ORIGINAL ARTICLE

NEUTROPHIL ACTIVITIES IN ADOLESCENTS WITH TYPE I DIABETES MELLITUS DEPENDING ON PERIODONTAL STATE

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ABSTRACT

The aim: To estimate the neutrophil activities in adolescents with type 1 diabetes mellitus (T1DM) depending on periodontal state.

Materials and methods: A total of 93 individuals aged 12-16 years, including 62 T1DM patients and 31 healthy (H) controls, were included. Both groups were categorized into subgroups depending on their periodontal state. Phagocytic activity of neutrophils (PAN) the index of neutrophil activation (INA), and the percent of formazan-active neutrophils were evaluated using the spontaneous and the induced nitroblue tetrazolium (sNBT and iNBT) tests into oral rinses.

Results: PAN was significantly higher in the healthy (H) controls with gingivitis compared with the individuals with gingival health ($p < 0.0001$). This parameter decreased significantly in the T1DM subjects, especially with periodontitis, compared with the H controls ($p < 0.0001$). The percent of formazan-active neutrophils and INA in the sNBT test increased in the T1DM patients with gingival health and continued to raise as periodontal state of adolescents with T1DM worsened ($p<0.0001$). The parameters of the iNBT test in the T1DM adolescents decreased with the periodontal disease development ($p < 0.0001$) that may demonstrate that superoxide production exhausts in diabetes, especially associated with periodontal disease.

Conclusions: The sNBT test in studied adolescents showed that both periodontal disease and T1DM increase the rate of activated neutrophils ($p<0,05$).

KEY WORDS: periodontitis, diabetes mellitus, phagocytosis, neutrophils, gingivitis

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INTRODUCTION

The effect of systemic diseases on the periodontal state has been studied in a number of researches [1-3]. Type 1 diabetes mellitus (T1DM) has long been called "juvenile diabetes" and today is considered the most common type of endocrine diseases in adolescents [4]. Recent studies showed that T1DM patients are at higher risk of developing periodontal diseases than non-T1DM individuals [5], and periodontal disease is one of the early manifestations of diabetes in oral cavity of children [6].

Neutrophils have a lot of activities to form the initial response against pathogenic insults: adherence and migration, degranulation and release of inflammatory mediators, phagocytosis and apoptosis [7]; also they represent the most of leukocytes of gingival crevice [8]. Clinical studies have reported that the neutrophil activities impaired in blood [9] and crevicular fluid [10] in the adults with periodontitis. Furthermore, impaired chemotaxis, phagocytosis and increased production of free radicals were confirmed in blood of the diabetic patients [11].

Increased level of glucose in saliva and crevicular fluid in diabetes strengthens proliferation of periodontopathic bacteria. To phagocytize bacteria of dental biofilm neutrophils can use both oxygen-dependent and oxygen-independent means [12]. The recent review points to the strengthening of superoxide generation in the patients with periodontitis associated with diabetes mellitus due to both hyperglycemia and periodontitis [13]. However, the overproduction of intracellular superoxide not only defends, but attacks periodontal tissues [14].

The nitroblue tetrazolium test is one of the methods to find release of superoxide radicals by stimulated and non-stimulated neutrophils. Nitroblue tetrazolium is easily phagocytized by cells and reduced to formazan with the hexose monophosphate shunt activation [15]. Unstimulated neutrophils from diabetic patients produce a great amount of superoxide anions and reduced NBT more efficiently [16]. Neutrophils migrate from crevicular fluid or periodontal pocket into the oral cavity and can be evaluated in oral rinses. To the best of our knowledge, no report about the evaluation of the phagocytic activity of neutrophils and the NBT test in oral rinse of adolescents with periodontal disease associated with T1DM.

THE AIM

To estimate the neutrophil activities in adolescents with T1DM depending on periodontal state.

MATERIALS AND METHODS

A group of adolescents was examined who were patients of the endocrinological ward of Paediatric community

hospital in the town of Poltava (Ukraine). The study was conducted in accordance with the ethical standards of the Helsinki Declaration (1975, revised in 2002). Ethical approval was obtained from the Bioethical Committee of Ukrainian medical stomatological academy (renamed to Poltava state medical university in 2021), and informed consent was obtained from all the study subjects (their parents).

The study comprised of 62 subjects with severe manifestation of T1DM (30 males and 32 females) aged 12-16 years. Duration of the disease was under 1 year in 18 subjects, from 1 to 5 years in 26 subjects, and over 5 years in 18 ones. Periodontal examination was performed in compliance with the guidelines for periodontal screening [17]. Patients with T1DM were categorized into three subgroups: 18 subjects with gingival health, 23 subjects with gingivitis, 21 ones with periodontitis. Healthy (H) controls included 16 adolescents with gingival health and 15 ones with gingivitis (15 males and 16 females). Exclusion criteria were history of any systemic disease other than diabetes for the patients with T1DM, presence of smoking, and history of antibiotic therapy within 6 months prior to study.

Phagocytic activity of neutrophils (PAN) and the nitroblue tetrazolium (NBT) test were examined into oral rinses. The oral rinse samples were obtained from all adolescents before the breakfast and the insulin injection. Three preliminary oral rinses with 10 ml of physiological saline for 30 s (PS, 0.9% NaCl) were repeated with 5 min break. Phagocytic activity of neutrophils was examined in the first and the second rinses which immediately delivered to the laboratory. The rinses centrifuged at 2000 rpm for 10 minutes. The Candida albicans cells in the yeast phase only were mixed with the neutrophil sediment and kept for 30 min at 37°C. The suspension was centrifuged at 1500 rpm for 5 min. Then the supernatant was removed and smears from the sediment were fixated in the methanol for 5 min, air dried, and stained with 10% Giemsa solution. The percentage of phagocytic neutrophils was calculated from the observations of 100 viable cells under the light microscopy.

The third oral rinses with PS weren't used and the forth oral rinses were taken for the NBT test evaluation. The samples (2 ml) were placed in the disposable tubes, immediately delivered to the laboratory, and PS was added to bring the level to the 10 ml. INA and the percent of formazan-active neutrophils among 100 cells were evaluated by the spontaneous and the induced NBT (sNBT and iNBT) tests [15] with modification of Saiapina L.M. [18]. The index of neutrophil activation (INA) was calculated the formula: a+2b+3c+4d/100, where a, b, c, d –number of active neutrophils in 0, I, II, III grades activation accordingly. The grade of activation is evaluated by the square of formazan granules (0 – cells without formazan, 1 – 25% of cell`s square was stained, II – 75%, III – 100%).

Taking in consideration groups comparisons in the studied groups, P-value below 0.0125 was calculated with Bonferroni coefficient (0,05/4 where 4 – number of group comparisons) and considered significant for neutrophil activities. Two-way analysis of variance (ANOVA) and the F-test were used for determining whether factory-to-factory variation was significant for diabetes and the periodontal state, where P-value below 0.05 accepted.

RESULTS

Analyzing the levels of the phagocytic activity of neutrophils in oral rinses the authors found that PAN was significantly higher in the H individuals with gingivitis than in group of individuals with gingival health ($p < 0.0001$) (Table I). Significantly lower PAN in oral rinses were demonstrated in all groups of the T1DM subjects than in the H controls with gingival health ($p < 0.0001$). A similar trend was found for the T1DM subjects with gingivitis, the median of which was significantly lower PAN compared with the diabetic subjects with gingival health ($p < 0.0001$). When the T1DM subjects with periodontitis were compared with the T1DM subjects with gingival health and gingivitis differences between comparisons continued ($p < 0.0001$).

Table II shows comparisons of the sNBT and iNBT test parameters between the healthy controls and the patients with T1DM. The percent of formazan-active neutrophils in sNBT test was lower in the H individuals with gingival health as compared with the H individuals with gingivitis ($p < 0.0001$). This parameter was higher in the T1DM subjects with gingival health than in the H controls with same state ($p < 0.0001$). The T1DM subjects with gingivitis had higher percent of formazan-active neutrophils compared with the H controls with gingival health and gingivitis ($p < 0.0001$), and the T1DM subjects with gingival health ($p = 0.0005$). Also, the percent of formazan-active neutrophils in the T1DM subjects with periodontitis was significantly higher from that in the H controls ($p < 0.0001$), the T1DM subjects with gingival health ($p < 0.0001$) and gingivitis ($p =0.0122$).

INA in sNBT test was higher in the H individuals with gingivitis compared with the individuals with gingival health ($p < 0.0001$). The T1DM subjects with gingivitis had higher INA in this test compared with the H controls ($p < 0.0001$) and the T1DM subjects with gingival health ($p < 0.001$). INA in the sNBT test in the T1DM subjects with periodontitis was higher from those in the H controls, the T1DM subjects with gingival health ($p < 0.0001$), but similar to the T1DM subjects with gingivitis ($p=0.08$).

The percent of formazan-active neutrophils in the iNBT test was similar in the H controls with gingival health and gingivitis ($p = 0.13$) and significantly decreased in the T1DM individuals with gingival health compared with the H controls with same status ($p = 0.0001$). The T1DM individuals with gingivitis had lower percent of formazan-active neutrophils compared with the H controls and the T1DM individuals with gingival health ($p < 0.0001$). Same trend was found in the T1DM individuals with periodontitis ($p < 0.0001$). In addition, there was significant difference ($p < 0.0001$) regarding the percent of formazan-active neutrophils in the iNBT test between the T1DM subjects with gingivitis and periodontitis.

Table I. Comparison of the phagocytic activity of neutrophils in oral rinses between the healthy controls and the patients with T1DM

Phagocytic activity	H controls with gingival health (n=16)	H controls with gingivitis (n=15)	T1DM patients with gingival health (n=18)	T1DM patients with gingivitis (n=23)	T1DM patients with periodontitis (n=21)
Mean±SD	30.08±0.96	35,02±1.28[A]	27.21±1.13[A]	21.23±0.97[A,B,C]	17.68±1.07[A,C,D]

*Categorical variables are presented as n (%). The p-values were computed with the t independence test. A: significant difference at p<0.0125 compared with the H controls with gingival health; B: significant difference compared with the H controls with gingivitis; C: significant difference compared with the T1DM patients with gingival health; D: significant difference compared with the T1DM patients with gingivitis.

Table II. Comparisons of the NBT test parameters between the healthy controls and the patients with T1DM

Parameter	H controls with gingival health (n=16)	H controls with gingivitis (n=15)	T1DM patients with gingival health (n=18)	T1DM patients with gingivitis (n=23)	T1DM patients with periodontitis (n=21)
Formazan-positive cells in the sNBT test (%)	28.26±2.51	38,96±3.45[A]	42.17±3.73[A]	51.06±4.11[A,B,C]	54.35±4.22[A,C,D]
INA in the sNBT test (%)	1.59±0.08	1.82±0.08[A]	1.92±0.09[A]	2.16±0.09[A,B,C]	2.21±0.10[A,C]
Formazan-positive cells in the iNBT test (%)	66.9±2.81	68,67±3.61	63.20±3.19[A]	59.42±3.12[A,,B,C]	57.32±2.21[A,C,D]
INA in the iNBT test (%)	2.60±0.08	2.67±0.09	2.58±0.09	2.42±0.10[A,B,C]	2.29±0.10[A,C,D]

*Categorical variables are presented as n (%). The p-values were computed with the t independence test. A: significant difference at p<0.0125 compared with the H controls with gingival health; B: significant difference compared with the H controls with gingivitis; C: significant difference compared with the T1DM patients with gingival health; D: significant difference compared with the T1DM patients with gingivitis.

INA in the iNBT test was not significantly higher in the H controls with gingivitis compared with the individuals with gingival health (p = 0.04), and T1DM did not lead to a difference in INA between the individuals with gingival health (p > 0.05). The T1DM individuals with gingivitis had lower INA in this test compared with the H controls and the T1DM individuals with gingival health (p < 0.0001). The T1DM subjects with periodontitis showed significantly lower INA in comparison with the H controls (p < 0.0001), the T1DM subjects with gingival health (p < 0.0001), and the subjects with gingivitis (p=0.002).

Healthy control groups of patients with and without gingivitis and T1DM groups of patients with and without gingivitis were statistically treated with two-way ANOVA. Data was not statistically significant in this test using the phagocytic activity of neutrophils related the gingival state (F=0,009, p>0,1) and existence of diabetes as measurement variable (F=2,32, p>0,1). Similar not significance was found for the percent of formazan-positive cells in sNBT test related to the gingival state (F=117, p=0,05), but dates were significant related to existence of diabetes as measurement variable (F=206, p<0,05). Data was statistically significant in this test using INA in the sNBT test related to the gingival state (F=4489, p<0,01), and related to existence of diabetes as measurement variable (F=2209, p=0,013). Formazan-positive cells and INA in the iNBT test do not present statistically significant data related to studied factors (all p>0,1).

DISCUSSION

Hyperglycemia caused by diabetes mellitus can alter periodontal tissues in many ways. A high level of glucose in saliva and crevicular fluid stimulates growth of cariogenic and periodontopathic bacteria [19]. Dental plaque induces chronic activation of neutrophils to gingival crevice, and neutrophils use superoxide production to phagocytized bacteria. However, oxygen radicals, cytokines, bactericidal proteins, and matrix-degrading enzymes released by neutrophils could not only protect periodontal tissues against damage, but could activate the damaging pathways [7]. Apart from this, the periodontopathic gram-negative bacteria are able to induce the cytokines which causes insulin resistance [20, 21]. Thus, periodontitis can block glycemic control and hamper glycemic control can further stimulate periodontal disease.

NBT test and phagocytosis assay are indirect markers of oxygen-depend antimicrobial activity of neutrophils. An increased superoxide production in blood was found in localized juvenile periodontitis [22]. The adult diabetic subjects with chronic generalized periodontitis demonstrated a lower phagocytosis degree and an increase in the NBT test in blood [23]. However, the response of peripheral neutrophils, which was analyzed in those studies, may be different from neutrophils of crevicular fluid or periodontal pocket.

In the present study, PAN in oral rinse increased in the healthy adolescents with gingivitis as the host immune response to microorganisms. Impaired neutrophil activities such as phagocytosis, superoxide generation, and chemotaxis in the diabetic patients with periodontitis [24] are considered as their hyperactivity [25]. Genetic polymorphism related to the neutrophil function may help periodontal pathogens to evade the neutrophil response or may lead to the neutrophil hyperactivity [12]. The reduced PAN in the T1DM patients with gingival health in the current study may have caused by diabetes and predisposed to the periodontal disease. PAN decreased as periodontal

state of subjects with T1DM worsened and had the lowest value in the subjects with periodontitis, so these results correlate with previous findings [24]. The findings of a two-way ANOVA suggest that diabetes did not have a greater impact on PAN than gingival state.

The percent of formazan-active neutrophils and INA in the sNBT test were used in this study for screening of the activated neutrophils. These parameters increased in the healthy patients with gingivitis and continued to raise as periodontal state of adolescents with T1DM worsened, which was in accordance with previous study [24]. In addition, the findings of a two-way ANOVA suggest that diabetes had a greater impact on the percent of formazan-active neutrophils than gingival state, and both diabetes and gingival state impacted on INA in the sNBT test.

The iNBT test assesses the functional ability of neutrophils to finish phagocytosis. The authors found significantly lower parameters of the iNBT test in the T1DM patients as compared with healthy controls, and these parameters worsened with periodontal disease development. Obtained results correlate with the study of Ahkamova et al. [25], where higher parameters of the iNBT test decreased in the adult patients with the severe stage of chronic periodontitis. This impact could be explained by prohibition of superoxide release of neutrophils by bacteria of periodontal pockets. Also a high extracellular glucose concentration reduces oxygen production from activated neutrophils [26]. However, our findings of a two-way ANOVA test suggest that diabetes did not have a greater impact on the iNBT-test than gingival state.

Obviously, periodontopathic bacteria and hyperglycemia both modify the neutrophil activities. Finally, the presence of T1DM is related to impaired phagocytic activity and superoxide production. Future researches may develop a novel pathogenic strategy in the patients with T1DM associated with periodontal disease based on the neutrophil activities' regulation.

CONCLUSIONS

PAN decreases with development of periodontal disease both in healthy adolescents and adolescents with T1DM ($p<0,0125$). Parameters of the sNBT test increased in healthy adolescents with gingivitis and patients with T1DM who had different periodontal state ($p<0,0125$), showing the reactive oxygen overproduction of neutrophils. The decreased parameters of the iNBT test in subjects with T1DM and gingival health ($p<0,0125$) may demonstrate that superoxide production exhausts in diabetes, and these indices worsened with development of periodontal disease ($p<0,0125$). The sNBT test in studied adolescents showed that both periodontal disease and T1DM increase the rate of activated neutrophils ($p<0,05$).

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