Oxidative function of neutrophils in periodontitis patients with type 1 diabetes mellitus

Renata Šadzevičienė, Jonas Žekonis¹, Gediminas Žekonis¹, Pajauta Paipalienė
Clinic of Dental and Oral Pathology, ¹Clinic of Prosthetic Dentistry, Kaunas University of Medicine, Lithuania

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

Summary. Background. The involvement of reactive oxygen species in periodontal diseases is unclear. The aim of the present study was to explore the oxidative function of neutrophilic leukocytes of the peripheral venous blood in patients with severe periodontitis and type 1 diabetes mellitus and in the control subjects with healthy periodontal tissues and without systemic diseases.

Materials and methods. The leukocytes for the present investigation were obtained from peripheral venous blood of 38 patients with severe periodontitis and type 1 diabetes mellitus and 27 control subjects. The maximal luminol-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 luminol-dependent chemiluminescence of neutrophils in patients with severe periodontitis, stimulated with non-opsonized Escherichia coli and Staphylococcus aureus, is mainly lower than that in the control group subjects (p<0.001). Luminol-dependent chemiluminescence of neutrophils reached its maximal value at the same time in both the diseased and the healthy patients.

Conclusion. In periodontitis, local non-opsonized bacteria might stimulate neutrophilic leukocytes to release oxygen species. However, these cells in patients with periodontitis are characterized by a lower intensity of luminol-dependent chemiluminescence (p<0.001) compared to analogous findings in people with healthy periodontal tissues.

This might indicate insufficient microbicidal activity of these cells in patients with periodontitis.

Introduction

Epidemiological studies indicate that 5–20% of the population suffer from severe forms of periodontitis (1). Active periodontitis occurs in a susceptible host (2). The susceptibility of the host is partly hereditary (such as inadequate or unregulated immune response) but can also be influenced by environmental and behavioral factors such as viral infections, smoking, and stress (3, 4).

It has been recognized for many years that some systemic diseases, especially diabetes mellitus, may influence periodontal health. The relationship between diabetes mellitus and periodontal disease has initiated much research and debate over the past decade (5–8).

Diabetes mellitus is a chronic metabolic disorder that affects more than 100 million people worldwide (9). 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 diabetes mellitus, especially in patients with poorly controlled disease or hyperglycemia (10).

Most authors (11–14) think that metabolic disturbances in periodontal tissues may lower the resistance of diabetes to infections and thus influence 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 (15). Periodontal disease is no exception to the potential influence of ROS. There is a clearly defined substantial role for reactive oxygen metabolites in periodontitis (16, 17), but little research has been performed in this area (16).

The yields of superoxide ions and other ROS can be measured by means of luminol-dependent chemi-

Correspondence to R. Šadzevičienė, Clinic of Dental and Oral Pathology, Kaunas University of Medicine, Eivenių 2, 50009 Kaunas, Lithuania. E-mail: irenaned@med.kmu.lt
luminescence (CL) (18, 19).

It is known (20) that the intensity of leukocyte CL depends on the nature and concentration of substances stimulating these cells. For leukocyte stimulation, chemical materials, opsonized microorganisms, and lipopolysaccharide are mostly used. In medical literature we found no findings concerning studies of luminol-dependent CL of peripheral venous blood (PVB) leukocytes from periodontitis patients, stimulated by non-opsonized microorganisms. It is possible that in the environment of the gingival crevice neutrophilic leukocytes contact with non-opsonized microorganisms. Hence, the response to non-opsonized microorganisms can play a decisive role in development of periodontitis.

The aim of the present study was to investigate the generation of ROS by leukocytes of PVB in patients with aggressive periodontitis and type 1 diabetes mellitus and in control subjects with healthy periodontal tissue using the method of luminol-dependent chemiluminescence; the stimulation of these cells was performed using non-opsonized Escherichia coli (E. coli) and Staphylococcus aureus (S. aureus).

Materials and methods

The patients for the study were selected from a large number of individuals with pathology of periodontal tissues and type 1 diabetes mellitus; the patients were treated at the Clinic of Endocrinology of Kaunas University of Medicine Hospital. The patients were examined clinically and radiographically and were diagnosed with periodontitis. Only patients with marked signs of periodontitis were selected and included in our study; the selection was performed using Russell’s (21) periodontal index (PI).

In performing investigations of luminol-dependent CL, leukocytes were obtained from peripheral venous blood of 38 diabetes mellitus patients suffering from severe periodontitis and from peripheral venous blood of 27 control subjects without any systemic pathology and with healthy periodontal tissues. The age of patients under study ranged from 18 to 50 years.

ROS generation was investigated using the luminol-dependent chemiluminescence method as proposed by L. G. Korkina et al. (19). Chemiluminescence measurements were performed at the Department of Biochemistry of Kaunas University of Medicine using a liquid scintillation counter “Delta-300.”

Luminol and Hanks’ balanced salt solution (HBSS) were obtained from Sigma Chemical Co. St. Lois. Mo (USA). Plastic vials and other disposable pieces of plastic ware were obtained from Care Roth GmbH and Co. KG.

E. coli and S. aureus samples were grown at the Laboratory of Microbiology of Kaunas University of Medicine Hospital. Specimens of E. coli and S. aureus cultures for the investigations were used within 24 hours.

Preparation of leukocyte specimens. For the assessment of ROS, leukocytes were obtained from PVB of 38 patients with aggressive periodontal diseases and type 1 diabetes mellitus and from PVB of 27 healthy control subjects. Ten milliliters of PVB 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 one hour at the temperature of 37°C. Then the supernatant layer of plasma rich in leukocytes was aspirated and diluted with HBSS (pH 7.2) 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 ROS generation. Cuvettes containing leukocyte suspension were placed in a thermostat with water, gradually adding 0.01 ml of luminol (final luminol concentration 50 μM) and taking measurements of the maximal level of chemiluminescence of non-stimulated neutrophilic leukocytes (NL). After 5 min, 0.01 ml of non-opsonized E. coli or S. aureus suspension was added, and the light reaction was followed after 15, 30, 45, 60, 75, and 90 min. Two CL parameters were calculated from the measurements:

1. Peak maximum cpm 1×10⁶ neutrophils;
2. Peak times values (time in minutes after starting the liquid scintillation counter required to reach the peak maximum).

Chemiluminescence of leukocytes make up the main part of the total amount of blood or of leukocyte suspension CL. Therefore, the intensity of CL 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 CL fraction (19), using the following equation:

\[ I_{(NL)} = I_{(leuk)} \times 100 \text{ V/ven} \]

where \( I_{(NL)} \) is \( 1 \times 10^8 \) NL chemiluminescence (cpm); \( I_{(leuk)} \) – CL of the leukocyte suspension; \( v \) – the volume of suspension (ml); \( c \) – the amount of leukocytes; \( n \) – NL percentage; and \( V \) – the volume of the cuvette (ml) used for photometric analysis.

Statistical analysis. The statistical significances of differences between groups were analyzed using Student’s t-test. Data are expressed as mean±standard deviation.
Results

Clinical data. The differences in mean age between the patients with severe periodontitis and the control group subjects was not significant (p>0.05) (Table 1). As seen in Table 1, periodontal tissues in the control group were healthy, while such tissues in the study group showed significant inflammatory changes.

Laboratory data. The results of the luminol-dependent CL studies are presented in Table 2. It should be noted that the maximal value of luminol-dependent CL of the peripheral blood leukocytes stimulated by non-opsonized E. coli and S. aureus in the control group subjects exceeded analogous CL values of periodontitis patients with type 1 diabetes mellitus (p<0.001).

It is interesting to note that CL of PVB leukocytes stimulated by S. aureus in both the patients with periodontitis and the controls was of essentially lower intensity, compared to the case of stimulation with E. coli.

In both the patients with periodontitis and the healthy controls, CL of PVB leukocytes stimulated by either E. coli or S. aureus reached its maximal value after approximately 30 minutes.

Discussion

The determined clinical parameters (Table 1) showed significantly increased PI in the group of periodontitis patients with type 1 diabetes mellitus in comparison with the control group subjects. Bearing in mind that the interactions between bacteria and the immune system play a central role in the etiology of periodontal disease (22, 23), it was expected that our selection of subjects with severe periodontal lesions of inflammatory origin in the group of patients with periodontitis and type 1 diabetes mellitus would provide clear data on the interaction between NL and bacteria, as compared with analogous data for the control group subjects. A similar method for the arrangement of study groups has been used by other authors (22, 24).

This study showed a decreased maximal value of luminol-dependent CL of neutrophilic leukocytes in patients with periodontitis and type 1 diabetes mellitus, stimulated with non-opsonized E. coli and S. aureus, compared to the control group patients with healthy periodontal tissues. We found no similar data in medical literature, except for our previous findings (25) on the decrease in the luminol-dependent NL

<table>
<thead>
<tr>
<th>Investigation groups</th>
<th>n</th>
<th>Sex (female)</th>
<th>Sex (male)</th>
<th>Age (years)</th>
<th>Russell’s PI (points)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periodontitis patients with type 1 diabetes mellitus</td>
<td>38</td>
<td>21</td>
<td>17</td>
<td>36.9±2.8</td>
<td>5.63±0.41</td>
</tr>
<tr>
<td>Controls</td>
<td>27</td>
<td>15</td>
<td>12</td>
<td>29.2±3.2</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 1. Investigation data of the subjects and clinical evaluation

<table>
<thead>
<tr>
<th>Investigation groups</th>
<th>n</th>
<th>1×10⁶ NL chemiluminescence (cpm) after stimulation</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>non-opsonized E. coli</td>
<td></td>
</tr>
<tr>
<td>Periodontitis patients with type 1 diabetes mellitus</td>
<td>38</td>
<td>96,612±6,561</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Controls</td>
<td>27</td>
<td>182,280±9,421</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 2. Maximal luminol-dependent chemiluminescence of peripheral venous blood neutrophilic leukocytes (NL) in the investigated groups of patients

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

<table>
<thead>
<tr>
<th>Investigation groups</th>
<th>n</th>
<th>E. coli</th>
<th>S. aureus</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periodontitis patients with type 1 diabetes mellitus</td>
<td>38</td>
<td>30.0±2.3</td>
<td>30.0±2.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Controls</td>
<td>27</td>
<td>30.0±2.2</td>
<td>30.0±3.3</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>p</td>
<td></td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

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chemiluminescence in patients with periodontitis and without any systemic pathology under the influence of non-opsonized *E. coli*.

It is known (26–28) that luminol-dependent CL is conditioned by myeloperoxidase (MPO) system of NL. Therefore the findings obtained in our experiments on control subjects concerning the markedly increased luminol-dependent CL of peripheral blood leukocytes affected by non-opsonized bacteria might be indicative of an elevated activity of MPO system in these cells under the influence of non-opsonized bacteria. This fact has been confirmed by our previously reported findings (29) about the intensification of the degradation by NL myeloperoxidase in subjects with healthy periodontal tissues affected by non-opsonized *E. coli*. A decreased luminol-dependent CL under experimental conditions could indicate a decreased microbicidal activity of these cells (30).

**Conclusions**

Non-opsonized *Escherichia coli* and *Staphylococcus aureus* enhanced the luminol-dependent chemiluminescence of peripheral blood neutrophilic leukocytes in patients with periodontitis and type 1 diabetes mellitus and in the control group subjects. The luminol-dependent chemiluminescence of neutrophilic leukocytes from periodontitis patients stimulated by non-opsonized *Escherichia coli* and *Staphylococcus aureus* is mainly (p<0.001) lower than that in the control group subjects. This could indicate insufficient microbicidal activity of neutrophilic leukocytes in patients with periodontitis.

**Sergančiųjų parodontitų ir I tipo cukriniu diabetu neutrofilinių leukocitų oksidacinė funkcija**

Renata Šadzevičienė, Jonas Žekonis1, Gediminas Žekonis, Pajauta Paipalienė

*Kauno medicinos universiteto Dantų ir burnos ligų klinika*

1Dantų ir žandikaulių ortopedijos klinika

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

**Santrauka.** Aktyviųjų deguonies formų įtaka priedančio audinių uždegiminėms ligoms išsivystyti nėra visiškai aiški.

**Darbo tikslas.** Ištirti sergančiųjų I tipo cukriniu diabetu, kuriems rasta sunkių priedančio audinių uždegimės kilmės pažeidimų, bei kontrolinės grupės asmenų, kuriems nerasta sisteminių patologijos, priedančio audiniais sveiki, neutrofilinių leukocitų oksidacinė funkcija.

**Medžiaga ir metodai.** Tyrimui atrinkti 38 asmenys, sergantys I tipo cukriniu diabetu ir sunkios formos parodontitu. Kontrolinę grupę sudarė 27 sveiki asmenys. Iš periferinio veninio kraujo buvo išskirti leukocitai. Jie buvo stimuliuoti neopsonizuotomis *E. coli* ir *S. aureus* bakterijomis, matuoti nuo liuminolio priklausomos chemiluminescencijos maksimumai.

**Rezultatai.** Stimuliuojant neopsonizuotomis *E. coli* ir *S. aureus* bakterijomis, sergančiųjų I tipo cukriniu diabetu ir sunkios formos parodontitu asmenų periferinio veninio kraujo neutrofilinių leukocitų nuo liuminolio priklausomos chemiluminescencijos maksimumai buvo statistiškai reikšmingai mažesni (p<0.001) nei kontrolinės grupės tiriančių. Ir sergančiųjų, ir kontrolinės grupės tiriančių neutrofilinių leukocitų nuo liuminolio priklausomos chemiluminescencijos maksimuma pasiekė panašų laikų.

**Išvada.** Neopsonizuotomis bakterijomis lokalai galima stimuliuoti sergančiųjų parodontitu periferinio veninio kraujo neutrofilinių leukocitų oksidacinį aktyvumą. Tačiau sergančiųjų parodontitu neutrofilinių leukocitų nuo liuminolio priklausomos chemiluminescencijos maksimumai buvo iš esmės (p<0.001) mažesni nei analogiški rodikliai kontrolinės grupės tiriančių. Tai galėtų byloti apie šių lastelių nepakankamą baktericidinį aktyvumą sergant parodontitu.

Adresas susirašinėti: Š. Šadzevičienė, KMU Dantų ir burnos ligų klinika, Eivenių 2, 50009 Kaunas
El. paštas: irenaned@med.kmu.lt

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