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Pecularities of Generation of Superoxide Anion of Peripheral Blood Leukocytes in Periodontitis

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SUMMARY

The aim of the present study was to explore oxidative function of periodontitis patients and healthy subjects blood neutrophils stimulated with opsonized zymosan, nonopsonized *E.coli* and *E.coli* LPS by method of lucigenin-dependent chemiluminescence.

The leukocytes for present investigation were obtained from peripheral venous blood of 16 periodontitis patients and 10 healthy subjects. Superoxide anions production was measured by a lucigenin-enhanced chemiluminescence assay.

Lucigenin-dependent chemiluminescence stimulated with opsonized zymosan, nonopsonized *E.coli* and *E.coli* LPS neutrophils of peripheral blood (1361±169, 2422±337 and 4073±893 cpm respectively) was significantly higher than analogous chemiluminescence of control subjects (492±56, 651±103 ir 381±67 cpm respectively).

The lucigenin-dependent chemiluminescence of nonstimulated PMN of periodontitis patients (390±65 cpm) was significantly higher (p £ 0,001) than analogous of control subjects (137±13 cpm).

The results indicate, that oxidative function of neutrophils of periodontitis patients was increased.

Keywords: periodontitis, neutrophils, chemiluminescence, lucigenin.

Inflammatory diseases of periodontal tissues mostly cause damage to the hard and soft tissues, which surround and support the tooth. Structural complexity of the components of periodontal tissues, including gingival bacteria and the products of their metabolism, as well as a variety of response factors of the macroorganism hinder the disclosure of etiopathogenetic mechanism of inflammatory diseases of the periodontal tissues [1].

In recent years some factors that have an influence on the development of gingivitis and periodontitis have been revealed [2]. On summing up investigations of recent years, R.C.Williams [3] has presented an up-to-data model of inflammatory diseases of periodontal tissues placing particular emphasis on the immune inflammatory response of the macroorganism to the invading microbes of dental plague.

Neutrophil leukocytes (NL) perform the protective role of periodontal tissues as they have a variety of substances necessary for destruction of pathogenic microorganisms. When the number of NL decreases or their functional efficiency gets disturbed, diseases of periodontal tissues set in [4]. Neutrophil leukocytes perform the protective role of macroorganism through different consecutive stages. Its final stage is phagocytosis of microorganisms, destructing them in the phagolysosome [5]. Along with phagocytosis, microbes, virus-infected cells, and cancer cells can be destroyed by NL, releasing their lysosomal granular content into the extracellular environment [6]. It has been established that NLs on leaving healthy periodontal tissues spontaneously release certain amount of active forms of oxygen [7].

Active forms of oxygen produced in the respiratory burst of NL (superoxide anion (O_2–), hydrogen peroxide (H_2O_2), hydroxy-radical (·OH), and singlet oxygen ($^1O_2$) [8, 9] make up an effective bactericidal NL system. Intensity of the respiratory burst depends on the nature of NL stimulator and concentration [10]. Active forms of oxygen, being potent oxidants, not only cause destruction of bacteria [11], but they can also damage the tissues of the macroorganism [12].

In recent years, most of scientists [13, 14], who investigate various diseases and oxidative metabolism of leukocytes, focus attention on superoxide anion generated by NL. Then, by Haber-Weiss reaction:

$$H_2O_2 + O_2– \rightarrow OH– + ·OH$$

or as a result of Fenton reaction:

$$H_2O_2 + Fe^{2}– \rightarrow Fe^{3}– + OH– + ·OH$$

hydroxyl-radical or some other similar toxic forms of oxygen can be produced [15].

Superoxide anion determination is mostly done by the method of lucigenin-dependent chemiluminescence (CL) [11, 13].

The aim of the present study was to investigate the generation of superoxide anion in NL of peripheral venous blood of patients with periodontitis and of healthy subject by the method of lucigenin-dependent chemiluminescence. Stimulating leukocytes with opsonized zymosan, nonopsonized E.coli, and E.coli 055:BB endotoxin (LPS).

MATERIALS AND METHODS

In performing investigations of superoxide anion generation, neutrophil leukocytes (NL) were obtained from peripheral venous blood of 16 patients suffering from periodontitis and that of 10 healthy subjects of the control group. The age of patients under study ranged from 18 to 50 years.

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The difference in mean age and sex of patients and of those in the control group was not significant (p>0,05).

Superoxide anion generation was investigated by lucigenin-dependent chemiluminescence (CL) method as proposed by L.G.Korkina et al. [12]. Chemiluminescence measurements were performed at the Department of Biochemistry of Kaunas University of Medicine using a liquid scintillation counter "Delta-300".

Lucigenin, zymosan, lypopolysacharides (LPS), as well as Hanks balanced salts solution (pH=7,3) were obtained from Sigma Chemical Co. (USA), and *E.coli* sample ATCC 25922 was grown at the Microbiology Laboratory of Kaunas Medicine University Clinics. Specimens of *E.coli* culture were used for investigations in the course of 24 hours. Zymosan was opsonized according to the method of R.Zeiger et al [16].

Preparation of leukocyte specimens

For assessment of superoxide anions, leukocytes were obtained from peripheral venous blood of patients with periodontal diseases and from that of healthy control subjects. Ten milliliters of venous blood were taken in the morning before meals. Blood clotting was controlled with heparin (20 units/ml). Plastic test tubes containing blood were positioned at an angle of 45 degrees and were kept for 1 hour at 37ţC. Then the supernatant layer of plasma rich in leukocytes was aspirated and diluted with Hanks balanced salt solution (HBSS) up to 5 ml. Then, this cell suspension in portions of 1 ml was taken into cuvettes used for chemiluminescence investigation, putting aside only 1 ml of the suspension for counting up the number of leukocytes and the percentage of their composition.

Investigation of NL superoxide anion generation

Cuvettes containing leukocyte suspension were placed in a thermostat with water, gradually adding 0,1 ml of lucigenin (final lucigenin concentration 50 μM), and taking measurements of non-stimulated NL chemiluminescence level. After 5 minutes, 0,1 ml of opsonized zymosan (its final concentration 2,5 mg/ml) was added into the cuvettes, then 0,1 ml of non-opsonized E.coli suspension ($1X10^9$ cells/ml) or 0,1 ml of LPS solution (its final concentration being 0,2 mg/ml).

Leukocytes make up the main part of total amount of blood or of leukocyte suspension chemiluminescence. Therefore, the intensity of chemiluminescence of stimulated or non-stimulated NL directly depends on the amount of NL in the medium, because CL intensity, as triggered by NL, can be calculated from the total leukocyte chemiluminescence fraction [12], using the equation:

$$I_{(NL)}=I_{(leuk)}X100V/vcn,$$

where $I_{(NL)}$ is $1X10^9$ NL chemiluminescence (imp/min), $I_{(leuk)}$ is chemiluminescence of leukocyte suspension, v is the amount of suspension (ml), c is the amount of leukocytes, n is NL percentage, and V is the volume of the cuvette (ml) used for photometric analysis.

INVESTIGATION DATA

Data on lucigenin-dependent CL (the latter reflecting the level of generated superoxide anion) of NL of peripheral venous blood taken from patients with periodontitis and from subjects with intact periodontium tissues are presented in Table 1. The data shows that lucigenin-dependent CL of non-stimulated $1X10^9$ NL of peripheral venous blood taken from patients with periodontitis, reaches 387±65 cpm, and it has been found to be statistically reliably (p≤0,001) higher than that of analogous cases in the control group (137±14 cpm).

It is also evident that stimulation of NL of blood of patients with periodontitis and of subjects with intact periodontium by means of opsonized zymosan, nonopsonized *E.coli*, or LPS, in the main intensifies lucigenin-dependent CL. Acted by opsonized zymosan, $1X10^9$ NL of peripheral venous blood of patients with periodontitis, lucigenin-dependent CL reaches 1361±169 cpm, and it is virtually (p≤0,001) more distinct than that of analogous tissues of healthy control subjects (492±56 cpm).

Nonopsonized *E.coli* intensified much more lucigenin-dependent CL of NL of peripheral venous blood taken from periodontal patients and from control healthy subjects. However, in the group of patients with periodontitis, it was in the main (p≤0,001) higher (2422±337 cpm) than in the analogous control group (651±103 cpm).

It is of interest to note that the above-mentioned difference was particularly distinct due to the effect of *E.coli* LPS. If lucigenin-dependent CL of NL $1X10^9$ of peripheral venous blood of patients suffering from periodontitis reaches its maximum 4073±893 imp/min, the analogous CL of healthy control subjects is only 381±67 imp/min. It is necessary to note that the NL reaction of peripheral venous blood of subjects in the control group to *E.coli* LPS was much weaker than the reaction to non-opsonized *E.coli* (p≤0,001).

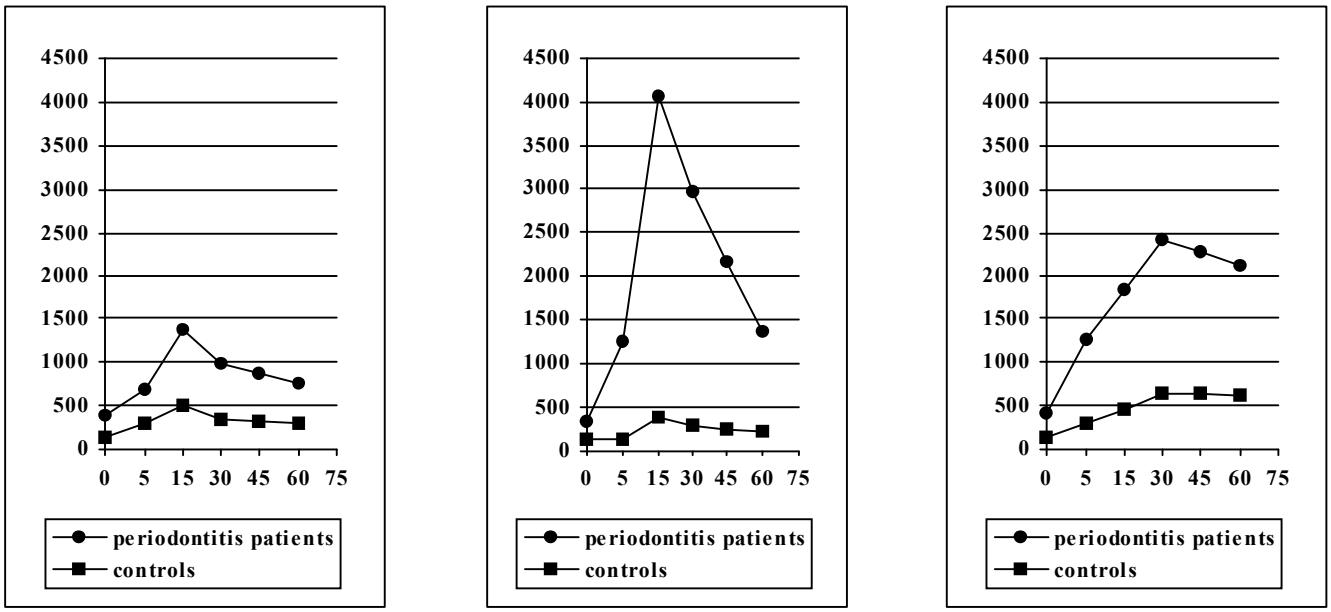

Figure 1. *Dependence of lucigenin-enhanced chemiluminescence upon the state of periodontal tissues and various bacterial stimuli.*

Table 1. *Lucigenin-dependent chemiluminescence of peripheral venous blood NL of investigation groups patients.*

Investigation groups	n	1×10^9 NL chemiluminescence (cpm)			
		Non-stimulated	After stimulation		
			opsonized zymosan	non-opsonized *E.coli*	LPS
Periodontitis patients	16	387±65	1361±169	2422±337	4073±893
Controls	10	137±14	492±56	651±103	381±67
p		≤0,001	≤0,001	≤0,001	≤0,001

Lucigenin-dependent CL changes in NL of peripheral venous blood of both groups of patients under study due to opsonized zymosan, nonopsonized *E.coli*, and *E.coli* LPS are presented in Fig 1. It shows that due to the effect of opsonized zymosan and *E.coli* LPS, the above-mentioned CL reaches its maximum after 15 minutes, while under the effect of non-opsonized *E.coli*; it takes about 30 min to reach its maximum after stimulation.

DISCUSSION

Periodontal disease is an inflammatory disorder caused by bacteria of dental plaque [17]. Recent investigations have shown that some dental plaque bacteria can penetrate deep into the tissues [18], their toxins penetrating biological membranes [19]. NL cells have been found to be the very first to migrate into tissues as a response of the macroorganism to the invaded microorganisms [20]. As NL cells migrate to the infected site, their activity undergoes some changes manifested in biological effects, including elevated production of active forms of oxygen. An increase in the activity of NL is an important non-specific immunological factor of the organism surveillance, hindering the penetration of microbes. At the same time this factor can cause damage to the tissues of the organism [21]. This is particularly characteristic of the action of NL superoxide anion released during the respiratory burst into the surrounding [22].

When drawing a comparison between the effect of lucigenin-dependent CL, which is induced by opsonized zymosan, non-opsonized *E.coli*, and LPS (see Table 1 and Fig.1) of NL of peripheral venous blood taken from patients with periodontitis with that of PVB of control subjects, we have established a marked difference between these groups: lucigenin-dependent CL of NL of peripheral venous blood of patients with periodontitis was 3 to 10 times higher than that of analogous subjects of the control group.

We have found no data on investigations of lucigenin-dependent CL of NL of peripheral venous blood (PVB) taken from patients suffering from periodontitis. It is known that [23] lucigenin-dependent CL of NL indicates only to the fact that superoxide anion is produced by NL cells and on undergoing a series of intermediate reactions, superoxide anion can attain various toxic forms of oxygen.

It is supposed [5] that active forms of oxygen produced *in vivo* can inactivate antiproteinases present in biological fluids, thus increasing the activity of proteases. In recent years, lots of research works [24] have been published, placing emphasis on the importance of proteases in causing inflammatory diseases of the periodontium. Besides, active oxygen forms can activate NL-produced metal proteinases. This makes it possible to presume that such active forms of oxygen produced by NL are particularly important factors causing tissue damage [25].

It is expected that performing investigations in this research area will make it possible to better understand the causative factors of diseases of the periodontal tissues, and to work out effective methods of prevention and treatment of such diseases.

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