

## Generation of superoxide anion by peripheral blood leukocytes in periodontitis patients with type 1 diabetes mellitus

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**Key words:** diabetes mellitus, periodontitis, neutrophils, chemiluminescence.

**Summary.** *The involvement of reactive oxygen species in periodontal diseases is unclear. The aim of present study was to explore oxidative function of neutrophil leukocytes of patients with severe periodontitis who have type 1 diabetes mellitus, and control subjects with healthy periodontal tissues and without systemic diseases.*

**Materials and methods.** *The leukocytes for present investigation were obtained from peripheral venous blood of 38 patients with severe periodontitis who have type 1 diabetes mellitus and 27 control subjects. The maximal lucigenin-dependent chemiluminescence and peak time values of neutrophils stimulated with non-opsonized Escherichia coli ATCC 25922 and Staphylococcus aureus 256 were measured.*

**Results.** *The maximal lucigenin-dependent chemiluminescence of neutrophils of patients with severe periodontitis stimulated with non-opsonized E. coli and S. aureus was much higher than that in control subjects ( $p < 0.001$ ).*

*In both affected and healthy patients, chemiluminescence of neutrophil leukocytes reached its peak value at similar time. The maximum value of chemiluminescence of leukocytes stimulated with non-opsonized E. coli in both studied groups was reached statistically significantly earlier than when stimulating with non-opsonized S. aureus bacteria ( $p < 0.001$ ).*

**Conclusion.** *In periodontitis, local non-opsonized bacteria might stimulate neutrophil leukocytes to release reactive oxygen species, which can cause inflammation and destruction of periodontal tissue.*

### Introduction

It has been recognized for many years that some systemic diseases (especially diabetes mellitus) may influence periodontal health. The relationship between diabetes mellitus (DM) and periodontal disease has initiated much research and debate over the past decade, and there are several relatively recent reviews available (1–4).

Diabetes mellitus is a chronic metabolic disorder that affects more than 100 million people worldwide (5). The incidence of periodontitis increases among diabetic subjects after puberty and as the patients' population ages. Periodontal disease may be more frequent and severe in diabetic individuals with more advanced systemic complications. There is a relationship between periodontal disease and DM especially in patients with poorly controlled disease or hyperglycemia (6).

According to the majority of authors (7–10), metabolic disturbances in periodontal tissues may lower the resistance of diabetics to infections and thus in-

fluence the initiation, development and progression of inflammatory periodontal disease.

There is an increasing body of evidence now available that implicates reactive oxygen species (ROS) in the pathogenesis of a variety of diseases, in addition to providing an important function in normal metabolic reactions (11). Periodontal disease is no exception to the potential influence of ROS since many forms of the disease result in the destruction of the extracellular matrix of the supporting connective tissues in response to a variety of agents of both host and bacterial origin (12, 13).

In recent years, most scientists (14, 15) who investigate various diseases and oxidative metabolism of leukocytes focus attention on superoxide anion generated by neutrophil leukocytes (NL).

The aim of the present study was to investigate to generation of superoxide anion by leukocytes of peripheral venous blood (PVB) of patients with aggressive periodontitis who have type 1 DM, and control

subjects with healthy periodontal tissue by the method of lucigenin-dependent chemiluminescence (CL); for leukocytes stimulation, non-opsonized *E. coli* and *S. aureus* were used.

### Materials and methods

In performing investigations of superoxide anion generation, NL were obtained from peripheral venous blood of 38 type 1 DM patients suffering from severe periodontitis and 27 control subjects with healthy periodontal tissues. Severity of periodontitis in patients was determined using A. L. Russell's (16) periodontal index (PI). The age of the studied patients ranged from 18 to 50 years.

Superoxide anion generation was investigated by lucigenin-dependent CL method as proposed by L. G. Korkina *et al.* (17). Chemiluminescence measurements were performed at the Department of Biochemistry of Kaunas University of Medicine using a liquid scintillation counter "Delta-300".

Lucigenin and Hank's balanced salt solution were obtained from Sigma Chemical Co (USA). Plastic vials and other disposable pieces of plasticware were obtained from Carl Roth GmbH and Co KG.

*E. coli* sample ATCC25922 and *S. aureus* 256 were grown at the Laboratory of Microbiology of Kaunas University of Medicine Hospital. Specimens of *E. coli* and *S. aureus* were used for investigations in the course at 24 hours.

*Preparation of leukocyte specimen.* For assessment of superoxide anions, leukocytes were obtained from peripheral venous blood of patients with periodontitis who have type I DM and 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 37°C. Then the supernatant layer of plasma rich in leukocytes was aspirated and diluted with Hank's balanced salt solution (pH 7.2) up to 9 ml. Then, this cell suspension in portions of 2 ml was taken into cuvettes used for chemiluminescence investigation, putting on one side only 1 ml of the suspen-

sion 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 0.05 ml of lucigenin (final lucigenin concentration 50 µM) and taking measurements of non-stimulated NL chemiluminescence level. After 5 minutes, 0.01 ml of non-opsonized *E. coli* or *S. aureus* suspension (final concentration  $6 \times 10^6$  cells/ml) was added in cuvettes.

Leukocytes make up the main part of the total amount of blood or leukocyte suspension chemiluminescence. Therefore, the intensity of chemiluminescence of stimulated and 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 (17), using the equation:

$$I_{(NL)} = I_{(leuk)} \times 100 \frac{V}{vcn}$$

Where  $I_{(NL)}$  is  $1 \times 10^6$  NL chemiluminescence (cpm),  $I_{(leuk)}$  – CL of leukocyte suspension,  $v$  – the amount of suspension (ml),  $c$  – the amount of leukocytes,  $n$  – NL percentage and  $V$  – the volume in the cuvette (ml) used for photometric analysis.

The CL response was expressed as mean  $\pm$ SD peak values of relative light units/min (cpm) per  $1 \times 10^6$  neutrophils.

CL was recorded after 15, 30, 45, 60, 75, 90 and 120 minutes. Two CL parameters were calculated from the measurements:

1. Peak maximum cpm  $1 \times 10^6$  neutrophils
2. Peak time values (time in minutes after starting the liquid scintillation counter required to reach the peak maximum).

*Statistical analysis.* The statistical significances of differences between groups were analyzed by Student's *t*-test. A value of  $p < 0.05$  was considered significant.

### Results

*Clinical data.* The differences in mean age between the groups (Table 1) were not significant ( $p > 0.05$ ).

**Table 1. Investigation data of the subjects and clinical evaluation**

Investigation groups	n	Sex		Age (years)	Russell's PI (points)
		female	male		
Periodontitis patients with type 1 diabetes mellitus	38	21	17	36.9 $\pm$ 2.8	5.63 $\pm$ 0.41
Controls	27	15	12	29.2 $\pm$ 3.2	0

The Russell's PI in the control group subjects equaled to 0, and in the periodontitis patients with type 1 DM it was very high.

*Laboratory data.* The result of the maximal lucigenin dependent CL (the latter reflecting the level of generated superoxide anion) of NL of PVB taken from patients with severe periodontitis who have type 1 DM, and control subjects with healthy periodontal tissue, are presented in Table 2.

The data show that stimulation of NL of PVB in both studied groups by non-opsonized *E. coli* and *S. aureus* intensified lucigenin-dependent CL. But the lucigenin-dependent CL of NL of PVB taken from patients with severe periodontitis who have type 1 DM exceeded analogous CL of patients in the control group ( $p < 0.001$  and  $p < 0.002$ , respectively).

It is noteworthy that under the influence of *S. aureus*, NL in the peripheral group of the studied groups generated less superoxide anion compared to its generation under the influence of non-opsonized *E. coli*. The difference was statistically reliable ( $p < 0.001$ ).

Peak times CL from peripheral venous blood NL of investigated groups is shown in Table 3. The presented data show that the shortest time during which the peak CL point was reached in the studied groups was in case of NL under the influence of non-opsonized *E. coli*. This was statistically significantly different ( $p < 0.05$ ) from the period of time during which the peak CL point was reached under the influence of non-opsonized *S. aureus*.

**Discussion**

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 reactive 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 some 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 non-opsonized *E. coli* and *S. aureus* (see Table 2) on NL of PVB taken from patients with periodontitis who have type 1 DM and that of control subjects, we have established a marked difference between these groups: lucigenin-dependent CL of NL of PVB of patients with periodontitis who have type 1 DM was higher than that in the analogous subjects of the control group. We have found no data on investigations of lucigenin-dependent CL of NL of PVB taken from patients suffering from periodontitis who have type 1 DM.

Our findings show that under the influence of *S.*

**Table 2. Maximal lucigenin-dependent chemiluminescence of peripheral venous blood neutrophil leukocytes (NL) of investigated groups of patients**

Investigation groups	n	1×10 <sup>6</sup> NL chemiluminescence (cpm) after stimulation	
		non-opsonized <i>E. coli</i>	non-opsonized <i>S. aureus</i>
Periodontitis patients with type 1 diabetes mellitus	38	95371±8130	36961±5805
Controls	27	995±124	449±97
p		<0.001	<0.002

**Table 3. Peak time values in minutes of lucigenin-dependent chemiluminescence**

Investigation groups	n	<i>E. coli</i>	<i>S. aureus</i>	p
Periodontitis patients with type 1 diabetes mellitus	38	30.0±2.1	45.0±2.5	<0.001
Controls	27	30.0±2.4	45.0±3.3	<0.001
p		>0.05	>0.05	

*aureus*, PVB leucocytes in the blood of both patients with periodontitis and controls generated essentially lower levels of superoxide anions than under the influence of *E. coli*. Literature data (23) confirm that the intensity of leukocyte CL depends on the nature of substances stimulating NL.

However, we found only one source (24) indicating that *S. aureus* induced the production of reactive oxygen metabolite of NL from DM patients. It must be noted that the condition of periodontal tissues was not taken into consideration during the aforementioned study. In addition to that, we found individual sources, according to which hyperglycemic conditions observed in diabetes mellitus are associated with oxidative stress (25). Other authors (26) stated that insulin-dependent DM was most likely the result of oxidative stress, due to high local levels of oxygen radicals on the beta-cells of the pancreas, which eventually led to their destruction.

Still other authors indicated that the cause of numerous complications of DM was ROS (27, 28), while periodontitis has been defined as “the sixth complication of diabetes mellitus” (29).

It is supposed (30) that reactive forms of oxygen produced *in vivo* can inactivate anti-proteinases present in biological fluids, thus increasing the activity of proteases. In recent years, a number of studies (31) have been published, placing emphasis on the importance of proteases in causing inflammatory diseases

of the periodontium. Besides, reactive oxygen forms can activate NL-produced metal proteinases. This makes it possible to presume that such reactive forms of oxygen produced by NL are particularly important factors causing tissue damage (32).

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.

### Conclusions

Lucigenin-dependent chemiluminescence showed that non-opsonized *E. coli* and *S. aureus* bacteria increased the generation of superoxide anion of neutrophil leukocytes in the peripheral venous blood of both the patients with periodontitis who have type 1 diabetes mellitus and the healthy controls.

Under the influence of *E. coli* and *S. aureus*, maximum lucigenin-dependent chemiluminescence of neutrophil leukocytes among patients with periodontitis essentially exceeded ( $p < 0.001$  and  $p < 0.002$ , respectively) analogous findings among subjects in the control group.

Such an intensive production of superoxide anion under the influence of non-opsonized bacteria in the neutrophil leukocytes of the peripheral blood of patients with periodontitis may play a role in the development of this disease of periodontal tissues.

## Superoksido anijonų išsiskyrimas iš periferinio kraujo leukocitų sergant parodontitu ir pirmojo tipo cukriniu diabetu

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**Raktažodžiai:** cukrinis diabetas, parodontitas, neutrofilai, chemiliuminescencija.

**Santrauka.** Darbo tikslas. Aktyvių deguonies formų įtaka priedančio audinių uždegiminėms ligoms išsivystyti nėra visiškai aiški. Šio darbo tikslas – ištirti asmenų, sergančių pirmojo tipo cukriniu diabetu ir turinčių didelių priedančio audinių pažeidimų, bei kontrolinės grupės asmenų, neturinčių sisteminės patologijos ir kurių priedančio audiniai sveiki, neutrofilinių leukocitų oksidacinę funkciją.

**Medžiaga ir metodai.** Tyrimui atrinkti 38 asmenys, sergantys pirmojo tipo cukriniu diabetu ir sunkios formos parodontitu, bei 27 sveiki asmenys. Iš periferinio veninio kraujo tyrimams buvo išskirti leukocitai. Jie buvo stimuliuoti neopsonizuotomis *E. coli* ir *S. aureus* bakterijomis ir buvo matuojama nuo lucigenino priklausoma chemiliuminescencija.

**Rezultatai.** Stimuliuojant neopsonizuotomis *E. coli* ir *S. aureus* bakterijomis, sergančiųjų pirmojo tipo cukriniu diabetu ir sunkios formos parodontitu asmenų periferinio veninio kraujo leukocitų chemiliuminescencijos maksimumai buvo statistiškai reikšmingai ( $p < 0,001$ ) didesni nei kontrolinės grupės asmenų. Ir sergančiųjų ir sveikų asmenų neutrofilinių leukocitų chemiliuminescencija maksimumą pasiekė panašiu laiku. Abiejų grupių tiriamųjų leukocitų, stimuliuojant neopsonizuotomis *E. coli*, chemiliuminescencijos maksimumas buvo pasiektas statistiškai reikšmingai ( $p < 0,001$ ) anksčiau negu stimuliuojant neopsonizuotomis *S. aureus* bakterijomis.

*Išvada.* Asmenims, sergantiems periodontitu, neopsonizuotos bakterijos gali stimuliuoti neutrofilinius leukocitus ir sukelti aktyvių deguonies formų generaciją, o tai gali sąlygoti priedančio audinių uždegimą bei jų destruktiją.

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