

REACTIVE OXYGEN AND NITROGEN SPECIES ROLE IN EXPERIMENTAL PERIODONTITIS DEVELOPMENT

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Introduction. Activation of lipid peroxidation is one of the trigger mechanisms of periodontium injury, which is primary caused by cellular damage. Reactive oxygen and nitrogen species (RONS) are able to cause damage to a cell as well as final products of lipid peroxidation, including unsaturated aldehydes and other metabolites.

Objective. The aim of the research was to determine the role of RONS and accumulation of lipid peroxidation derivatives in initial development and formation of chronic inflammatory process in periodontium.

Methods. Experimental periodontitis was modeled in animals by injection of complex mixtures of microorganisms diluted in egg protein into periodontal tissues. The results of biochemical studies of free radical processes activity in blood serum were evaluated by content of diene, triene conjugates, TBA-active products and total quantity of metabolites of nitric oxide ($\text{NO}_2^- + \text{NO}_3^-$), which were determined on the 7th, 14th and 30th days of the experiment.

Results. Generation of active forms of oxygen is more influential, providing longevity of inflammatory process. This pays attention to typical dynamics of changes in active processes of lipid peroxidation in the development and course of experimental periodontitis. The study of inflammatory process with a bacterial-immune component in the rats' periodontal complex proved accumulation of lipid peroxidation and nitric oxide metabolites in blood serum.

Conclusions. The preservation of increased lipid peroxidation and nitric oxide metabolites in blood serum of the experimental animals with acute periodontitis conduce enhance of alteration and delayed healing that result in its sequel into chronic periodontitis.

KEY WORDS: periodontitis; nitric oxide metabolites; TBA-active products; diene conjugates; triene conjugates.

Introduction

Improvement of the established and creation of new methods of generalized periodontitis treatment is one of the urgent matters of contemporary dentistry [1, 2]. Inflammatory processes in the complex of periodontal tissues are the most common among inflammatory complications of maxillofacial area [3, 4]. It is indisputable that pathogenic infection is crucial in this case, as well as their combinations, in particular Staphylococcus and Streptococcus, which develop in cases of reduced resistance of oral tissues to infectious agents. [5, 6]. The etiology and pathogenesis of periodontal diseases are complicated and studied insufficiently, however, the infectious factor and ability of immune protective mechanisms (local cellular unspecific

and general adaptive) are essential; the features of pathological process development, its subsequent treatment and prophylaxis affects depend on them [7]. It is unclear as well as the mechanisms, due to which different by nature and character of action local and general factors lead to inflammatory and destructive lesions of periodontal tissues [8]. The investigation of inflammatory process mechanisms in the tissues of periodontal complex is one of the current issues of contemporary dentistry due to a relatively wide spread and unfavorable prognosis, because the frequency of periodontal disease in world is within 5–20 % and increases with age up to 75 % [9, 10].

The development of inflammatory-destructive changes in periodontal tissues is caused by disturbances of microcirculation and transcapillary exchange with underlying severe hypoxia. Activation of reactive oxygen and nitrogen species (RONS) and exhaustion of antioxidant defense in biological tissues is the most serious of all effects and outcomes of

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hypoxia. Activation of lipid peroxidation (LPO) is a trigger mechanism for oxidative stress with cellular metabolism disorders, which are primarily caused by damage of cellular and subcellular membranes [11].

Activation of LPO and decrease of antioxidant protection contribute to accumulation of deleterious free cholesterol, lysophosphatides, phosphatidylcholine, that changes the dynamic stability of cellular membranes due to pathological process development in periodontal complex [13].

All these facts about the influence of oxidative stress on the pathogenesis of periodontitis are present in the activity of lipid peroxidation as potential predictors of escalation of inflammatory lesions in periodontal disease. The disturbance of antioxidant protection in the patients with hypertension, which was proved by changes in the activity of catalase, ceruloplasmin and saturation of transferrin by iron, and the increase in the level of diene conjugates and TBA-active products in serum, which leads to the development of endogenous intoxication syndrome in the patients with general periodontitis. One of the parameters that allow estimating the state of free radical processes is the content of lipids hydroperoxides and TBA-active products formed by oxidation of unsaturated fatty acids, and aldehyde and ketone derivatives, which are developed by the action of active radicals on the amino acid residues in protein molecules [14].

The components of bacterial toxins (especially lipopolysaccharide) and proinflammatory cytokines (mainly TNF- α , IL-1 and interferon gamma-IFN- γ) produced by the affected tissues stimulate the production of nitric oxide (NO) by the inducible nitric oxide synthase (iNOS) in different cell types [15]. It is proved that parodontopathogenic bacteria are capable of inducing NO formation by inducible NO synthase. Excessive NO formation, which occurs when iNOS is stimulated by proinflammatory cytokines and endotoxins of pathogenic microflora of oral cavity, leads to nitrooxidative stress which, together with the activation of lipoperoxidation and oxidative modification of proteins, can cause increased disintegration of connective tissue components and progressing of periodontitis [16].

The aim of this investigation was to determine the pathogenic influence of RONS and accumulation of lipid peroxidation derivatives in regard to initial development and formation

of chronic inflammatory process in periodontal complex.

Methods

The experiments were carried out using white clinical healthy male rats, 150–200 g in weight, in environments of vivarium, on a standard diet balanced for the basic elements. The research related to animals' use has been complied with all the relevant national regulations and institutional policies for the care and use of animals. The investigations were conducted following the general rules and regulations of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986), the General Ethics on Animal Experimentation (Kyiv, 2001).

The experimental animals were randomly selected and divided into 4 groups: the 1st – intact animal, controls (n=10); the 2nd – animals with experimental periodontitis on the 7th day of study (n=8); the 3rd – animals with experimental periodontitis on the 14th day of study (n=8); the 4th – animals with experimental periodontitis on the 30th day of study (n=8). Experimental periodontitis (EP) was caused by introduction of complex mixtures of microorganisms diluted in egg protein into periodontal tissues [17]. Simultaneously with the injections of the pathogen a complete Freund's adjuvant was injected in the rat paw to enhance the immune response. When conducting studies with animals of group 4, on the 14th day, repeated administration of the pathogen and injection of adjuvant was carried out. At the 7th and 14th days the experimental animals were euthanized by total heart bloodletting and previous thiopental anesthesia. Serum samples were taken for further research.

In blood serum the level of diene (DC) and triene conjugates (TC), TBA-active products and total quantity of metabolites of nitrogen (II) oxide were determined. The concentration of diene conjugates (DC) and triene conjugates (TC) was evaluated by the method based on the fact that the extracted heptane-isopropyl hydroperoxide mixture had an appropriate absorption maximum: DC at a wavelength of 232 nm; TK at a wavelength of 275 nm [18]. The total nitric oxide metabolites in blood plasma: nitrite anion (NO₂⁻) and nitrate anion (NO₃⁻), were determined by photometry using a Gray reagent (sulfanilamide solution and N-naphthyl ethylenediamine dihydrochloride in 30 % glacial acetic acid), which was used as a color reagent

giving raspberry coloring in the presence of nitrogen oxide metabolites in a liquid [19]. The method of determining the concentration of TBA-active products consisted in the ability of malonic dialdehyde to interact with tiobarbituric acid in an acidic medium to form a colored complex which intensity is adequate to the content of TBA-active products [20]. The results were statistically analyzed by means of nonparametric indexes [21]. The data were presented in the arithmetic mean (M) ± standard deviation of the mean value (m) for a specific number of the animals (n). Changes were considered statistically significant at p<0.05. Excel 2010 (Microsoft Corporation) and Statistica 10.0 (StatSoft, USA) software were used.

Results

These studies were performed in accordance with the suggested and patented patterns for experimental periodontitis [22], which presented the influence of bacterial and immune disorders on the mechanisms of inflammation development in periodontal complex. The study of experimental periodontitis is associated with the fact that this type and values of bacterial-immune inflammation has not investigated before.

The results of the research proved that in the early period of inflammation development in periodontal complex, which included the period from the 1st to the 7th day of the experiment, there was an excessive accumulation of lipid peroxidation products in serum, as evidenced by increased concentration of DC (in 2.20 times, p<0.01) and TC (in 1.93 times, p<0.01)

respectively, compared with the control group of experimental animals (Table 1, Fig. 1). On the 14th day of experimental periodontitis model, there was a significant decrease of DC (in 1.53 times, p<0.01) and TC (in 1.52 times; p<0.01) in serum compared to the group of animals studied on the 7th day of the experiment, but these indices were higher than those of the intact animal group (in 1.44 times, p<0.01 and by 1.26 times, p<0.01, respectively).

In the further observation, on the 30th day of inflammatory process development in the tissues of periodontal complex, the content of DC in blood serum slightly increased in comparison with the indices on the 7th day, but the data were statistically insignificant (p>0.05), but on the 14th day this index increased in 1.53 times (p<0.01). When comparing it with the indices of the control group, it was found out that the content of this metabolite in serum was significantly higher (in 2.21 times, p<0.01).

The content of triene conjugates changed the same during the period of monitoring, however, the increase in their concentration in blood serum was less significant – in 1.53 times (p<0.01), compared with the indices on the 14th day, and in 1.94 times (p<0.01), compared with the control group. When comparing them with the results of the group of animals with experimental periodontitis on the 7th day of the experiment, the changes were found to be statistically insignificant (p>0.05).

When determining the ratio of DK/TC content (Table 2) in blood serum, it was proved that that index significantly increased on the 7th day of the study (in 1.15 times; p<0.01)

Table 1. Concentration of diene and triene conjugates in serum of the rats in different periods of experimental periodontitis (M±m)

Form of experiment	Duration of experiment (days)	Number of animals	DC, conditioned, units/ml	TC, conditioned, units/ml
Control, intact animals	-	10	2.383±0.071	2.756±0.022
Animals with periodontitis	7	8	5.250±0.242 p1<0,01	5.310±0.187 p1<0,01
	14	8	3.431±0.089 p1<0.01, p2<0.01	3.485±0.107 p1<0.01, p2<0.01
	30	8	5.266±0.141 p1<0.01, p2>0.05, p3<0.01	5.338±0.140 p1<0.01, p2>0.05, p3<0.01

Notes: p1 – statistical significance of differences relative to the intact animals;
p2 – statistical significance of differences relative to the animals with experimental periodontitis on the 7th day of the research;
p3 – statistical significance of differences relative to the animals with experimental periodontitis on the 14th day of the research.

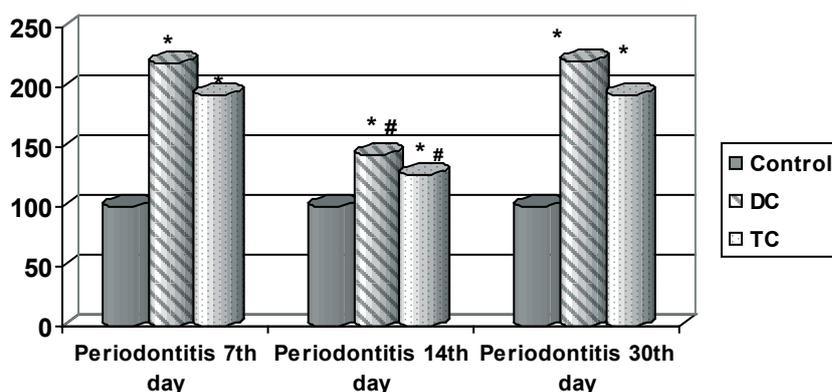


Fig. 1. Changes in the indices of lipid peroxidation in rats' serum in the experimental periodontitis follow-up (% of the control).

Notes: * - statistically significant differences relative to the intact animals ($p < 0.01$);

- statistically significant differences relative to the animals with periodontitis on the 7th day of the experiment ($p < 0.01$);

• - statistically significant differences relative to the animals with periodontitis on the 7th day of the experiment ($p > 0.05$);

° - statistically significant differences relative to the animals with periodontitis on the 14th day of the experiment ($p < 0.01$).

compared to the control group and remained on the same level throughout the duration of the experiment: it was higher on the 14th (in 1.16 times, $p < 0.01$) and on the 30th day (in 1.15 times, $p < 0.01$) of the indices of the intact animals. When comparing the same ratios in the rats at different periods of the experiment, in particular on the 7th, 14th, 30th days, the differences were statistically insignificant ($p < 0.05$).

As a result of the study of the main indices of lipid peroxidation - the content of TBA-active products, significant changes were also evidenced (Table 3). In particular, it was found out that on the 7th day of experimental periodontitis development in the rats, this serum level was higher in 4.22 times ($p < 0.01$) compared to the control group.

On the 14th day of the experimental periodontitis model, a gradual decrease in the level of TBA-active products (in 1.34 times, $p < 0.01$) was evidenced in blood serum in comparison with the group of animals with

inflammatory process in periodontal tissues on the 7th day of the experiment, but these indices were still increased compared to the intact group of animals (in 3.16 times, $p < 0.01$), that proved a significant activation of free radical lipid oxidation processes during the entire period of inflammation development. The studies on the 30th day of the experiment proved that the content of TBA-active products in serum gradually decreased (in 1.49 times, $p < 0.01$ and in 1.11 times, $p < 0.01$) respectively, compared to the groups of animals with experimental periodontitis on the 7th and 14th days of the experiment. At the same time, it was higher (in 2.84 times, $p < 0.01$) than in the intact group of white rats (Fig. 2).

At the early stage of experimental periodontitis development, that is on the 7th day, there was a significant increase in the content of nitric oxide metabolites ($\text{NO}_2^- + \text{NO}_3^-$), which were classified as unstable products of free radical oxidation in serum (in 6.86 times, $p < 0.01$), but on the 14th day this index changed

Table 2. Correlation of diene and triene conjugates in serum of the rats in different periods of experimental periodontitis development (M \pm m)

Form of experiment	Control, intact animals	Animals with periodontitis		
Duration of experiment (days)	-	7	14	30
Number of animals	10	8	8	8
DC / TC	0.86 \pm 0.03	0.99 \pm 0.02 $p_1 < 0.01$	1.00 \pm 0.04 $p_1 < 0.01$, $p_2 > 0.05$	0.99 \pm 0.01 $p_1 < 0.01$, $p_2 > 0.05$, $p_3 > 0.05$

Notes: p_1 - statistically significant differences relative to the intact animals;

p_2 - statistically significant differences relative to the animals with experimental periodontitis on the 7th day of the research;

p_3 - statistically significant differences relative to the animals with experimental periodontitis on the 14th day of the research.

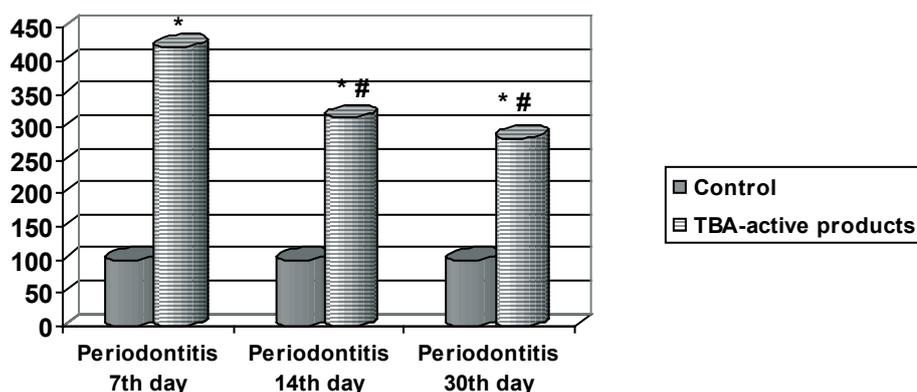


Fig. 2. Changes in the indices of TBA-active products in rats' serum in the experimental periodontitis follow-up (% of the control).

Notes: * – statistically significant differences relative to the intact animals ($p < 0.01$);

– statistically significant differences relative to the animals with periodontitis on the 7th day of the experiment ($p < 0.01$);

° – statistically significant differences relative to the animals with periodontitis on the 14th day of the experiment ($p < 0.01$).

reversely, that is it decreased (in 1.31 times, $p < 0.01$) compared with the animals on the 7th day of the experiment, but was increased compared to the intact group of animals (in 5.25 times, $p < 0.01$) (Table 3, Fig. 3).

Characterizing the changes in the content of products of metabolism of nitric oxide in blood serum of the experimental animals with periodontitis, it should be noted that this active form of oxygen on the 30th day of the experiment along with the lipoperoxidation indices of previous studies also significantly increased (in 3.64 times, $p < 0.01$) compared to the results of the animals of control group. However, the data were lower than those in rats on the 7th (in 1.88 times, $p < 0.01$) and 14th days (in 1.44 times, $p < 0.01$) respectively.

Discussion

Introduction of complex mixtures of microorganisms diluted in egg protein into periodontal tissues caused hyperergic inflammatory process with significant changes in soft tissue of lower jaw accompanied by edema and hyperemia of mucous membrane and the manifestations were the same as the changes in humans [23]. Inflammatory process in periodontal tissues was accompanied by cellular infiltration of surrounding tissues and destructive changes in periodontal complex [24, 25].

The obtained data proved that generation of active forms of oxygen at a sufficiently high level, activation of free radical lipid oxidation were present during the entire period of

Table 3. The content of TBA-active products and metabolites of nitrogen (II) oxide (NO₂-+NO₃-) in serum of the rats in different periods of experimental periodontitis development (M±m)

Form of experiment	Duration of experiment (days)	Number of animal	TBA-active products, mcmol/l	NO ₂ -+NO ₃ -, mcmol/l
Control, intact animals	-	10	2.555±0.092	0.028±0.001
Animals with periodontitis	7	8	10.774±0.122 p1<0.01	0.192±0.006 p1<0.01
	14	8	8.066±0.143 p1<0.01, p2<0.01	0.147±0.003 p1<0.01, p2<0.01
	30	8	7.255±0.103 p1<0.01, p2<0.01, p3<0.01	0.102±0.002 p1<0.01, p2<0.01, p3<0.01

Notes: p1 – statistically significant differences relative to the intact animals;

p2 – statistically significant differences relative to the animals with experimental periodontitis on the 7th day of the research;

p3 – statistically significant differences relative to the animals with experimental periodontitis on the 14th day of the research.

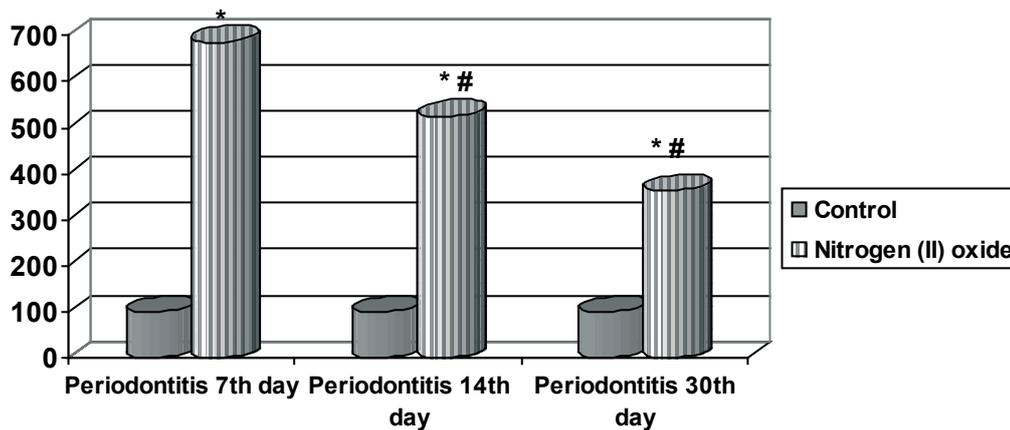


Fig. 3. Changes in the indices of metabolites of nitrogen (II) oxide in rats' serum in the experimental periodontitis follow-up (% of the control).

Notes: * - statistically significant differences relative to the intact animals ($p < 0.01$);

- statistically significant differences relative to the animals with periodontitis on the 7th day of the experiment ($p < 0.01$);

° - statistically significant differences relative to the animals with periodontitis on the 14th day of the experiment ($p < 0.01$).

inflammatory reaction development, but the highest degree was during the peak of the inflammatory process that corresponded to a more severe clinical picture in this group of animals. In a later period of periodontitis, despite a slight decrease in the intensity of LPO, a complete reduction of the inflamed process in periodontal tissues did not take place, which may point to its chronicity.

The indices of lipid peroxidation activity: the content of TBA-active products in serum proved that irrespective of the period of their study, during the development of bacterial-immune experimental periodontitis, the formation and accumulation of intermediate toxic products of lipid peroxidation in serum took place at different stages of its chain branching. Also, the inflammatory reaction in periodontal complex in the acute period of development became a source of formation of reactive oxygen species, which were capable of triggering a cascade of free radical processes involving NO-radical metabolites. Active form of oxygen on the 30th day of the experiment proved the continuation of NO generation, the enhancement of free radical

processes activity and the disturbance of dynamic equilibrium with the antioxidant defense system.

Conclusions

The inflammatory process with bacterial-immune component in periodontal complex is accompanied by increase of lipid peroxidation and nitric oxide metabolites in the blood serum that affects the course and completion of the inflammatory process.

A significant increase of diene and triene conjugates level and TBA-active products in blood serum in the acute period (on the 7th day of the experiment) and a temporary decrease on the 14th day, as well as further increase on the 30th day of the experiment evidence the increased generation of reactive oxygen species and their derivatives for the entire period of inflammation development.

The preservation of increased lipid peroxidation and nitric oxide metabolites in blood serum of the experimental animals with acute periodontitis conduce to enhance of alteration and delayed healing that result in its sequel into chronic periodontitis.

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