4-4.0 mUI/l); FT3 5.48 pg/mL (2.0-5.6 pg/mL); FT4 16.9 ng/mL (9.0-17.1 ng/mL); AbTPO 2.5 UI/mL (normal range < 50 UI/ml); AbTg 6.4 UI/mL (normal range < 50 UI/ml); calcitonin was 1.03 pg/mL (normal



**Fig. 1.** Transverse (A) and longitudinal (B) sonograms reveal a heterogeneous mass (2.6 cm × 1.3 cm × 1.9 cm) with boundary hypoechoic rim and internal hyperechoic foci located at the posterior right lobe of the thyroid. Doppler sonography showing no blood-flow signals in the lesion (C).

range < 10 pg/ml). A thyroid US was performed by using an ACCUVIX A30, S80 Ugeo (Samsung Medison Co. Ltd., Seoul, Korea) equipped with a high-frequency (10-18 MHz) linear-array probe on both the transverse and longitudinal planes of the



**Fig. 2.** *A)* FNAC smears showing several muscular fibers and a few mature squamous cells in a background of erythrocytes and bacteria. *At higher magnification, B)* vegetable cells with dark nuclei and *C)* clear-cut striated muscle fibers.

patient's neck region in hyperextension, while lying in a supine position.

In the mid-portion of the posterior right thyroid lobe, the test revealed the presence of a 2.6 x 1.3 x 1.9 cm heterogeneous mass with internal echogenicity hypoechoic margins and internal hyperechoic spots, which were interpreted as microcalcifications, with no appreciable flow during the doppler evaluation (Fig.1). Based on these US features, the TIRADS (thyroid imaging reporting and data system) score was 4c, hence high suspicious of malignancy. Therefore, US-guided fine-needle aspiration cytology (FNAC) was performed. The FNAC showed mature squamous cells, several vegetable and striated muscular fibres (meat fibres) intermingled with numerous bacteria, erythrocytes, macrophages and rare granulocytes (Fig. 2). No thyrocytes or colloids were observed. The cytological diagnosis of esophageal diverticulum

was proposed. No FNAC-related complications were observed.

To confirm the diagnosis, the patient underwent a barium-swallow study that showed the presence of an outpouching filled with barium projecting to the right of and posterior to the oesophagus at pharyngoesophageal junction level (Fig. 3). The patient was then recommended to undergo an endoscopic surgical treatment of the Zenker's diverticulum.

## DISCUSSION

Palpable and non-palpable thyroid nodules are very common. During sonography they are present in up to 67% of the adult population (4). Around 5% of patients affected by thyroid nodules harbor a malignant lesion and, hence, the primary aim is to exclude malignancy (5). Ultrasound (US) has a pivotal role in thyroid nodule evaluation and in the differentiation between benign and malignant lesions (6). In fact, US features such as microcalcifications, marked hypoechogenicity, longer than wide shape, thick irregular or lobulated margins and microcalcifications are correlated with malignancy (6). Recently, these US features have been combined with the TI-RADS score to differentiate nodules presenting different malignancy risks (7). Being close to the thyroid gland, during neck US, a Zenker diverticulum (ZD) can be mistaken for a thyroid or parathyroid mass or a recurrent lymph node in patients who have undergone thyroidectomy for thyroid cancer (8).

To avoid an unnecessary invasive procedures, such as FNA, it is advisable to analyze the thyroid mass accurately in order to detect the typical sonographic features of ZD. The most frequent cause of mistaken diagnosis is the presence of central hyperechoic foci resembling microcalcifications. To differentiate between ZD air microbubbles and thyroid microcalcifications in a mass suspected of being connected to the oesophagus, a real-time sonography is recommended during swallowing or drinking water to demonstrate the change of the internal echogenicity and shape of the lesion (9). A review of the literature (Table I) showed that the

**Table I.** Clinical and ultrasound features and cytology outcome of 55 patients affected by pharyngoesophageal diverticula mimicking a thyroid mass described in the literature.

| N. of cases | Sex        | Age   | Symptoms (GE) | Location | Max size (cm) | Ultrasound characteristics                                                                                                             | FNAC             | Ref.          |
|-------------|------------|-------|---------------|----------|---------------|----------------------------------------------------------------------------------------------------------------------------------------|------------------|---------------|
| 1           | F          | 50    | +             | L        | -             | Round mass, hyperechoic rim                                                                                                            | -                | 13            |
| 1           | F          | 66    | -             | L        | -             | Echogenic mass, sonolucent rim                                                                                                         | -                | 12            |
| 2           | F          | 66    | +             | L        | 2.5           | Hypoechoic                                                                                                                             | -                | 14            |
|             | F          | 61    | +             | L        | 2.5           | Hypoechoic                                                                                                                             | -                |               |
| 1           | F          | 63    | +             | L        | 1.7           | Homogeneous internal echo, hyperechoic foci                                                                                            | -                | 15            |
| 1           | F          | 91    | -             | L        | -             | Hyperechoic heterogeneous                                                                                                              | -                | 16            |
| 1           | M          | 65    | +             | R        | 1.0           | Hypoechoic, hyperechoic foci                                                                                                           | -                | 17            |
| 1           | F          | 58    | -             | L        | 2.5           | Hypoechoic, hyperechoic foci                                                                                                           | -                | 18            |
| 1           | F          | 65    | -             | L        | 1.2           | Hypoechoic margins                                                                                                                     | E, Sq, I, B, V   | 19            |
| 6           | F          | 61    | -             | L        | 1.6           | Isoechoic mass with internal echogenic foci and boundary hypoechoic zone                                                               | -                | 20            |
|             | F          | 56    | -             | L        | 2.0           | Isoechoic mass with internal echogenic foci and boundary hypoechoic zone                                                               | -                |               |
|             | F          | 70    | -             | L        | 3.3           | Hypoechoic mass with internal echogenic foci and boundary hypoechoic zone                                                              | -                |               |
|             | M          | 65    | -             | R        | 1.0           | Hypoechoic mass with internal microcalcifications and peripheral echogenic line                                                        | -                |               |
|             | F          | 60    | -             | L        | 0.7           | Round mass with peripheral arc-shaped echogenic line without posterior shadowing                                                       | -                |               |
|             | F          | 26    | -             | L        | 0.8           | Round mass with peripheral arc-shaped echogenic line without posterior shadowing                                                       | -                |               |
| 1           | F          | 63    | -             | L        | 1.5           | Heterogeneous echogenicity                                                                                                             | E, Sq, V, B, A   | 21            |
| 1           | M          | 54    | -             | L        | 1.1           | Hypoechoic, hypoechoic margins                                                                                                         | Sq, A, V         | 22            |
| 1           | M          | 75    | -             | L        | 1.6           | -                                                                                                                                      | BR, M, Sq B      | 23            |
| 1           | F          | 54    | +             | L        | 2.0           | Hypoechoic with hyperechoic internal echo                                                                                              | B, V, Fu, Sq, Fo | 24            |
| 9           | M          | 72    | -             | L        | 1.0           | Isoechoic mass with boundary hypoechoic rim and internal small, punctuate, echogenic foci                                              | -                | 2             |
|             | M          | 75    | -             | L        | 1.7           |                                                                                                                                        | -                |               |
|             | M          | 74    | -             | L        | 1.6           |                                                                                                                                        | -                |               |
|             | M          | 79    | -             | L        | 1.1           |                                                                                                                                        | -                |               |
|             | M          | 61    | -             | L        | 1.5           | A lesion containing a central hyperechoic area associated with a comet-tail artifact                                                   | -                |               |
|             | M          | 70    | -             | L        | 1.3           |                                                                                                                                        | -                |               |
|             | M          | 71    | -             | L        | 0.9           |                                                                                                                                        | -                |               |
|             | M          | 53    | -             | L        | 1.4           | Hyperechoic and heterogeneous mass with stronger echoes anteriorly, but there was no acoustic shadowing or reverberation artifacts     | -                |               |
| 4           | F          | 55    | -             | L        | 3.0           | heterogeneous echogenicity Hypoechoic margins                                                                                          | N, T, B          | 1             |
|             | M          | 50    | -             | L        | 1.2           | Microcalcifications                                                                                                                    | Sq               |               |
|             | M          | 55    | -             | L        | 1.2           | Hyperechoic                                                                                                                            | -                |               |
|             | F          | 59    | -             | L        | 1.7           | heterogeneous echogenicity Hypoechoic margins                                                                                          | -                |               |
| 1           | F          | 50    | +             | L        | -             | Hypoechoic Uncompleted margins                                                                                                         | -                | 26            |
| 1           | F          | 50    | -             | L        | -             | Heterogeneous and spongiform                                                                                                           | Sq, I, V         | 27            |
| 1           | F          | 74    | +             | L        | 3.1           | Isoechoic mass                                                                                                                         | -                | 28            |
| 1           | M          | 69    | -             | L        | 1.5           | Microcalcifications                                                                                                                    | V, B, I, A, Fo   | 29            |
| 5           | F          | 47    | +             | L        | 4.0           | Irregular, hyperechoic zone                                                                                                            | -                | 30            |
|             | F          | 39    | +             | L        | 3.6           | Hypoechoic                                                                                                                             | -                |               |
|             | M          | 54    | +             | L        | 1.8           | Hypoechoic, post acoustic shadow                                                                                                       | -                |               |
|             | F          | 35    | +             | L        | 4.0           | Patchy hyperechoic                                                                                                                     | -                |               |
|             | F          | 37    | +             | L        | 4.0           | Microcalcifications                                                                                                                    | -                |               |
| 14          | 6 M<br>8 F | 33-79 | 8+<br>6-      | L        | 0.7-3.3       | Four major nodules showed heterogeneous echoic striae, no posterior margins. Seven nodules showed margins with posterior reverberation | -                | 31            |
| 1           | M          | 49    | -             | R        | 2.6           | Isoechoic mass with hypoechoic rim and internal small hyperechoic foci                                                                 | Sq, E, M, V, B   | Present study |

B: bacterial flora; Sq: squamous cells; A: amorphous material; T: thyrocytes; M: muscular fibers; I: inflammatory cells; V: vegetable fibers; E: erythrocytes; Fo: food; BR: birefringent material; Fu: fungus; N: necrotic material; L: left side of the neck; R: right side of the neck; GE: gastro enteric symptoms.





**Fig. 3.** Barium-swallow study demonstrated the Zenker diverticulum projecting to the right and posterior of the esophagus.

majority of ZD occur on the left side of the neck, and only 2 out of 55 have been described on the right side (1, 2, 10-28). In our case, the ZD showed US features compatible with the first type of Lixin classification described above, and located on the right side of the neck (2). This rare localization did not arouse suspicion of ZD, and led us to consider it erroneously as a thyroid nodule with a TI-RADS score of 4c, and therefore eligible for FNAC. The absence of thyrocytes and colloid in the cytological smears excluded that the sample was thyroid tissue and corroborated the hypothesis of a ZD (18, 26). This was confirmed by means of a barium-swallow esophagram.

In conclusion, in the sonographic evaluation of a thyroid mass, the possible occurrence of an unexpected Zenker diverticulum should be taken into consideration, by detecting the connection to the oesophagus. In this case dynamic US during

swallowing or drinking water should always be carried out even if the nodule is located on the right side of the neck in an asymptomatic patient.

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## LETTER TO THE EDITOR

**ORTHODONTIC FORCES MODULATE INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-5 CHANGES IN GINGIVAL CREVICULAR FLUID**

A. BALLINI<sup>1</sup>, L. SANTACROCE<sup>2</sup>, S. CANTORE<sup>1</sup>, R. SAINT<sup>3</sup>, F. MASTRANGELO<sup>4</sup>,  
A. DESIATE<sup>5</sup> and S. SCACCO<sup>1</sup>

<sup>1</sup>Department of Basic Medical Sciences, Neurosciences and Sense Organs, University of Bari Aldo Moro, Bari, Italy; <sup>2</sup>Ionian Department, University of Bari Aldo Moro, Taranto, Italy; <sup>3</sup>Department of Periodontology and Oral Implantology, Pravara Institute of Medical Sciences, Loni, Maharashtra, India; <sup>4</sup>Department of Oral Sciences, San Raffaele Research Institute, Vita e Salute University, Milan, Italy; <sup>5</sup>Department of Interdisciplinary Medicine, University of Bari Aldo Moro Bari, Italy

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All Authors contributed equally to this paper

**Orthodontic tooth movement results from the response of the periodontal tissue to orthodontic force, which leads to modeling and remodeling of the surrounding alveolar bone. The response is considered to occur through the activation of specific signaling pathways, many of which are known, all acting to ultimately result in tooth movement. Much is known about the actions of these two cells, and the signaling pathways that affect them, both in bone and orthodontic literature, however, to date, little work has been carried out to examine the effect of the insulin-like growth factor binding proteins (IGFBP) in orthodontics. Therefore, we investigated the presence of IGFBP-5 in the gingival crevicular fluid (GCF) of 6 healthy subjects, and assessed the effects of orthodontic treatment on the levels and molecular state of this protein.**

To the Editor,

Movement of teeth in the oral cavity occurs naturally in a variety of ways, in fact prior to eruption, tooth buds migrate due to their own devices or through the growth of surrounding dental structures (bone, teeth, etc.) (1). Upon entering the oral cavity, teeth find a final position by both the eruptive mechanism followed by the pressures of surrounding tissues (cheeks, tongue, etc.) (2), and once reaching the level of occlusion they are subjected to masticatory forces which can result in tooth movement within the bony tooth socket (3).

Migration can also occur if there are changes in the equilibrium of forces that maintain the current position (i.e., trauma to surrounding musculature or dentition)

(4). Furthermore, there are a variety of additional circumstances where tooth movement occurs, but the focus here was on the tooth movement that does not occur naturally, specifically the movement that occurs upon application of continuous mechanical forces (i.e., orthodontic tooth movement) (5, 6).

After the initiation of tooth movement, fluid flow transfers, mineralized and non-mineralized matrix strains and micro-damage dominate the early stages of orthodontic tooth movement and ultimately lead to cellular strain (7).

When mechanical forces are applied to teeth, the cells/extracellular matrix of the periodontal ligament (PDL) and alveolar bone respond synergistically, resulting in

*Key words: gingival crevicular fluid, orthodontic forces, insulin like growth factor binding protein*

*Mailing address*

Prof. Luigi Santacroce,  
Ionian Department,  
University of Bari Aldo Moro, Via Duomo, 259  
74123 Taranto, Italy  
Tel./Fax: +39 0805478486  
e-mail: luigi.santacroce@uniba.it

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tooth movement (8-11) that induces changes in non-cellular elements such as cytokines, growth factors, neurotransmitters, to initiate and sustain the remodeling activity ultimately facilitating tooth movement (5).

The strain produced by mechanical forces in the microenvironment surrounding dental and paradental cells causes changes in cellular shape and elicits release of signaling molecules from the affected cells (5), molecules that bind with cell adhesion complexes on other cells, or the same cells that released the factors, and initiate many cellular responses (6).

This complex cellular/non-cellular orchestration ultimately results in the bone remodeling necessary for tooth movement, and can initially be characterized as an acute inflammatory process, so that the initial phase of tooth movement is dominated by characteristics of such a phenomenon, predominately exudative, in which plasma and leukocytes leave capillaries in the areas of strain and the production of various cytokines which interact directly or indirectly with the native periodontal cells (7).

Insulin-like growth factor-1 (IGF-1) plays an important role in the regulation of bone metabolism, and has been shown to stimulate osteoblastic cells, inducing their proliferation and collagen synthesis (8). Upon administering IGF-1 to neonatal mouse bone cell cultures, a dose-dependent stimulation of bone resorption was observed, and in isolated osteoclast cultures the addition of exogenous IGF-1 produced no increase in osteoclastic cell activation, suggesting the promotion of osteoblast-mediated activation of osteoclast function (8-10).

IGF-1 has also been shown to regulate the OPG/RANK/RANKL axis by suppressing OPG and increasing RANKL expression respectively in bone cell cultures (11), in addition, using *in situ* hybridization, IGF-1 and IGF-1 receptor mRNAs are localized in osteoblast-like and fibroblastic cells subjected to tensile force (12-14).

The connection between these findings and orthodontics is that IGF-1 has been identified in gingival crevicular fluid (GCF) during tooth movement and seems to be responsible for cellular recruitment and activation in mechanical load situations (15-17).

The six members of the insulin-like growth factor-binding protein family (IGFBP-1-6) are important

components of the insulin-like growth factor (IGF) axis, and they serve to regulate the activity of both IGF-I and -II polypeptide growth factors (18).

Overexpression of IGFBP-5 in the osteoblastic cell line MC3T3-E1 also delayed or decreased the appearance of mRNA for bone-formation markers (e.g. osteocalcin and osteopontin) and inhibited the formation of bone nodules in confluent cell cultures (19).

The implications of this in orthodontics are potentially very meaningful, and some literature suggests that exogenous factors may be capable of enhancing traditional orthodontic appliance load effects on the tissues and enhancing tooth movement. The aim of the present study was to assess the concentration of IGFBP-5 gingival crevicular fluid (GCF) during orthodontic treatment.

## MATERIALS AND METHODS

### *Patients*

Six healthy subjects (aged 18-20 yrs; 3 males and 3 females) undergoing orthodontic treatment with fixed appliances in a private dental practice were enrolled in this study. The patients were selected for having healthy gingival tissues around the teeth exposed to orthodontic force, as demonstrated by a clinical periodontal examination including measurement of plaque index, gingival bleeding, and probing depth; moreover, they were free from periodontal diseases as assessed by panoramic radiographs. All patients gave formal informed consent to participate in the study, according to the declaration of Helsinki.

### *Sample collection and clinical procedure*

GCF samples were collected from the 6 orthodontic patients by inserting a sterile paper strip into the gingival crevice of the upper right incisor and leaving it there for 30 s. None of the subjects had a history of systemic disease, smoking or steroid medication.

Prior to the procedure, the tooth surface was gently dried with cotton rolls; the tooth was also kept dry during the 30-sec procedure to avoid contamination with saliva. GCF was collected immediately before fixed force appliance (time 0) and 6 h and 7 days after the application. The volume of GCF recovered was immediately measured using a Periotron System (Periotron 6000, Harco Electronics, USA) previously calibrated with

sterile water. The samples were then placed in 300  $\mu$ l of 20 mM phosphate buffer pH 6.0, containing 0.15 M NaCl, 1.0 mM EDTA, 1 mM dithiothreitol, 0.1% Tween 20 and 10  $\mu$ l/ml protease inhibitor cocktail (Sigma). Samples were vortexed for 30 min, the strips were removed, and the solutions were centrifuged for 5 min at 3000xg.

#### *Immunochemical detection of IGBP-5 in GCF*

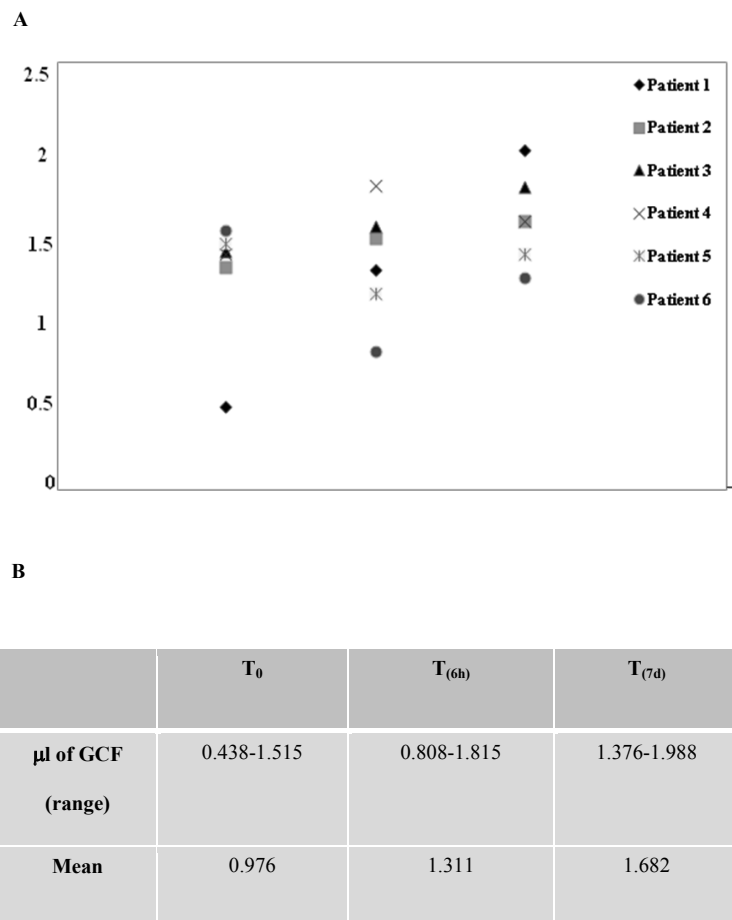
GCF specimens from each patient were concentrated to 50  $\mu$ l (Ultrafree-0.5, Millipore). After adding 16.7  $\mu$ l of Laemmli buffer, under non-reducing conditions, SDS-PAGE separation on 13% acrylamide was carried out.

Proteins were transferred to nitrocellulose membrane for Western blotting. Membranes were blocked with 5% non-fat dry milk in TBST (10 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.2% Tween 20) for 1 h at room temperature and then incubated with polyclonal rabbit anti-IGFBP-5 IgG (Santa

Cruz Biotechnology) diluted 1:500 in phosphate-buffered saline (PBS) overnight at 4°C. After 3 washes in TBST, the membrane was incubated with horseradish peroxidase-labeled mouse anti-rabbit IgG (Amersham Pharmacia Biotech) diluted 1:5000 for 2 h at room temperature. Bound antibody was detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Intensity of ECL-detected bands was measured by densitometry (Quantity One Software, BioRad). To permit quantitative measurement of band intensities, we determined the linear range of the ECL-film response by performing, under identical conditions, a Western blot of pooled human serum, obtained from a healthy donor, considered a positive control for IGFBP-5.

Intensity of IGFBP-5 was measured in samples of serially diluted serum corresponding to 0.01-1  $\mu$ l. After assuring that band intensities of the experimental samples



**Fig. 1.** Graphical (A) and numerical (B) representation of gingival crevicular fluid (GCF) volumes in 6 subjects before and during orthodontic treatment. GCF volumes were determined using a Periotron.

were within linear range, we expressed IGFBP-5 levels as densitometric intensity *per*  $\mu$ l of GCF recovered from the gingival cervice.

## RESULTS

The volumes of GCF collected from the patients in observation times after application of mechanical force, measured at time 0, after 6 hours and after 7 days increased in function of time (Fig. 1 A-B). We observed that the fixed force application appeared to produce a time-dependent fragmentation of IGFBP-5 with the appearance of intermediate bands on day 7 of treatment.

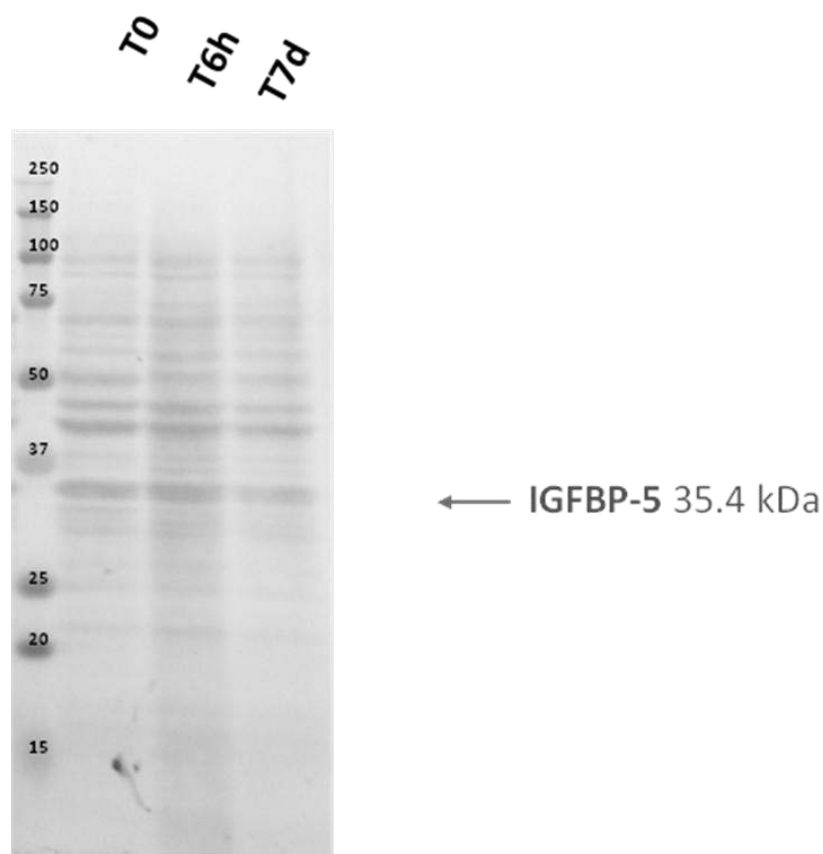
Densitometric quantification of IGFBP-5 was possible because band intensities were always within

the linear range of the enhanced chemiluminescence (ECL)-film response, determined using a positive control consisting of human serum (data not shown).

When normalized to control values for each patient (time 0), the mean of IGFBP-5 had decreased by 10% at 6 h and by 40% at 7 days of treatment (Fig. 2)

## DISCUSSION

Various molecules are capable of passing through gingival sulcular epithelium and filter their way into GCF, and the majority of these molecules are associated with remodelling of periodontal tissues during situations such as normal maintenance, periodontal diseases and orthodontic treatment (6).



**Fig. 2.** IGF binding protein-5 (IGFBP-5) expression in healthy subjects, prior to and during orthodontic treatment. Immunochemical detection (Western blot) of IGFBP-5 in gingival crevicular fluid (GCF) collected from 6 healthy subjects undergoing orthodontic treatment. Lane 1, immediately before fixed force appliance (0 h); Lane 2, 6 h after start of treatment; Lane 3, 7 days after start of treatment. The major band (35.4 kDa) corresponds to the glycosylated, intact form of IGFBP-5; lower molecular weight bands represent IGFBP-5 fragments. Positions of molecular weight markers (kDa) are indicated on the left. The figure reports the results from an 18-year-old patient and is representative of results for all other subjects.

The collection of GCF is a non-invasive procedure, which provides for the periodontist and orthodontist useful diagnostic information on the nature and extent of the periodontal health or disease, as well as the patient's response to mechano-therapy, and in our observations increase in function of time (1).

It is probably the result of an increase in the inflammatory process caused by the extension of the application of mechanical force, a hypothesis in accordance with studies that indicate an increase in the amount of CGF released during the application of the orthodontic force (13, 15-16).

Direct evidence for modified or enhanced cellular activities during orthodontic tooth movement can be found in GCF of treated teeth. In one theory, growth factors are also released during inflammation and repair the cells of periodontal ligament and bone, another theory states that the growth factors may be secreted by bone cells and stored (bound to bound matrix), and furthermore they are likely to be released and activated during bone resorption (5, 6).

The first indication that IGFBPs interacted with biomolecules other than IGFs came with the observation that gel chromatography of heparinized plasma in a heparin-containing buffer caused a 70–80% shift in immunoreactive IGF from a high-molecular-mass complex (140 kDa) to an elution position characteristic of free IGF (19).

Following this initial study, later work by the same group using immunoblotting and immunohistochemical techniques identified IGFBP-5 associated with ECM preparations. A key finding in this study was that IGFBP-5 enhanced the growth response of human fibroblasts (plated on either ECM or Type IV collagen) to added IGF-I. IGFBP-5 has also been reported to bind to osteoblast and osteoclast-like cells, although, in this instance, no binding was observed to the ECM derived from these cells (20).

At present, more adult patients seek orthodontic treatment as compared to earlier days, and according to some studies, orthodontic tooth movement can be enhanced but some investigators have reported contradictory results.

Some evidence shows that low-level laser irradiation accelerates the bone remodelling process

during orthodontic treatment and other shows that some molecules such as TRAIL are involved in periodontal and, consequently, in orthodontic tooth movement (21-23).

Invariably both the orthodontist and the periodontist need to be well equipped with thorough understanding of each other's fields, to initiate the best possible treatment for the patient, and for that reason, studies involving the biology of periodontal tissues, and their reactions to mechanical, chemical or pathological stimuli is an exciting field as we are trying unravel several queries to meet demands of this ultramodern era.

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#### RETRACTION STATEMENT

As the authenticity of the authorship of the article “Mechanism of age-related changes of bone marrow mesenchymal stem cells in senescent osteoporosis” published in Vol. 30 No. 2 has been questioned, the Editor has decided to retract this article from the Journal.