Activity of Neutrophil β-Glucuronidase in Diabetic and Nondiabetic Patients With Chronic Generalized Periodontitis and Healthy Subjects

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Key words: *periodontitis; diabetes mellitus; β-glucuronidase.*

Summary. *Objective. The aim of the study was to establish the dynamics of β-glucuronidase activity in subjects suffering from type 1 diabetes and chronic untreated generalized periodontitis, subjects suffering from chronic untreated generalized periodontitis only, and control subjects not suffering from generic diseases with healthy periodontal tissue.*

Material and Methods. The study involved 165 19–50-year-old subjects who were divided into three groups: healthy subjects (n=55), subjects with chronic untreated generalized periodontitis (n=55), and subjects with type 1 diabetes and chronic untreated generalized periodontitis (n=55). Neutrophilic leukocytes of peripheral venous blood were exposed to bacterial stimuli: opsonized zymosan, nonopsonized Staphylococcus aureus, and prodigiosan. The activity of β-glucuronidase was determined by the spectrofluorimetry method.

Results. The diagnostic value of changes in β-glucuronidase activity of neutrophilic leukocytes markedly increased in all study groups after stimulation of neutrophilic leukocytes by opsonized zymosan, nonopsonized Staphylococcus aureus, and prodigiosan as compared to control media not exposed to any stimulus (P<0.001). The strongest relationship (canonical correlation coefficient eta, 0.993) between the intensity of periodontal pathology markers and the activity of β-glucuronidase of neutrophilic leukocytes in incubated media in patients with type 1 diabetes mellitus and periodontitis was found under the effect of nonopsonized Staphylococcus aureus.

Conclusions. If periodontal impairment is severe, diabetes mellitus possibly causes a faster destruction of the periodontal tissue and presents a higher risk of periodontitis for patients with diabetes.

Introduction

Diabetes mellitus (DM) is a syndrome of abnormal carbohydrate, fat, and protein metabolism that leads to acute and chronic complications due to the absolute or relative lack of insulin (1). Hyperglycemia is the most common characteristic of diabetes and plays a central role in mediating an adverse effect on vascular cells during the progression of diabetic vascular complications. In diabetic microangiopathy, hyperglycemia induces biochemical and molecular changes in microvascular cells that ultimately lead to retinal, renal, and neural complications and extends to other complications, including advanced periodontal disease (2). Many studies suggest that periodontal disease is at least two-fold more severe in individuals with diabetes than those without diabetes. The American Diabetes Association has reported that individuals with uncontrolled diabetes (defined as 200 mg/dL of glucose on three consecutive readings) are at increased risk of infections, abnormal wound healing, and consequent prolonged recovery time. Moreover, individuals with diabetes may be more likely to develop periodontal and cardiovascular diseases than healthy individuals. History of poorly controlled chronic periodontal disease can alter diabetic/glycemic control (3). In population with poor oral hygiene, diabetes has a strongly negative influence on oral health: patients with diabetes have fewer teeth, more plaque, and a higher prevalence of moderate-to-severe periodontal disease than those without diabetes (4).

Periodontal disease is a chronic inflammatory disorder caused by the invasion of anaerobic bacteria into periodontal tissues including gingival connective tissue, periodontal ligament, and alveolar bone. Diabetic patients tend to suffer from periodontitis with severe alveolar bone loss caused by lowered immune reaction and delayed tissue recovering.

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Toxins of periodontal pathogens such as *P. gingivalis* lipopolysaccharide (P-LPS) and several cytokines (TNF-α, IL-1 and IL-6) stimulate osteoclast differentiation in gingival connective tissue. Then, alveolar bone resorption progresses and the resultant tooth loss falls oral functions. It is well documented that the prevalence of periodontitis is 2- to 3-fold higher in diabetic patients than nondiabetic subjects. Recently, many studies have demonstrated that periodontitis affected diabetic condition, in which toxins of periodontal pathogen like P-LPS and TNF-α possibly elevated insulin resistance by inhibiting glucose incorporation into smooth muscle cells. It was shown that serum C-reactive protein level was increased in periodontitis patients and periodontal treatment improved the level of HbA(1C) in diabetic patients. These data indicate that periodontal pathogens influenced systemic conditions and these are partly improved by periodontal therapy. In addition, periodontal pathogens possibly promotes atherosclerosis formation. Further investigations are necessary to clarify the relationship between diabetes and periodontal disease (5).

Gingival crevicular fluid (GCF) can be found in the physiologic space (gingival sulcus), as well as in the pathological space (gingival pocket or periodontal pocket) between the gums and teeth (6). Dental plaque is widely recognized as the main etiological factor in periodontal disease. Microorganisms seem to produce destruction directly through their endotoxins and indirectly through activation of the host cells to produce a variety of biologically active substances: cytokines, arachidonic acid metabolites, and proteolytic enzymes. This suggests that alterations in the host immunoinflammatory response to potential pathogens may play a predominant role.

Polymorphonuclear neutrophil (PMN) dysfunction is associated with diabetes (7). Diabetes may result in impairment of neutrophil adherence, chemotaxis, and phagocytosis, which may facilitate bacterial persistence in the periodontal pocket and significantly increase periodontal destruction. While neutrophils are often hypofunctional in diabetes, patients with diabetes may have a hyperresponsive monocyte/macrophage phenotype, resulting in significantly increased production of proinflammatory cytokines and mediators. However, it is observed that even if DM is well controlled, periodontal diseases are more severe and occur more frequently in diabetic than nondiabetic persons. It might be assumed that a change in the host immunoinflammatory response of neutrophilic leukocytes (NL) and monocytes/macrophages plays a significant role in the development of periodontal disease. Circulating neutrophils are an essential component of the human innate immune system (8).

Neutrophilic leukocytes, with their numerous biologically active substances and secretory granules, which comprise the main part of leukocytes in the gingival crevice, play the most important role in the protection of periodontal infections.

A number of lysosomal enzymes of neutrophilic leukocytes can be found not only inside the cell but also can be secreted extracellularly, killing microorganisms that are not destroyed during phagocytosis due to their size or for other reasons (9). Neutrophilic leukocytes can be stimulated by bacterial lipopolysaccharides and other toxins, cytokines, and the 3 fraction of activated complement (C3). Their outer membranes bear numerous receptors specific to inflammatory mediators, bacterial metabolites, lymphokines and monokines, opsonization, endothelium, and proteins of tissue matrix. β-Glucuronidase (βG) is a lysosomal acid hydrolase, which plays a significant role in the degradation of connective tissue ground substance. The increased amount of βG in gingival crevicular fluid is related to the severity of inflammatory periodontal disease and to pocket depth and is known as a biomarker of the activity of inflammatory periodontal disease. Elevated proinflammatory mediators in periodontal environment may play a role in the increased periodontal destruction seen in many people with diabetes (8, 10).

The aim of this study was to investigate the biochemical parameters of neutrophilic leukocytes affected by stimuli of bacterial origin in diabetic and nondiabetic patients that may be associated with the severity and extent of periodontal disease.

Material and Methods

The study involved 165 subjects of both sexes aged from 19 to 50 years from the Hospital of Lithuanian University of Health Sciences (former Kaunas University of Medicine). Three groups were formed by assigning 55 patients to each group: healthy 30 men and 25 women (group HD), nondiabetic 30 men and 25 women with chronic generalized untreated periodontitis (group PD), and diabetic (type 1 diabetes) 30 men and 25 women with chronic generalized untreated periodontitis (group DM). All patients in the group DM were treated with insulin for 3–8 years at the Clinic of Endocrinology, Lithuanian University of Health Sciences. The patients showed no evidence of diabetes complications (organ failure).

Written informed consent was obtained from all patients. A standard proforma contained the following data: name, age, sex, medical and past dental history. Clinical attachment loss for each patient was recorded. Inflammation of periodontal tissue was rated by the Russell periodontal index-PI (11): from 0.0 to 0.2 points for healthy subjects and 4.0 to 8.0 points for diabetic and nondiabetic persons

with chronic generalized untreated periodontitis of high severity. Each patient was examined using a mouth mirror and Williams graduated periodontal probe under artificial light. Inclusion criteria were as follows: number of teeth, 20; sites involved should have a clinical attachment loss of ⩾2 mm in the groups PD and DM. Exclusion criteria were as follows: history of any periodontal destruction for the HD group; history of any systemic disease for the HD and PD groups; history of any systemic disease other than type 1 diabetes mellitus for the DM group; pregnancy or lactation; presence of any habits such as smoking and alcoholism; history of antibiotic therapy within 6 months before the study.

The medium of neutrophilic leukocytes (MNL) was obtained from peripheral venous blood. The activity of βG was estimated in the MNL affected by bacterial stimuli: nonopsonized *S. aureus*, opsonized zymosan, and prodigiosan. *S. aureus* 256 strains were cultured in the Laboratory of Microbiology, Lithuanian University of Health Sciences.

For the preparation of peripheral venous blood neutrophilic leukocytes, peripheral blood (5 mL) was taken from subjects who had abstained from morning meals by means of a sterile vacuum test tube containing heparin (20 IU/mL). The test tube was positioned at an angle of 45 degrees and kept for 1 hour at 37°C. Subsequently, plasma was collected, and leukocyte count was standardized to 1×10^9/L cells by the using Hank's balanced salt solution. Every test tube was filled with 0.2 mL of plasma and 0.02 mL of stimuli of bacterial origin, the concentration of which in the phosphate buffer was equal to 2 μg/mL. The prepared medium was placed into a thermostat at 37°C and kept for 1 hour.

Phagocytes were activated by using opsonized zymosan, nonopsonized *S. aureus*, and prodigiosan. The activity of β-glucuronidase (nanomoles of substrate hydrolyzed in 1 mL of serum within 1 h at 37°C) in MNL was determined using 4-methylumbelliferyl-β-D-glucuronide as a substrate; a HITACHI MPF-2A spectrofluorometer (365 nm, excitation wavelength; 450 nm, emission wavelength) was employed for this purpose.

Statistical Analysis. Statistical analysis was performed with the SPSS 16 statistical program for Windows. Continuous variables are expressed as mean and standard deviation (SD). After testing for normality, parametric and nonparametric criteria – the ANOVA and Kruskal-Wallis tests – were used to compare the groups. For multiple comparisons, the Bonferroni or Dunn's test, respectively, was applied. A significance level of 0.05 was selected for testing statistical hypotheses. The difference between the means was considered significant when type II error was $\beta\leqslant0.2$ and type I error was $\alpha=0.05$.

Correlations between clinical of and biochemical data of the investigated groups were evaluated by the canonical correlation coefficient eta. Discriminant analysis was used to determine the system of statistically significant biochemical parameters for classifying the investigated groups.

The study design and completion conformed to the ethical guidelines for conducting studies approved by the Medical Academy, Lithuanian University of Health Sciences.

Results

The clinical and biochemical parameters of persons in the HD, PD, and DM groups are presented in Table 1.

As the received data on periodontal index did not satisfy the normality hypothesis, the nonparametric test was applied for the comparison of the study groups. The periodontal index in the group HD was lower than that in the groups PD and DM ($P<0.01$), whereas no significant difference was found comparing the groups PD and DM. The descriptive statistics of the factors is presented in Table 1.

No significant age difference was found comparing the study groups.

As the biochemical indices (βG-control medium of neutrophilic leukocytes, βG-ops. zymosan, βG-non-ops. *S. aureus*, βG-prodigiosan) satisfied the

Table 1. Descriptive Statistics of Clinical and Biochemical Parameters in the Study Groups

Variable	Group HD, n=55		Group PD, n=55		Group DM, n=55	
	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range
Age, years	32.91 (10.55)	19–57	40.84 (9.55)	19–59	37.27 (10.71)	19–59
Periodontal index	0.05 (0.08)	0–0.2	7.82 (0.51)	6–8	7.80 (0.49)	6–8
Activity of βG in CMNL, μmol/(L·min)	0.99 (0.01)	0.98–1	1.25 (0.06)	1.14–1.36	2.06 (0.54)	1.15–2.97
Activity of βG stimulated by opsonized zymosan, μmol/(L·min)	19.31 (0.94)	17.77–20.85	35.22 (1.13)	33.1–37.34	46.48–3.04	41.28–51.68
Activity of βG stimulated by nonopsonized *S. aureus*, μmol/(L·min)	11.40 (0.63)	10.36–12.44	17.96 (0.07)	17.84–18.08	56.41–3.96	49.6–63.22
Activity of βG stimulated by prodigiosan, μmol/(L·min)	8.54 (0.62)	7.5–9.58	14.76 (0.67)	13.53–15.99	52.75–8.02	38.73–66.77

HD, healthy donors; PD, patients with periodontal disease; DM, patients with type 1 diabetes mellitus and inflammatory periodontal disease; βG, β-glucuronidase; CMNL, control medium of neutrophilic leukocytes.

Table 2. Differences in the Means of Biochemical Indices in the Study Groups

Activity of βG	Mean Difference Between PD and HD				Mean Difference Between DM and HD				Mean Difference Between DM and PD			
	Δ	%	P	β*	Δ	%	P	β*	Δ	%	P	β*
CMNL	0.26	26.3	0.00	0.00	1.07	108.1	0.00	0.00	0.81	71.1	0.00	0.00
Stimulated by opsonized zymosan	15.91	82.4	0.00	0.00	27.17	140.7	0.00	0.00	11.26	34.0	0.00	0.00
Stimulated by nonopsonised *S. aureus*	6.56	57.5	0.00	0.00	45.01	394.8	0.00	0.00	38.45	215.5	0.00	0.00
Stimulated by prodigiosan	6.22	72.8	0.00	0.00	44.21	517.7	0.00	0.00	37.99	280.8	0.00	0.00

*Computed using α=0.05.
HD, healthy donors, PD, patients with periodontal disease; DM, patients with diabetes mellitus and inflammatory periodontal disease; βG, β-glucuronidase; CMNL, control medium of neutrophilic leukocytes.

normality hypothesis, ANOVA was applied for the comparison of means. The results of the comparison of means are presented in Table 2.

As shown in Table 2, the means of all variables (βG-CMNL, βG-ops. zymosan, βG-non-ops. *S. aureus,* βG-prodigiosan) in the HD, PD, and DM groups are statistically significantly different (α=0.05, β<0.001). The means of each index increased comparing the HD group with the PD group and the PD group with the DM group. Table 2 shows that the greatest differences in the means were found comparing the groups HD and DM: the activity of βG stimulated by prodigiosan and nonopsonized *S. aureus* was higher by 517.7% and 394.8%, respectively, in the group DM that group HD (α=0.05, β<0.001).

Fig. 1 provides the coefficients of canonical correlation eta for the evaluation of relation between the HD, PD, and DM groups and indices βG-CMNL, βG-ops. zymosan, βG-non-ops. *S. aureus,* βG-prodigiosan. It can be concluded that the study groups had the strongest correlation with the index βG-non-ops. *S. aureus* (eta=0.993), a slightly weaker one with βG-ops. zymosan (eta=0.985), βG-prodigiosan (eta=0.973), and βG-CMNL (eta=0.828).

To evaluate the diagnostic qualities of the studied indices βG-CMNL, βG-ops. zymosan, βG-non-ops. *S. aureus*, βG-prodigiosan, a discriminant analysis was performed. Fig. 2 provides a representation of the groups in the axes of canonical functions.

Both Fig. 2 and the results of discriminant analysis show that these indices can be used for diagnosis with a 100% accuracy and distinguishing between the HD, PD, and DM groups. It is noteworthy that a 100% accuracy in discrimination is maintained by using only one of the following indices: βG-ops. zymosan, βG-non-ops. *S. aureus*, βG-prodigiosan.

The activity of βG in the control medium of neutrophilic leukocytes obtained from peripheral venous blood of the subjects of the PD and DM groups was significantly higher than in healthy subjects (P<0.001). A significant increase (P<0.001) in βG activity was observed in the MNL of the group

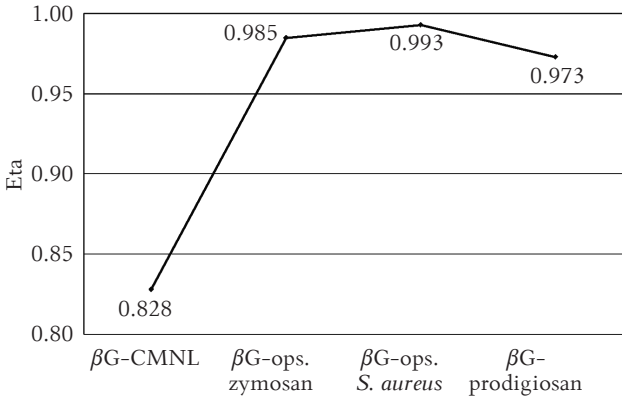

Fig. 1. Coefficients of canonical correlation eta

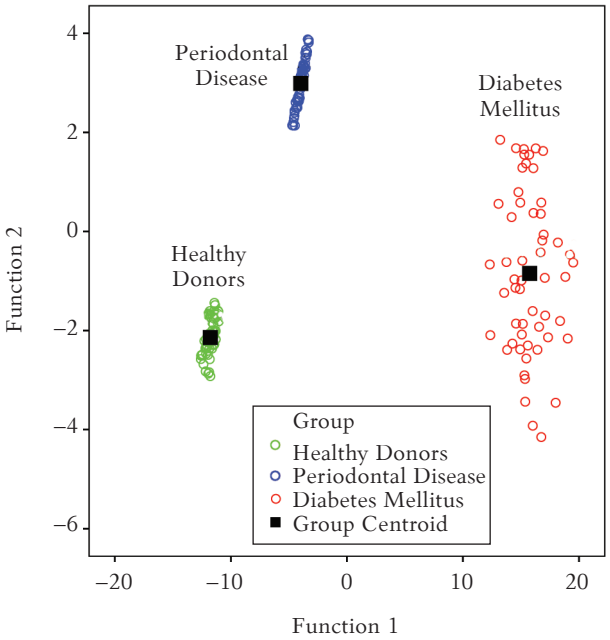

Fig. 2. Scatterplot of the first two discriminant function values for healthy donors, patients with periodontal disease, and patients with type 1 diabetes mellitus and inflammatory periodontal disease

PD, stimulated by opsonized zymosan, nonopsonized *S. aureus,* and prodigiosan, as compared to the control medium. However, there was no significant difference in βG activity ($P>0.05$) between the PD group patients' MNL exposed to prodigiosan and nonopsonized *S. aureus*. A higher βG activity ($P<0.001$) was registered in the MNL stimulated by opsonized zymosan, nonopsonized *S. aureus,* and prodigiosan in the group DM as compared with the control medium. However, there was no difference ($P>0.05$) in βG activity in the DM group patients' MNL exposed to prodigiosan and nonopsonized *S. aureus*. Interestingly, the βG activity in the MNL stimulated by opsonized zymosan, nonopsonized *S. aureus,* and prodigiosan was more than twice higher in the group DM than group HD.

Discussion

As it has been shown by studies in recent years, periodontal disease is initiated by subgingival infection with selective gram-negative bacteria, but the presence of microorganisms alone is not the only factor responsible for periodontal destruction (12). Biofilm-related products released into the periodontal pocked include bacterial endotoxins, chemotactic peptides, and organics acids (13). Inflammation results in ulceration of the epithelial pocket lining, providing ready access of these compounds into the gingival tissues. This results in further stimulation of the host response, activation of host enzymes including matrix metalloproteinases, and release of proinflammatory cytokines and prostaglandin E2, among others (14). This cascade of events leads to eventual destruction of periodontal tissues.

The responses of the host to periodontopathic microorganisms are thought to be critically important. NLs are the principal cells of the host defense system and the primary protective cells against periodontal diseases. Circulating neutrophils are an essential component of the human innate immune system. The inflammatory lesions were dominated by PMNs apparently being unable to efficiently clear bacterial pathogens (8, 15). Released granule components from infiltrating leukocytes, such as lysosomal enzymes and reactive oxygen species, which are normally intended to degrade ingested microbes, can also lead to tissue destruction and amplification of the inflammatory response. The activity of gram-negative bacteria, present in dental plaque, results in the release of contents of lysosomal granules into noncellular environment by NLs with the subsequent suppression of bacterial adhesion and growth as well as destruction of bacteria in noncellular environment.

Not only local but also systemic factors play an important role in the development of periodontal disease. Systemic and hormonal changes have been implicated as factors contributing to periodontal diseases. Diabetes has been identified as a risk factor for periodontal disease, and diabetic patients can experience periodontal destruction at an earlier age than nondiabetic individuals.

Impaired neutrophil chemotaxis, defective phagocytosis of *P. gingivalis* by neutrophils, the intracellular killing capacity of neutrophils were observed in diabetic patients, and superoxide released by PMNs in diabetic patients was drastically increased. These above defects in the neutrophils of diabetic patients could be a possible mechanism to render them more susceptible to periodontal diseases (15).

Extensive research has examined which periodontal diseases might influence glycemic control in diabetes (16).

Persistent poor glycemic control has been associated with the incidence and progression of diabetes-related complications, including gingivitis, periodontitis, and alveolar bone loss (17). Nationwide US surveys have demonstrated that people with diabetes, especially poorly controlled diabetes, have a significantly higher prevalence of severe periodontitis (3, 18). Several mechanisms have been proposed to explain the increased susceptibility to periodontal diseases, including alterations in host response, subgingival microflora, collagen metabolism, vascularity, gingival crevicular fluid, and heredity patterns (18).

Our findings support data from other studies showing the relationship of increased βG activity in sulcus fluid and periodontal tissue lesions (7). Differences in βG activity were determined between the HD group and the PD, DM groups in the medium of neutrophilic leukocyte degranulation according to enzyme activity induced by opsonized zymosan, nonopsonized *S. aureus*, prodigiosan. The activity of βG in MNLs when affected by opsonized zymosan, nonopsonized *S. aureus,* and particularly prodigiosan is dependent on periodontal status and systemic factors of patients.

The data of our study show that in patients of the PD and DM groups, βG activity was significantly increased ($P<0.001$) when NLs were affected by opsonized zymosan, nonopsonized *S. aureus*, and prodigiosan as compared to βG activity in CMNL. The highest βG activity ($P<0.001$) was documented in the DM group when NLs were affected by prodigiosan, and it was higher than that in the control medium of DM patients and analogous values in the PD and HD groups.

Based on our findings, we can state that neutrophilic leukocytes in peripheral venous blood of patients of the PD and DM groups, while reacting to dental plaque bacteria and their toxins, secrete lysosomal enzymes of a higher activity in a noncellular environment and may possibly cause destruction of periodontal tissue. The findings have

demonstrated a significant relationship between glycemic control and periodontal health in medium, measuring probing depths and bleeding, and alveolar bone loss. Poorly controlled diabetes is a risk factor for periodontal disease, and it was confirmed by our study. The positive correlation between glycemic control, HbA1c and severity of periodontal disease was consisted with the findings from other reported studies (19). The reasons for poorer periodontal health among patients with poor glycemic control could be explained by the hyperglycemic status resulting in the accumulation of advanced glycation end-products, as these products in turn lead to a cascade of inflammatory reactions leading to the release of inflammatory mediators like IL-1, IL-6, TNF-α, C-reactive protein, thereby enhancing the periodontal breakdown process (7, 20, 21).

Conclusions

β-Glucuronidase activity was increased after stimulation of neutrophilic leukocytes with opsonized zymosan, nonopsonized *Staphylococcus aureus*, and prodigiosan. β-Glucuronidase activity after stimulation of neutrophilic leukocytes by stimuli of bacterial origin was considerably increased in periodontitis patients. β-Glucuronidase activity was more increased in diabetic patients with periodontitis than in periodontic patients without diabetes.

A direct correlation (canonical correlation coefficient eta, 0.993) was established in all study groups between the intensity of periodontal pathology markers and the activity of β-glucuronidase in neutrophilic leukocytes stimulated by nonopsonized *Staphylococcus aureus*. The effect of other antigens – opsonized zymosan and prodigiosan – on β-glucuronidase activity was less pronounced (eta=0.985 vs. eta=0.973).

The results of discriminant analysis show that the dynamics of the activity of β-glucuronidase in neutrophilic leukocytes is 100% accurate in reflecting changes in the course of periodontitis and may be used for monitoring the process.

Statement of Conflict of Interest

The authors state no conflict of interest.

Sergančiųjų lėtiniu išplitusiu periodontitu ir cukriniu diabetu bei nesergančių cukriniu diabetu ir sveikų asmenų neutrofilinių leukocitų β-gliukuronidazės aktyvumas

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Raktažodžiai: periodontitas, cukrinis diabetas, β-gliukuronidazė.

Santrauka. *Tyrimo tikslas*. Įvertinti žmonių, sergančių 1 tipo cukriniu diabetu ir periodonto ligomis; sergančiųjų tik periodonto uždegiminėmis ligomis; asmenų, nesergančių sisteminėmis ligomis ir periodonto ligomis, neutrofilinių leukocitų β-gliukuronidazės aktyvumo dinamiką.

Medžiaga ir metodai. Tyrimui buvo atrinkti 19–50 amžiaus 165 suaugusieji, po 55 asmenis į kiekvieną iš trijų tiriamųjų grupių: sveikų asmenų; sergančiųjų tik lėtiniu negydytu išplitusiu periodontitu; sergančiųjų 1 tipo cukriniu diabetu ir lėtiniu negydytu išplitusiu periodontitu. Periferinio veninio kraujo neutrofiliniai leukocitai buvo stimuliuojami bakterinės kilmės stimulais: opsonizuotu zimozanu, neopsonizuotu *Staphylococcus aureus*, prodigiozanu. β-gliukuronidazės aktyvumas buvo nustatytas spektrofluorimetrijos metodu.

Rezultatai. Neutrofilinių leukocitų β-gliukuronidazės aktyvumo pokyčių diagnostinė vertė žymiai padidėja visose tiriamųjų grupėse, paveikus opsonizuotu zimozanu, neopsonizuotu *Staphylococcus aureus*, prodigiozanu, palyginus su kontrolinėmis šių grupių terpėmis ($p<0,001$). Stipriausia β-gliukuronidazės aktyvumo ir patologinių periodonto pokyčių sąsaja nustatyta sergantiesiems 1 tipo cukriniu diabetu ir periodonto ligomis stimuliuojant neopsonizuoto *Staphylococcus aureus* (eta=0,993).

Išvada. Kai periodonto pažeidimai yra sunkūs, galbūt, cukrinis diabetas turi įtakos spartesnei periodonto destrukcijai ir patvirtina didesnę sergančiųjų cukriniu diabetu riziką sirgti periodontitu.

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