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ORIGINAL PAPER

THE INFLUENCE OF THYROID HORMONES ON MITOCHONDRIAL MECHANISMS OF BLOOD NEUTROPHILS' APOPTOSIS IN CASE OF EXPERIMENTAL PERIODONTITIS

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Abstract

The objective of the study. This study aims to investigate the role of mitochondria in blood neutrophils' apoptosis in case of periodontitis combined with hyper- and hypothyroidism in rats.
Material and methods. Experimental studies were conducted on male, nonlinear, white rats of around 4 months of age. Analysis of cell samples to determine reactive oxygen species was evaluated by the flow laser cytometry method, using 2.7-dichlorodihydrofluorescein diacetate. The percentage of neutrophils with low transmembrane mitochondrial potential and percentage of apoptotic neutrophils were evaluated by the flow laser cytometry method, using specific kits.
Results. We have found a statistically significant higher percentage of blood neutrophils with increased reactive oxygen species generation, low transmembrane mitochondrial potential and the percentage of FITC Annexin V- positive cells in rats with periodontitis and in rats with periodontitis combined with thyroid

Résumé

L'influence des hormones thyroïdiennes sur les mécanismes mitochondriaux de l'apoptose des neutrophiles sanguins en cas de parodontite expérimentale

L'objectif de l'étude. Cette étude vise à étudier le rôle des mitochondries dans l'apoptose des neutrophiles sanguins en cas de parodontite associée à une hyper et une hypothyroïdie chez le rat.
Méthodes. Des études expérimentales ont été menées sur des rats blancs, non lignés et blancs, âgés d'environ 4 mois. L'analyse des échantillons de cellules pour déterminer les espèces d'oxygène réactives a été évaluée par la méthode de la cytométrie au laser en flux, en utilisant du diacétate de 2,7-dichlorodihydrofluorescéine. Le pourcentage de neutrophiles à faible potentiel mitochondrial transmembranaire et le pourcentage de neutrophiles apoptotiques ont été évalués par la méthode de la cytométrie au laser en flux, à l'aide de kits spécifiques.

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dysfunction. At the same time, the changes were more pronounced in animals with hyperthyroidism.
Conclusion. One of the important signaling pathways of apoptosis triggering, in case of experimental periodontitis combined with thyroid dysfunction, is reactive oxygen species overproduction and disruption of the mitochondrial inner membrane, due to the decreased transmembrane potential, which is more pronounced in case of hyperthyroidism.

Keywords: periodontitis, thyroid hormones, mitochondria, cell death.

Résultats. Il a été établi l'augmentation statistiquement significative du pourcentage de neutrophiles dans le sang avec une augmentation de la génération d'espèces réactives de l'oxygène, un faible potentiel mitochondrial transmembranaire et le pourcentage de cellules FITC positives à l'annexine V chez les rats atteints de parodontite et chez les rats atteints de parodontite associée à un dysfonctionnement de la thyroïde. Dans le même temps, les changements étaient plus prononcés chez les animaux hyperthyroïdiens.
Conclusion. Ainsi, l'une des principales voies de signalisation de l'apoptose qui déclenche une parodontite expérimentale associée à un dysfonctionnement de la thyroïde est la surproduction d'oxygène réactif et la perturbation de la membrane interne des mitochondries en raison de la diminution du potentiel transmembranaire, qui est plus prononcée en cas d'hyperthyroïdisme.

Mots-clés: parodontite, hormones thyroïdiennes, mitochondries, mort cellulaire.

Introduction

Reactive oxygen species (ROS) are products of normal oxygen metabolism and have beneficial biological effects, in low levels and under normal conditions. Instead, higher concentrations present harmful effects to the body[1-2].

Periodontitis is an immune inflammatory response which arises from the interaction between the periodontal-pathogenic bacteria and the host[3]. Tissue destruction in periodontal diseases is considered to be the result of an altered inflammatory/immune response to microbial plaque and involves massive release of neutrophils, ROS and enzymes[4]. Gingival epithelial cells form the first line of defence in the gingival crevice. They have the key role as the protection mechanism of host oral structures from bacterial invasion. Thus, gingival epithelial cells produce an adaptive immune response[5] and release the chemotaxis factor for neutrophils[6], antimicrobial peptides[7] and pro-inflammatory cytokines, such as interleukin-8. On the other hand, over-expression of these pro-inflammatory cytokines causes collateral tissue damage. ROS produced by activated neutrophils, in response to periodontal-pathogenic bacteria, cause serious periodontal tissue lesions, in the context of periodontal disease[8]. Therefore, the balance between antioxidant mechanisms and ROS is very important in periodontal pathogenesis.

Several authors reported a positive correlation between periodontal tissue damage and high levels of ROS[9-10]. Hypoxia and inflammation induced higher expression of ROS in primary periodontal ligament fibroblasts[11]. Besides, the exposure of periodontal ligament cells to hydrogen peroxide decreased their viability, by promoting apoptosis[12]. Apoptosis involves a cascade of complex events, from external apoptotic signals, activating specific receptor complexes, to the execution of apoptosis, by activation of proteases and endonucleases. The commitment to apoptosis depends on the balance between proapoptotic and antiapoptotic signaling components within cells[13-14]. Hyperproduction of ROS and oxidative stress may result in a pathological transformation of mitochondria – the opening of the mitochondrial permeability transition pore and induction of cell death[15-17].

The objective of our study was to establish the role of mitochondria in blood neutrophils' apoptosis, in case of periodontitis combined with hyper- and hypothyroidism in rats.

Materials and methods

Experimental studies were conducted on male, nonlinear, white rats of around 4 months of age, who were housed at 25±3°C and humidity of 55±2%, under a constant 12h light and dark cycle. Water was available ad libitum. The experimental animals were divided into the following groups: I – control animals, who were administered intragastric a 1% solution of starch (n=12); II – animals with periodontitis (the rats of this group were injected into gum tissue with 40 µl of lipopolysaccharide (LPS) E. Coli

(«Sigma-Aldrich», США)) 7 times during 2 weeks (n=12)[18]; III– animals with periodontitis combined with hyperthyroidism. To model the experimental hyperfunction of the thyroid gland, animals received intragastric L-thyroxine daily in 1% starch solution, at a rate of 10 μg/day per 100 g of body mass, for 21 days (n=12)[19]. Starting from the eighth day of experiment, rats were injected into gum tissue with 40 μl of LPS E. Coli 7 times during 2 weeks; IV – animals with periodontitis combined with hypothyroidism. To model the experimental hypofunction of the thyroid gland, animals received intragastric Merkazolil daily in 1% starch solution, at a rate of 1 mg/day per 100 g of body mass, for 21 days (n=12)[19]. Starting from the eighth day of experiment, rats were injected into gum tissue with 40 μl of LPS E. Coli, 7 times during 2 weeks.

Animal euthanasia was carried out on the 22nd day of the experiment, by cardiac puncture under deep anesthesia. All procedures were conducted according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes[20].

The population of neutrophils was obtained by blood centrifugation at double density gradient 1.077 and 1.093 of ficoll-urografin. After 40 minutes of centrifugation at 4°C and the speed of 1500 rpm, two interphases were formed. Upper interphase (on the border of plasma – ficoll-urografin density 1.077) consisted of mononuclear cells – 80% of lymphocytes, 15-18% of monocytes and 2-3% of granulocytes. Lower interphase (on the border of solutions gradient density 1.077-1.092) was the population of neutrophils[21]. The number of viable cells present in a cell suspension was 98-99% (Trypan blue exclusion test).

To determine the level of neutrophils' apoptosis, the leukocyte blood suspension was resuspended in a pre-diluted (1:10) binding buffer from the kit of reagents „ANNEXIN V FITC" („Beckman Coulter", USA). The number of cells was counted with the help of Goryaev's calculating camera, under the microscope, and adjusted to the number of $1x10^6$ cells/mL. Then 100 μL of cell suspension was taken to test tube and 1 μL of annexin V-FITC solution and 5 μL of dissolved PI were added. Cells were mixed and incubated in binding buffer at room temperature. As a negative control, we have used unlabeled cells. The determination of apoptosis was conducted on flow cytometer Epics XL (Beckman Coulter, USA), using a software system. Results are expressed as the percentage of cells which have attached FITC Annexin V or PI. The cells are alive when they are FITC Annexin V and PI negative. The cells are apoptotic when they are FITC Annexin V positive and PI negative. The cells are in stage of irreversible apoptosis (necrosis) when they are FITC Annexin V positive and PI positive.

Analysis of cell samples to determine ROS (hydrogen peroxide) of neutrophils was evaluated by the flow laser cytometry method on flow cytometer Epics XL (Beckman Coulter, USA), using 2.7-dichlorodihydrofluorescein diacetate. The value of the studied parameter was expressed as a percentage (ratio of cells with ROS overproduction to general cell count×100%).

The number of neutrophils with low transmembrane mitochondrial potential ($\Delta\psi$) was evaluated by the flow laser cytometry method, using a kit of reagents «MitoScreen» («BD Pharmigen», USA) on flow cytometer Epics XL (Beckman Coulter, USA). The value of the studied parameter was expressed as a percentage (ratio of cells with low $\Delta\psi$ to general cell count×100%).

All of the data were processed using the software package Statistica 6.1 for Windows. Intergroup comparisons were performed using parametric methods and Student's t-test. Correlation analysis was performed by Pearson's method. Coefficient of linear correlation (r) and its reliability (p) were calculated, that was accordingly denoted in the tables (correlation matrices). If the index r=0, the linkage was considered as absent, in the range 0-0.29 – the linkage was considered as weak correlation, interval of index 0.30-0.69 described linkage as medium strength and interval 0.70-1.00 pointed to strong correlation interaction. The correlation coefficient was significant at $p<0.05$.

Results

The analysis of data indicates that production of ROS by blood neutrophils has significantly increased in rats of all experimental groups: in periodontitis by 63.3%, in periodontitis combined with hyperthyroidism by 127.3%, in periodontitis combined with hypothyroidism by 83.6% vs control group (Table 1). ROS generation in animals with periodontitis combined with hyperthyroidism was 39.2% greater vs group with periodontitis and 23.8% greater vs group with periodontitis combined with hypothyroidism.

The same trend is observed for changing the percentage of blood neutrophils with low transmembrane mitochondrial potential. Thus, the percentage of blood neutrophils with low $\Delta\Psi m$ in periodontitis significantly increased by 51.6%, in periodontitis combined with hyperthyroidism by 2.4 times, in periodontitis combined with hypothyroidism by 91.4% vs control group. The percentage of neutrophils with low $\Delta\psi$ in animals with periodontitis combined with hyperthyroidism was significantly greater, by 58.8%

Table 1. The indices of mitochondrial apoptosis pathway in rats with periodontitis without concomitant pathology and combined with hyper- and hypothyroidism (M±m, n=12)

Index	*Experimental groups*			
	Control group	*Periodontitis*	*Periodontitis combined with hyperthyroidism*	*Periodontitis combined with hypothyroidism*
Suspension of blood neutrophils				
The percentage of reactive oxygen species	17,28±1,10	28,22±0,87*	39,28±0,90* p_1<0.05	31,72±0,53* $p_{2,3}$<0.05
The percentage of neutrophils with low mitochondrial transmembrane potential	1,28±0,09	1,94±0,09*	3,08±0,10* p_1<0.05	2,45±0,11* $p_{2,3}$<0.05
The percentage of FITC Annexin V- positive neutrophils	2,08±0,14	3,16±0,14*	5,26±0,15* p_1<0.05	3,97±0,11* $p_{2,3}$<0.05

Note: * – significant differences compared to control animals, p<0.05;
2. p_1 – significant differences between the group of periodontitis with the group of periodontitis combined with hyperthyroidism;
3. p_2 – significant differences between the group of periodontitis with the group of periodontitis combined with hypothyroidism;
4. p_3 – significant differences between the group of periodontitis combined with hyperthyroidism with the group of periodontitis combined with hypothyroidism.

comparing to data of rats with periodontitis, and by 25.7% comparing to data of rats with periodontitis combined with hypothyroidism.

To investigate the apoptosis onset, the percentage of Annexin V-positive cells was determined. It was established the statistically significant increase of Annexin V-positive blood neutrophils percentage in rats of all experimental groups: in periodontitis by 51.9%, in periodontitis combined with hyperthyroidism by 2.5 times, in periodontitis combined with hypothyroidism by 90.9% vs control group. The percentage of Annexin V-positive cells in animals with periodontitis combined with hyperthyroidism was significantly greater, by 66.5% comparing to data of rats with periodontitis, and by 32.5% comparing to data of rats with periodontitis combined with hypothyroidism (Fig. 1).

The correlative analysis carried out between the percentage of ROS and the percentage of FITC Annexin V- positive neutrophils revealed a strong direct correlative linkage in animals of all experimental groups (Table 2); the correlative analysis carried out between the percentage of neutrophils with low mitochondrial transmembrane potential and

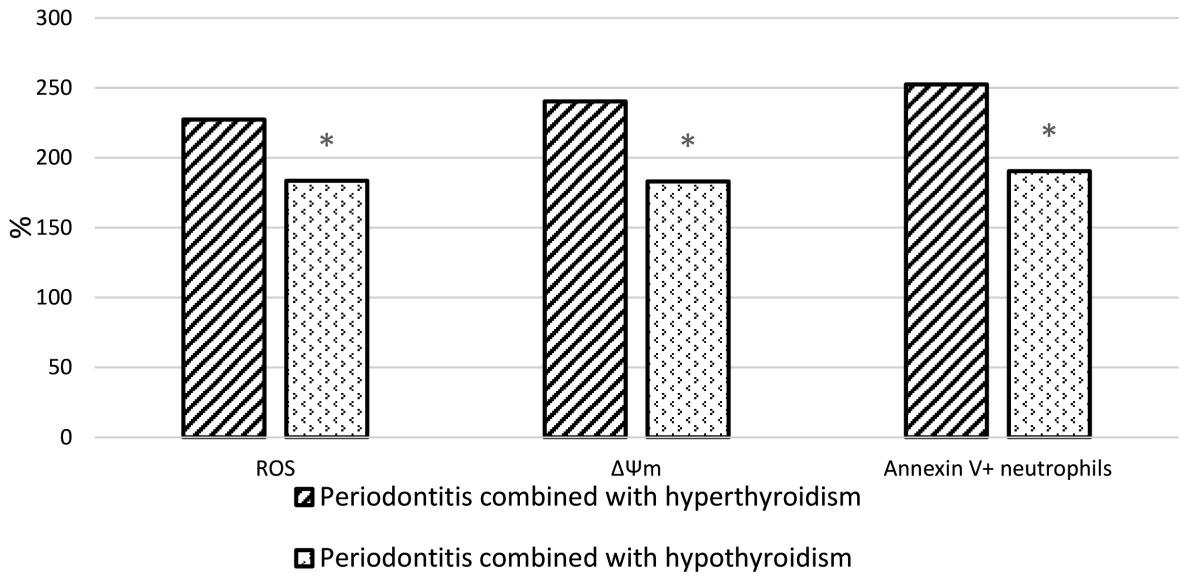

Fig. 1. Comparison of the indices of mitochondrial apoptosis pathway in rats with periodontitis combined with thyroid dysfunction.

Table 2. Correlative linkages between the percentage of FITC Annexin V- positive neutrophils with ROS and mitochondrial transmembrane potential in case of periodontitis without concomitant pathology and combined with hyper- and hypothyroidism (r_{xy})

Index	*The percentage of reactive oxygen species of neutrophils*		
The percentage of FITC Annexin V- positive neutrophils	*Periodontitis*	*Periodontitis combined with hyperthyroidism*	*Periodontitis combined with hypothyroidism*
	0,73*	0,75*	0,71*
	The percentage of neutrophils with low mitochondrial transmembrane potential		
	Periodontitis	Periodontitis combined with hyperthyroidism	Periodontitis combined with hypothyroidism
	0,60*	0,61*	0,68*

Note: * – significant differences of correlation coefficients, $p<0.05$

Table 3. Correlative linkages between the TSH, fT3, fT4 and the percentage of FITC Annexin V- positive neutrophils, ROS and ΔΨm in periodontitis without concomitant pathology and combined with hyper- and hypothyroidism (r_{xy})

Index	*Correlative linkages*	*Experimental groups*	
		Periodontitis combined with hyperthyroidism	*Periodontitis combined with hypothyroidism*
TSH	ROS	-0,79; p<0.01	0,55; p>0.05
	ΔΨm	-0,57; p<0.05	0,41; p>0.05
	FITC Annexin V+ neutrophils	-0,66; p<0.05	0,53; p>0.05
fT3	ROS	0,70; p<0.05	-0,20; p>0.05
	ΔΨm	0,60; p<0.05	-0,18; p>0.05
	FITC Annexin V+ neutrophils	0,41; p>0.05	-0,23; p>0.05
fT4	ROS	0,98; p<0.01	0,17; p>0.05
	ΔΨm	0,65; p<0.05	-0,34; p>0.05
	FITC Annexin V+ neutrophils	0,75; p<0.01	-0,17; p>0.05

the percentage of FITC Annexin V- positive neutrophils revealed a direct correlative linkage of medium strength in animals of all experimental groups.

The correlative analysis carried out between the percentage of ROS in neutrophils and serum concentration of TSH, fT3 and fT4 in animals with periodontitis combined with hyperthyroidism showed a strong negative correlative linkage with TSH (r=-0,79; p<0.01), a strong direct correlative linkage with fT4 (r=0,98; p<0.01) and direct correlative linkage of medium strength with fT3 (r=0,70; p<0.05) (table 3). A medium negative correlative linkage between the percentage of neutrophils with low mitochondrial transmembrane potential and TSH concentration in this group of animals has also been found (r=-0,57; p<0.05). Concerning the relationship between the ΔΨm and the thyroid hormones in animals with periodontitis combined with hyperthyroidism, direct correlative linkages of medium strength have been found (fT3 – r=0,60; p<0.05; fT4 – r=0,65; p<0.05). The correlative analysis carried out between the percentage of FITC Annexin V-positive neutrophils and thyroid hormones revealed negative correlative linkage of medium strength (r=-0,66; p<0.05) with TSH concentration and strong direct correlative linkage with fT4 (r=0,75; p<0.01). In animals with periodontitis combined with hypothyroidism, no reliable correlative linkages between the serum concentration of TSH, fT3, fT4 and the percentage of FITC Annexin V-positive neutrophils, ROS and mitochondrial transmembrane potential.

Discussion

Apoptosis, a form of programmed cell death, plays a critical role in the development and homeostasis of tissues. Imbalance between cell death and cell proliferation may cause premature death or uncontrolled cell expansion[22]. Neutrophils are actively involved into the inflammatory reaction development in the periodontium caused by microbial lipopolysaccharides. These cells make up the first line of non-specific antimicrobial protection. They are the first to be mobilized and sent to the place of inflammation or infection, and the elimination of pathogens depends on the increase in their phagocytic activity. Moreover,

endotoxins induced activation of monocyte/macrophagic system and inducible NO synthase is a key enzyme in the macrophage, that is potently induced in response to proinflammatory stimuli[23].

After stimulation of neutrophils, a cascade of oxidative reactions occurs inside them (respiratory burst) and a large number of free radicals are formed, which have a pronounced bactericidal effect. Neutrophilic granules contain a spectrum of substances intended to destroy the bacterial cell wall (lysozyme, lactoferrin) and hydrolytic enzymes: proteases, peptidases, oxidases, deoxyribonucleases and lipases[24]. It has been established that when neutrophils effectively phagocytize bacteria and perform intracellular killing of absorbed microorganisms, their degranulation is a „switch" of the cell's physiological function to cell death. Leukocyte elastase inhibitor upon cleavage by its cognate protease is transformed into leukocyte-DNase II, a protein with a pro-apoptotic activity. The caspase independent apoptotic pathway, in which leukocyte-DNase II is the final effector, interacts with other pro-apoptotic molecules like poly-ADP-ribose polymerase or apoptosis inducing factor[25-26].

It has been recognized that the mitochondria play a key role in cell-death pathways, by activating the mitochondrial permeability transition pore and causing the release of cytochrome C, proapoptotic factors, and the Ca^{2+} overload, that causes a nonselective permeability of the inner membrane[27].

Our results showed the significant increasing of apoptotic neutrophils and neutrophils with decreased mitochondrial transmembrane potential in rats with periodontitis. Parahonsky et al observed 39 patients with periodontitis. Analyzing the main marker of cell apoptosis – cytochrome C – it was found that the level of this protein increases in the initial stages of periodontitis and decreases in the later stages of the disease. Since the main cellular elements of the gingival crevicular fluid are leukocytes, it can be assumed that the detected cytochrome C is of leukocyte origin[28].

The thyroid hormones play an important role in many physiological processes, such as differentiation, growth, development, and the physiology of all cells. Modifications in its levels can produce several alterations, including modifications in the ROS production[27].

The metabolic effects of thyroid hormones are directly linked to ROS production and oxidative stress in various ways[29-30]. First, the general metabolic effect of thyroid hormones is a relative acceleration of the basal metabolism, that includes an increase of the rate of both catabolic and anabolic reactions. This results in increased energy expenditure, fuel mobilization, fuel oxidation for energy extraction, oxygen consumption, respiratory rate, and heat production and release. Although thyroid hormones do not directly determine the respiratory state of the mitochondria[31], stimulation by thyroid hormones promotes state 3 by augmenting ATP breakdown by different energy-consuming mechanisms in the cell, and thus increasing ADP availability. This would be expected to decrease ROS production. However, thyroid hormones also promote a reduction state in the cell, by increasing fuel availability and extramitochondrial production of ATP and NADH, which in turn promote reduction of the components of the mitochondrial respiratory chain and transition to state 4. Thyroid hormones have also been shown to stimulate the synthesis of elements of the respiratory chain, which further enhances the reductive state. Thyroid hormones also promote extramitochondrial ROS production by modifying the expression of genes coding for enzymes involved in ROS production and elimination. Finally, the increase in thyroid hormones levels has been shown to modify the composition of membrane phospholipids increasing the degree of unsaturation particularly in the mitochondrial membranes. Since unsaturation of fatty acids makes them more susceptible to free radical attack, this effect results in augmented lipid peroxidation in mitochondria[30].

There are data indicating that excess thyroid hormones cause apoptosis, due to enhance the expression of several death receptors and their ligands, such as TNF- FasL, proNGF, and proBDNF, resulting in activation of apical caspase-8, which is further amplified through the activation of the p75NRT-mediated pathways[32]. Mihara et al found that T lymphocytes, cultured with T3 and T4 in vitro, showed enhanced apoptosis, evidenced by DNA ladder formation and characteristic morphological changes. In addition, prolonged cultivation with thyroid hormones of the lymphocytes further enhanced the extent of apoptosis. Furthermore, the treatment with thyroid hormones of T lymphocytes induced reduction of mitochondrial transmembrane potential and production of reactive oxygen species, both of which are intimately associated with apoptotic cell death. These findings suggest that thyroid hormones have the potential to induce apoptotic cell death of human lymphocytes in vitro[33].

The authors observed that intracellular oxidation was increased and Bcl-2 (an antiapoptotic protein) was decreased in thyroid hormone-treated lymphocytes. Thus, the authors suggested that enhanced apoptosis of thyroid hormone-treated lymphocytes may be due to the enhanced ROS production and/or the reduction of antioxidant effects by decreasing

Bcl-2 protein expression[31]. Reduction of antiapoptotic Bcl-2 protein expression has also been found in most, but not all, patients with Graves' disease[34].

On the other hand, the results of the Klatka et al study revealed that thyroid hormones exert a negative or even no influence on cellular apoptosis ex vivo, as the treatment of hyperthyroidism in all patients leads to the increment of apoptotic cells[22]. Other authors showed that thyroid hormones inhibit apoptosis of early differentiating cerebellar granule neurons, through an increase in the amounts of Bcl-2 protein[35]. Huang et al also found that thyroid hormones significantly decrease apoptosis of cells, such as hippocampus neurons in rats[36].

There is some evidence that demonstrates the molecular mechanisms by which hypothyroidism itself may produce a protected state of the tissues, such as reducing the enzyme activity associated with the mitochondrial-respiratory chain, the decrease in adenine nucleotide translocase, reduced activity of cytochrome-C oxidase, and the resistance to forming the permeability transition-pore formation of the inner mitochondrial membrane[27].

Conclusions

One of the important signaling pathways of apoptosis triggering, in case of experimental periodontitis combined with thyroid dysfunction, is reactive oxygen species overproduction and disruption of the mitochondrial inner membrane, due to the decreasing of transmembrane potential, which is more pronounced in case of hyperthyroidism.

The conducted correlative analysis showed the presence of significant relationships between free triiodothyronine, free thyroxine and thyroid stimulating hormone and indices of mitochondrial apoptosis pathway in case of periodontitis only in hyperthyroid rats, which requires further investigations.

Compliance with Ethics Requirements:

„The authors declare no conflict of interest regarding this article"

„The authors declare that all the procedures and experiments of this study respect the ethical standards in the Helsinki Declaration of 1975, as revised in 2008(5), as well as the national law. Informed consent was obtained from all the patients included in the study"

„All institutional and national guidelines for the care and use of laboratory animals were followed"

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