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Salivary human beta-defensins and cathelicidin levels in relation to periodontitis and type 2 diabetes mellitus

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ABSTRACT

Objective: Type 2 diabetes mellitus (T2DM) is a well-defined risk factor of periodontitis and it can affect expression of human beta-defensins (hBDs) and cathelicidin (LL-37) as well. The aim of the present study was to evaluate the impact of periodontitis and T2DM on salivary concentrations of these antimicrobial peptides.

Material and methods: Unstimulated saliva samples, together with full-mouth periodontal recordings were collected from 92 individuals with periodontitis (63 with T2DM and 21 smokers) and 86 periodontally healthy controls (58 with T2DM and 21 smokers). Salivary hBD-1, -2, -3, LL-37, and advanced glycalization end products (AGE) concentrations were measured by enzyme-linked immunosorbent assay.

Results: Among the periodontitis patients, T2DM group demonstrated lower levels of hBD-1 ($p = .006$), hBD-2 ($p < .001$) and hBD-3 ($p < .001$), and higher levels of LL-37 ($p < .001$) compared to systemically healthy controls. When only periodontally healthy controls were included into the analysis, higher hBD-1 ($p = .002$) and LL-37 ($p < .001$) levels were found in T2DM patients in comparison to systemically healthy controls. Salivary LL-37 levels were associated with HbA1c and periodontitis, while hBD-2, hBD-3 and levels associated only with HbA1c.

Conclusion: In the limits of this study, hyperglycaemia can be proposed as a regulator of salivary hBD and cathelicidin levels. Periodontitis, on the other hand, affects only salivary LL-37 levels.

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Antimicrobial peptides; saliva; periodontitis; diabetes mellitus

Introduction

Antimicrobial peptides are small cationic molecules, which exist in almost all organisms [1]. There are over 45 antimicrobial peptides found in human body, of these human beta-defensins (hBDs) and cathelicidin (LL-37) play important role to establish homeostasis [2]. In the oral cavity, hBD 1-3 and LL-37 are found in gingival epithelium, gingival crevicular fluid (GCF) and saliva [3,4]. hBDs and LL-37 are multifunctional peptides and besides their well-known antimicrobial effect, they contribute to innate and adaptive immunity by enhancing phagocytosis, suppressing production of proinflammatory cytokines, and by regulating complement system [5]. They act as chemoattractants for immune cells and stimulate wound healing and angiogenesis [6]. Despite their well-defined effects on immunity, the question of how periodontitis and related factors do regulate the salivary antimicrobial peptides levels is left unexplained [7–10].

Periodontitis is a bacteria-induced inflammatory disease that destroys the tooth supporting tissues. The development and progression of periodontal diseases consist of a cascade of events modulated by the innate and adaptive immune systems [11,12]. Type 2 diabetes mellitus (T2DM) is chronic and metabolic disorder and characterized by insulin resistance. The prevalence of disease is estimated that 425 million adults (20–79 years) in Worldwide and to be foreseen to reach 629 million by 2045 [13]. It is defined as a modifiable risk factor for periodontitis as well [12,14]. We previously demonstrated elevated protein expressions of hBD-2, hBD-3 and LL-37 in gingival tissue samples of individuals with periodontitis and T2DM [15], while controversial results were also found when the relationship between T2DM and hBD-1 was analysed [16,17]. Association between elevated hyperglycaemia and decreased hBD secretion was shown *in vitro* [18]. Salivary levels of LL-37, on the other hand, were found to be elevated in T2DM patients [19]. Advanced glycalization end products (AGE), which are formed by nonenzymatic modifications of proteins by reducing sugars, are important pathophysiologic mechanism in the development of diabetic complications [20]. AGE has detrimental effect on periodontal tissues and wound healing. It elicits an intracellular reactive oxygen species, which subsequently activates mitogen-activated protein kinase and nuclear factor κB signalling, leading to the production and release of several pro-inflammatory cytokines [21,22]. In spite of the distinct effects of AGE on periodontal tissues and immunity, little is known about its

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relationship with antimicrobial peptide expression in periodontal tissues.

In the present study, the hypotheses were (1) periodontitis and T2DM impair salivary concentrations of antimicrobial peptides and (2) the relation between T2DM and antimicrobial peptides can be explained through AGE and HbA1c levels. Therefore, the aim of the present study was to profile the concentrations of salivary levels of antimicrobial peptides in relation to periodontitis and T2DM.

Material and methods

Ethical guidelines

This study was approved by the Ethical Committee of Sakarya University, Faculty of Medicine, Sakarya, Turkey (Protocol no:16214662/050.01.04/37) in accordance with Helsinki Declaration 1975, as revised in 2000. All participants were informed about study protocol and written informed consents were obtained from study participants.

Study population

Recruitment of the study population was performed at the Periodontology Department of Faculty of Dentistry, Sakarya University, in collaboration with Internal Medicine Department of Faculty of Medicine, Sakarya University. The participant recruitment and sample collection were performed between March 2018 and January 2019. Overall 178 participants were included the study. Demographic variables, including age, sex, family history, medical and dental treatment history, history of periodontal tooth loss (PTL), alcohol consumption, diabetes duration, current anti-diabetic medication and/or insulin doses, and presence of chronic diseases were obtained by interviews. Individuals reporting the intake of antibiotics or receival of periodontal treatment within 6 months, in pregnancy or lactating period, an existing or previous diagnosis of cardiovascular diseases (including angina, miyocardial infarction and stroke) genetic, renal, and hepatic disorders or HIV, or have a history of transplantation, participants who diagnosed with pre-diabetes after medical examination and were excluded from the study.

Information on smoking status was obtained from self-reports. Participants who smoked >10 cigarettes per day were defined as smokers ($n = 42$), and those who never smoked were defined as non-smokers ($n = 136$). Former smokers and individuals who were smoking randomly were excluded from the study.

Clinical examination

Periodontal examination performed by single calibrated specialist (DY) (*Kappa 0.91*). Full-mouth periodontal recordings, including plaque index (PI) [23], gingival index (GI) [24], probing pocket depth (PPD), and clinical attachment level (CAL) were recorded by using a manual periodontal probe (PW7, Hu-Friedy, IL, US.) for six sites per tooth except the third molars. Radiographic evidence of alveolar bone loss was evaluated by an optimal quality panaromic tomography. In the presence of superimposition, periapical radiographs were taken to correctly identify the presence and type of the bone loss (Orthopantomograph OP 100, Sirona Orthophos XG5, NY, USA).

Periodontitis was diagnosed according to the 2017 Classification of Periodontal and Peri-Implant Diseases and Conditions [25]. Briefly, subjects were diagnosed as periodontitis if they had bleeding on probing (BOP) $\geq$10% and interdental CAL was detectable at $\geq$2 non-adjacent teeth with PPD $\geq$4 mm. In the meantime periodontal health defined as BOP $<$10% of the surfaces and no sites with PPD $>$ 3 mm besides no clinical attachment or bone loss. The degree of periodontitis was characterized using the staging and grading system. Participants were diagnosed with stage II, if the radiographic bone loss exists at coronal third of the root (15–33%), interdental CAL was 3 to 4 mm at site of greatest loss, and no PTL. Participants were diagnosed as stage III, if the radiographic bone loss extends to mid-third of root or beyond, interdental CAL $\geq$5 mm at site of greatest loss, and PTL $\leq$4 teeth. As the previous periodontal records of study participants were not available, the grade of disease was determined by using bone level of the worst affected toot and age (BL/A) ratio. Individuals with 0.25 to 1.0 BL/A was diagnosed with Grade B and the participants with $>$1.0 of BL/A was diagnosed with Grade C. In the presence of certain risk factors like smoking and diabetes, grade shifted to a higher level. In accordance with this classification 51% (92 individuals) of participants were diagnosed with periodontitis and 49% (86 individuals) were periodontally healthy. Among the participants diagnosed with periodontitis; 75% (69 individuals) were diagnosed with stage 3 grade C periodontitis, 14% (13 individuals) were diagnosed with stage 2 grade B periodontitis and 11% (10 individuals) were diagnosed with stage 2 grade C periodontitis.

Metabolic examination of participants was performed by an expert physician (EUA). According to American Diabetes Association's 2018 guideline [13], study participants with fasting plasma glucose (FPG) $\geq$126 mg/dL (7.0 mmol/L, fasting is defined as no caloric intake for at least 8 h) and HbA1c $\geq$ 6.5% (48 mmol/mol) were diagnosed as having T2DM. In accordance with these criteria, 67% ($n = 121$) of the study participants were diagnosed with T2DM and rest of them were metabolically healthy.

Saliva collection

Non-stimulated saliva collection technique was applied. Saliva collection performed before periodontal and metabolic examinations. Participants were asked to split and to fill the calibrated, plastic, 5 mL tubes for five minutes. Samples were collected between 8 and 10 AM and at least one hour after the last food intake. After collection, the samples were centrifuged (6000 g, 5 min) and then were immediately stored at –80 °C until analysis.

Enzyme-linked immunosorbent assay for hBDs, cathelicidin and AGE

Commercially available sandwich ELISA kits were used to measure the salivary levels of hBD-1, -2, -3, LL-37 and AGE (SinoGeneClon Biotech Co., Ltd., HangZhou, China). All measurements were performed according to manufacturer's instructions. Briefly, 100 µl of standard or sample were added to each well and the plates were incubated for 30 min at 37 °C following washing procedure for five times. The prepared detector (100 µL) was added to the plates and incubated for 30 min at 37 °C. After that, 100 µL of the chromogen solution was added and incubated for 15 min at 37 °C. Finally, 100 µL of the stop solution was added, and the plates were read at 450 nm (Triturus, Grifols International, S.A., Barcelona, Spain.) detection limits of ELISA kits were 6 ng/L for hBD-1, 5 ng/L for hBD-2, 2 ng/L for hBD-3, 1 ng/ml for LL-37 and 26 ng/ml for AGE.

Statistical analyses

The primary outcome variables of the study were antimicrobial peptides (hBD-1, hBD-2, hBD-3 and LL-37). In post-hoc power analysis, Bonferroni Correction was performed, and alpha error was accepted as 0.025 in order to control Type I error. The power ranged from 67.8 to 99.1%. Post hoc power analysis was performed by G* Power 3.0.10 (Franz Faul, Universität Kiel, Kiel, Germany).

Normality of the continuous variables was tested with the Kolmogorov-Smirnov test. Data distributions of age, GI, PI, PPD, CAL, FPG, HbA1c, and AGE were normal, thus presented as means and standard deviations. Data distributions of hBDs and LL-37 were found to be skewed, therefore presented as medians and minimum–maximum values. All categorical variables were presented as frequencies and proportions (percentages) and evaluated by chi-square test. Intragroup comparisons were performed either using parametric ANOVA test or with the non-parametric Kruskal–Wallis (for multiple comparisons), depending on the data distribution of the tested parameter. $p < .05$ were considered statistically significant according to the Bonferroni correction. Spearman's correlation test was used for correlation analysis. Multinomial logistic regression was used to examine unadjusted and adjusted associations of salivary hBD and LL-37 concentrations with age, gender, periodontitis, smoking, AGE, and HbA1c. All analyses were conducted by using the IBM SPSS Statistics 24.0 (IBM Corporation, Armonk, NY, USA).

Results

Demographic, periodontal clinical and biochemical variables of study groups were presented in Table 1. A 47% of T2DM patients used intramuscular insulin injection for their diabetic treatment and mean diabetes duration was 6.82 ± 2.79 years. PI, GI, CAL and PPD values were significantly higher in individuals with periodontitis than periodontally healthy participants as expected ($p < .001$).

Salivary antimicrobial peptide levels were presented in Table 2. Among the systemically healthy individuals, periodontitis patients had higher levels of hBD-1 ($p = .005$) and hBD-3 ($p = .039$) in comparison to periodontally healthy controls. Among the T2DM patients, lower levels of hBD-1 ($p = .003$) and higher levels of LL-37 ($p < .001$) were observed in periodontitis patients in comparison to periodontally healthy controls. When only periodontitis patients were

Table 1. Demographic, clinical and biochemical data of study groups.

	SH + C ($n = 28$)	SH + P ($n = 29$)	T2DM + C ($n = 58$)	T2DM + P ($n = 63$)	p Value
Age (years)	43.9 ± 14.4	45.4 ± 12.9	49.4 ± 10.4	50.2 ± 9.9	.045*
Female (%)	17.3	15.3	33.7	33.7	.860**
Smoking (%)	85.3	85.3	66.9	62.5	.331**
GI	0.27 ± 0.25	2.23 ± 0.27	0.31 ± 0.20	2.29 ± 0.31	<.001*
PI	0.53 ± 0.42	2.56 ± 0.25	0.56 ± 0.34	2.55 ± 0.24	<.001*
PPD (mm)	2.26 ± 0.38	5.30 ± 0.53	2.31 ± 0.37	5.52 ± 0.53	<.001*
CAL (mm)	2.67 ± 0.69	5.80 ± 0.72	2.59 ± 0.65	6.29 ± 0.69	<.001*
FPG (mg/dL)	95.6 ± 10.1	97.8 ± 12.1	170 ± 60.2	167 ± 54.7	<.001*
HbA1c (%)	5.56 ± 0.45	5.41 ± 0.54	8.45 ± 1.63	8.41 ± 1.35	<.001*
AGE (ng/ml)	24.4 ± 38.5	46.8 ± 52.1	235 ± 360	332 ± 350	<.001*

Age, gingival index (GI), plaque index (PI), probing pocket depth (PPD), clinical attachment level (CAL), fasting plasma glucose (FPG), HbA1c and advanced glycalization end products (AGE) levels are expressed as mean and standard deviations.
*ANOVA.
**Chi-square.
SH + C: systemically health + control; SH + P: systemically health + periodontitis; T2DM + C: type 2 diabetes mellitus + control; T2DM + P: type 2 diabetes mellitus + periodontitis.

Table 2. Salivary human beta defensin (hBD) and cathelicidin (LL-37) levels of study groups.

	SH + C ($n = 28$)	SH + P ($n = 29$)	T2DM + C($n = 58$)	T2DM + P ($n = 63$)	p Value
hBD-1 (ng/L)	3 (1.07–19)	8.8 (2.1–31)	8.5 (0.02–23)	3 (0.09–34)	.001*
hBD-2 (ng/L)	2.5 (2.5–27)	9.5 (2.5–23)	2.5 (2.5–27)	2.5 (2.5–31)	.007*
hBD-3 (ng/L)	2.6 (1–21)	7.4 (1–23)	1 (0.46–17)	1 (0.46–54)	<.001*
LL-37 (ng/ml)	0.5 (0.02–3)	0.5 (0.02–5.5)	1.6 (0.01–6.2)	2.5 (0.59–7.5)	<.001*

hBD and LL-37 levels are expressed in medians (minimum and maximum levels in parenthesis).
*Kruskal–Wallis *H*.
SH + C: systemically health + control; SH + P: systemically health + periodontitis; T2DM + C: type 2 diabetes mellitus + control; T2DM + P: type 2 diabetes mellitus + periodontitis.

Table 3. Adjusted (age, gender, smoking status, periodontitis, glyceamic indicators) associations of salivary antimicrobial peptides with periodontitis and type 2 diabetes mellitus.

		hBD-1	hBD-2	hBD-3	LL-37
Middle tertile	AGE	1.001 (0.999–1.002), .447	1.001 (0.999–1.002), .470	1.00 (0.998–1.002), .830	**1.006 (1.001–1.011), .024**
	HbA1c	1.080 (0.862–1.354), .504	1.289 (0.992–1.676), .058	0.757 (0.564–1.017), .064	**2.204 (1.463–3.321), <.001**
	Periodontitis	0.475 (0.219–1.033), .060	1.715 (0.679–4.326), .254	2.017 (0.833–4.884), .120	2.104 (0.810–5.466), .127
Highest tertile	AGE	1.001 (1.000–1.002), .190	**1.001 (1.000–1.003), .047**	1.001 (1.000–1.003), .058	**1.008 (1.002–1.013), .004**
	HbA1c	0.895 (0.721–1.112), .317	**0.647 (0.500–0.837), .001**	**0.650 (0.508–0.832), .001**	**2.573 (1.658–3.994), <.001**
	Periodontitis	0.607 (0.294–1.252), .177	0.866 (0.433–1.729), .683	1.323 (0.655–2.675), .435	**7.258 (2.443–21.57), <.001**

Salivary antimicrobial peptide concentrations were converted into tertiles and the lowest tertile is taken as reference. Data is presented as odds ratio (95% confidence interval), *p* value. Bold data indicate a significant association.

included into the statistical analyses, T2DM patients demonstrated lower levels of hBD-1 ($p = .006$), hBD-2 ($p < .001$) and hBD-3 ($p < .001$), and higher levels of LL-37 ($p < .001$) compared to systemically healthy controls. When only periodontally healthy controls were included into the analysis, hBD-1 ($p = .002$) and LL-37 ($p < .001$) levels were elevated in T2DM patients in comparison to systemically healthy controls.

There was a strong significant positive correlation between salivary LL-37 levels and periodontal parameters and, AGE and HbA1c levels ($p < .001$). Positive correlation was also observed between the salivary hBD-3 and AGE levels ($p < .001$). Salivary hBD-2 levels were negatively correlated with FPG levels ($p < .001$)

According to the regression analysis (Table 3), hBD-2 and hBD-3 concentrations inversely associated with HbA1c levels. Adjusted associations indicated that the salivary LL-37 concentrations independently associated with AGE, HbA1c levels and periodontitis.

Discussion

To best of author's knowledge, this is the first study to show the association of HbA1c with salivary hBDs and LL-37 concentrations. Periodontitis, on the other hand, associated only with salivary LL-37 concentrations.

In the present study, FPG and HbA1c values were used to diagnose the T2DM. HbA1c is a demonstrative indicator to represent clinical status of DM [13] and also be predictor for the diabetes-associated risk for periodontitis [14]. Relatively high number of participants and inclusion of periodontitis risk factors are main strengths of the present study.

The cross-sectional design of our study did not allow us to monitor possible fluctuations in the secretions of hBDs and LL-37 in response to changes in the glycemic status, which can be considered as limitation. Salivary flow rate may affect the salivary antimicrobial peptide levels. To minimize the effect of salivary flow rate, antimicrobial peptide levels could be given in relation to total amount of protein. However, protein determination was not performed due to lack of sample material after several repetitions in ELISA tests. Sample size could not be calculated before the beginning of this study because there was not any study investigating the effect of periodontitis and T2DM on salivary hBD levels in literature to find an effect size. Therefore, achieved power (observed power) was calculated. Finally, most of individuals diagnosed with periodontitis had severe form of disease. Additional studies including participants with gingivitis and initial periodontitis may be useful to understand how those peptides change in the presence of gingival inflammation and would be valuable to see the trend how those defensive proteins increase as the severity of periodontal disease increase.

According to our results, elevated salivary LL-37 levels associate with periodontitis, while salivary levels of hBD-1, hBD-2, and hBD-3 were not related to periodontal status. Increased salivary and GCF hCAP18/LL-37 levels were previously presented in individuals with periodontitis [26–28]. Elevated LL-37 levels in participants with periodontitis could be explained by the rise in number and activation of neutrophils in periodontitis, as these cells are the main source of LL-37 [1–3]. On the contrary to the present findings, Davidopoulou et al. [10] found no difference in salivary LL-37 levels between periodontitis and the control groups. The inconsistency may be explained by relatively low proportion of individuals with severe periodontitis that were included in that study. According to our results, salivary hBD levels are not associated with periodontitis. Controversial findings were presented in terms of hBD levels in periodontitis-affected oral biologic fluids and tissues; RNA and protein expression profiles of hBDs in periodontitis were found to be elevated [9], steady [7,15] or suppressed [8]. On one hand, infection activates hBD expression. On the other hand, the disturbed epithelial structure and integrity of the infected gingiva limit the secretion and release of antimicrobial defensins. Very recently our group demonstrated that the gingival tissue levels of hBD-2 and hBD-3 are not prone to the shifts in the severity of inflammation but indeed show a negative correlation with total protease activities [29]. Highly elevated proteolytic activity in the disease-affected periodontium may limit the transition of tissue hBDs to saliva, as these peptides can be degraded by host- and bacterial enzymes [4,6]. Thus, it is possible to state that elevated RNA and protein expressions of hBDs at the early stages of infection and inflammation can be hindered by the disruption of tissue integrity or by their immediate degradation by disease-induced proteases.

We showed a positive association between salivary LL-37 with salivary AGE and HbA1c. In the literature, there is limited information related to the salivary LL-37 levels in individuals with T2DM [19]. In line with the present results, we previously demonstrated elevated LL-37 levels in gingival tissues of participants with T2DM [15]. Despite increased levels of antimicrobial LL-37, individuals with T2DM are prone to periodontal infection. This might be, at least partly, related to the cytotoxic effects of LL-37 on host cells at high concentrations [30]. On the other hand, present findings pointed out that the salivary levels of hBD-2 and -3 were decreased in participants diagnosed with T2DM. In the literature, Lan et al. [31] indicated

that hyperglycaemic conditions suppress the hBD-3 expression of human keratinocytes at mRNA and protein levels. They explained their findings via the inhibition of P38 mitogen-activated protein kinases (p38MAPK) signalling by excessive AGE formation. Another *in-vitro* study reported that even though AGE can induce a transient increase of p38MAPK signalling, yet at higher levels, AGE leads to reduced expression of antimicrobial peptides [32]. However, there are also other studies that presented increased levels of hBDs in individuals with T2DM [15,17]. These differences may relate to the variations in sample materials (gingiva, GCF, saliva) and diabetic control of participants. We speculate that hyperglycaemia can be one major determinant in the hBD expression profile in individuals diagnosed with periodontitis and T2DM.

Conclusion

Within the limitations of this study, hyperglycaemia seems to be an independent regulator of salivary hBD-1, hBD-2 and hBD-3 concentrations. Salivary LL-37, on the other hand, is affected by both local inflammatory (periodontitis) and systemic (T2DM) factors. Further studies to understand the underlying mechanism of these alteration and possible effect of antidiabetic medications on antimicrobial peptide expression are warranted.

Disclosure statement

No potential conflict of interest was reported by the authors.

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