Salivary Azurocidin and Tumor Necrosis Factor-α Levels in Patients with Stage III-IV, Grade C Periodontitis

Evre III-IV, Derece C Periodontitis Hastalarında Tükürük Azurosidin ve Tümör Nekroz Faktör-α seviyeleri

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ABSTRACT

Objective: Azurocidin, a neutrophil granule-derived antimicrobial protein, plays an important role in host-pathogen interactions and immunoregulation. Periodontitis is a polymicrobial inflammatory disease caused by the host response to subgingival microbiota. This study aimed to evaluate salivary azurocidin and tumor necrosis factor (TNF)-α levels in patients with stage III-IV, grade C periodontitis.

Methods and Materials: In total 78 systemically healthy individuals (40 periodontitis (stage III-IV, grade C) and 38 periodontally healthy) were enrolled. Saliva samples were collected and clinical periodontal measurements including probing depth (PD), clinical attachment loss (CAL), the percentage of sites with bleeding on probing (BOP %), gingival index (GI) and plaque index (PI) were recorded. Salivary azurocidin and TNF-α levels were measured by ELISA.

Results: Periodontitis group exhibited higher PD, CAL, BOP (%), GI and PI values compared to the periodontally healthy group ($p<0.001$). Salivary azurocidin and TNF-α concentrations in periodontitis group were significantly higher than healthy controls ($p<0.05$). There were positive correlations among azurocidin, TNF-α and all clinical periodontal parameters ($p<0.001$).

Conclusion: Present findings suggest that salivary azurocidin and TNF-α levels may involved in periodontal inflammation and may be useful in the diagnosis of periodontal diseases. Further studies are needed to enlighten the role of these molecules in the pathogenesis of periodontal disease.

Key words: *Periodontitis, inflammation, saliva, cytokine*

ÖZ

Amaç: Azurosidin, nötrofillerin granüllerinde sentezlenen, konak-patojen etkileşimlerinde ve immunregülasyonda önemli rol oynayan antimikrobiyal bir proteindir. Periodontitis, subgingival mikrobiyotaya karşı gelişen konak yanıtından kaynaklanan polimikrobiyal enflamatuvar bir hastalıktır. Bu araştırma, evre III-IV, derece C periodontitis hastalarında tükürük azurosidin ve tümör nekroz faktör (TNF)-α seviyelerini değerlendirmeyi amaçlamaktadır.

Yöntem: Çalışmaya sistemik sağlıklı toplam 78 birey (40 periodontitis (evre III-IV, derece C) ve 38 sistemik sağlıklı) dahil edildi. Tükürük örnekleri toplandı ve sondalama derinliği (SD), klinik ataşman kaybı (KAK), sondalamada kanama yüzdesi (SK %), gingival indeks (GI) ve plak indeksini (PI) içeren klinik periodontal ölçümler kaydedildi. Tükürük azurosidin ve TNF-α seviyeleri ELISA ile ölçüldü.

Bulgular: Periodontitis grubunun SD, KAK, SK (%), Gİ ve Pİ değerleri, periodontal sağlıklı gruptan anlamlı yüksek bulundu ($p<0.001$). Periodontitis grubunun tükürük azurosidin ve TNF-α konsantrasyonları, periodontal sağlıklı gruptan anlamlı yüksekti ($p<0.05$). Azurosidin, TNF-α ve tüm klinik periodontal parametreler arasında anlamlı pozitif korelasyonlar gözlendi ($p<0.001$).

Sonuç: Bu çalışmanın bulguları, tükürük azurosidin ve TNF-α seviyelerinin periodontal enflamasyon ile ilişkili olabileceğini ve periodontal hastalıkların tanısında yarar sağlayabileceğini düşündürmektedir. Bu moleküllerin, periodontal hastalık patogenezindeki rollerine ilişkin daha ileri çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: *Periodontitis, enflamasyon, tükürük, sitokin*

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INTRODUCTION

Periodontitis is a chronic inflammatory disease characterized by irreversible destruction of connective tissue attachment and alveolar bone, eventually leading to tooth loss[1]. It has been generally accepted that periodontal tissue breakdown results from the complex interaction between subgingival pathogenic bacteria and host immunoinflammtory response[2]. Periodontal pathogens, especially Gram-negative anaerobic bacteria[3], activate host cells to produce proinflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 and proteolytic enzymes, which in turn promote the destruction of periodontal tissues[2].

Neutrophils are the most abundant leukocytes recruited to the gingival sulcus in response to bacterial biofilm.[2,4] They respond to bacterial products by microbial killing via oxygen-dependent and oxygen-independent mechanisms.[4] The non-oxidative antibacterial mechanisms involve a diverse group of antimicrobial peptides[5] which play a role in periodontal inflammation and wound healing.[6,7] Azurocidin is also a 37 kDa cationic antimicrobial protein expressed in azurophil granules of neutrophils.[8] It is considered to be inactive serine protease homologou due to the lack of its proteolytic activity.[8,9] Azurocidin exhibits broad spectrum of antimicrobial activity, particularly against Gram-negative bacteria.[10] It also has chemotactic effects on monocytes/macrophages and T cells and enhances macrophage phagocytosis.[9] Moreover, the release of TNF-α and IL-6 from monocytes in response to lipopolysaccharide (LPS) is induced by azurocidin.[9,11]

Earlier studies have reported that azurocidin is a marker of a worse prognosis in sepsis and acute respiratory distress syndrome.[12] Elevated salivary azurocidin levels were observed in patients with type 1 diabetes mellitus with retinopathy and/or nephropathy.[13] Therefore, it might be potential screening targets for diabetes mellitus complications.[13] To date, there is limited data on the relationship between azurocidin and periodontal condition. Choi et al.[14] demonstrated for the first time the presence of azurocidin in gingival crevicular fluid (GCF) of chronic periodontitis patients by proteomic analysis. The authors validated the elevated GCF azurocidin levels in these patients by ELISA. Leppilahti et al.[15] found higher GCF azurocidin levels in chronic periodontitis compared to gingivitis and healthy sites which were also positively correlated with expression of collagenolytic matrix metalloproteinases. Guzman et al.[16] suggested that azurocidin might be the strongest candidate biomarker for periodontitis due to decreased GCF azurocidin levels following non-surgical periodontal treatment.

Saliva, an important physiological fluid, has been proposed as a non-invaziv diagnostic medium that could be used in the diagnosis of oral and systemic diseases.[17] Whole saliva is a complex mixture derived from not only major and minor salivary glands but also GCF that contains products of inflammation ongoing in periodontium.[18] It has been suggested that GCF is the primary source of periodontitis-associated cytokines in whole saliva.[19] Analyses of inflammatory biomarkers in saliva could serve as an attractive tool for the diagnosis of periodontal diseases.[18] Various enzymes, cytokines and biomarkers that are involved in periodontal tissue breakdown have been found to be elevated in saliva of periodontitis patients in comparison with periodontally healthy controls.[17-19] Considering the possible relationship between azurocidin and periodontal inflammation[14-16], it was hypothesized that salivary azurocidin and TNF-α levels increase in periodontitis patients. The aim of this study was to investigate whether salivary azurocidin levels could be used as a discriminatory biomarker to distinguish periodontally healthy and periodontal diseased individuals.

MATERIALS AND METHODS

Study Population and Clinical Examination

A total of 78 individuals (38 females and 40 males; age range 30-46 years) seeking dental treatment in the School of Dentistry, Adnan Menderes University, Aydın, Turkey, were recruited for the present study from March to October of 2019. The protocol was approved by the Ethics Committee of the School of Dentistry, Adnan Menderes University with the protocol number (No: ADÜDHF2019/053) and the research was conducted in compliance with the Declaration of Helsinki (version 2008). The purpose and procedures of the study were explained and written informed consent was received from each participant prior to participation.

A comprehensive medical and dental history was taken on all participants with an oral examination. All participants were never smokers. Each individual had at least 20 natural teeth. Patients were excluded from the study if they were suffering from any chronic inflammatory or immunological conditions such as diabetes mellitus, cardiovascular diseases, rheumatoid arthritis and mucocutaneous diseases. None of the females were pregnant or lactating. Individuals who took

medications such as antimicrobials, anti-inflammatory drugs and immunosuppressive agents within 4 months before study entry were also excluded. Participants had not undergone nonsurgical/surgical periodontal therapy in the preceding 12 months. Individuals who had orthodontic appliances, removable partial dentures and restorative and endodontic treatment requirements were not included in this study.

Probing depth (PD), clinical attachment loss (CAL), gingival index (GI)[20], the percentage of sites with bleeding on probing (BOP %) and plaque index (PI)[21] were assessed during periodontal clinical examinations. All measurements were obtained in 6 sites per tooth, except the third molars, using a manual periodontal probe (William's periodontal probe, Hu-Friedy, Chicago, IL) by a single trained examiner (BA). PD and CAL were determined by the distance from reference point to bottom of pocket using reference as gingival margin and cement-enamel junction respectively. As an indicator of oral hygiene, PI is determined by the Quigley-Hein Plaque Index as modified by Turesky[21]. The alveolar bone resorption was assessed on the digital panoramic radiograph in each participant.

According to the diagnostic criteria proposed by the 2017 International Workshop on the Classification of Periodontal and Peri-implant Diseases and Conditions[22] participants were categorized into two distinct groups based upon their periodontal status: 1) 40 patients with generalized stage III-IV, grade C periodontitis 2) 38 periodontally healthy individuals. The severity of periodontitis was defined by the amount of CAL. These patients had at least two interdental sites (at two non-adjacent teeth) with CAL ≥ 5 mm. Care was taken to ensure that CAL was not caused by non-periodontal causes such as dental caries extending in the cervical area of the tooth, gingival recession of traumatic origin, the presence of CAL on the distal aspect of a second molar due to extraction of a third molar, an endodontic lesion draining through the marginal periodontium and the occurrence of a vertical root fracture.The complexity of periodontitis was defined by PD and furcation involvement. These patients had PD ≥ 6 mm at least two interdental sites (at two non-adjacent teeth) and also Class II-III furcation involvement. Considering the extend and distribution of periodontitis, these patients had CAL at ≥ 30 of teeth involved.

The grading of periodontitis was evaluated indirect evidence of progression through % bone loss/ age. Radiographic alveolar bone loss (RBL) of each natural tooth was assessed by using the panoramic radiograph. It was recorded by measuring the mesial and distal of the remaining teeth (except the 3rd molars) from the cemento-enamel junction of the tooth up to the highest point of the proximal alveolar bone crest. The most extensive bone loss (%) (RBL expressed as percentage of root length) was determined and % bone loss/age were calculated. When this value was > 1.0, participants were included in Grade C.

Periodontally healthy individuals in the control group had an intact periodontium (without detectable CAL or RBL) or a reduced periodontium in a non-periodontitis patient. In this group, PD was ≤ 3 mm and BOP was < 10 % in the whole mouth.

Saliva Collection

All samples were collected before clinical measurements. Unstimulated whole saliva obtained from the participants in the morning between 8:00 am-9:30 am after an overnight fast. A modification of the method described by Navazesh[23] was used for saliva collection. All individuals were asked to avoid any oral care practice such as brushing, interdental cleaning and rinsing with mouthwash before collection. Each participant was requested first to rinse the mouth completely with tap water for 2 minutes and to wait for 10 minutes. Then, the participants were asked to let the saliva pool in their floor of the mouth and to allow the saliva to drain passively into a sterile plastic container for 5 minutes. Saliva samples were held on ice and then stored at -40°C, until the analysis.

Measurement of Azurocidin and TNF-α Levels in Saliva Samples

On the day of analysis, frozen saliva samples were thawed on ice and centrifuged at 10000 x g for 15 minutes at 4 °C. Azurocidin and TNF-α concentrations in saliva samples were determined by the enzyme-linked immunosorbent assay (ELISA) using commercial kits (Human Azurocidin and TNF-α ELISA kits, Sunred Biotechnology, Shanghai) in accordance with the manufacturer's guidelines. The minimum detection limits in the assays were 0.25 ng/ml for azurocidin and 3 ng/L for TNF-α. Absorbance was measured at 450 nm with 650 nm as a reference wavelength by an automated ELISA plate reader (DTX 880 Multimode Reader, Beckman Coulter, Miami, FL). Cytokine concentrations were determined from the standard curve. Salivary concentrations of azurocidin and TNF-α were presented as ng/ml and ng/L, repectively.

Statistical Analysis

The sample size was calculated using a specialized software package (G*Power version 3.0.8, Heinrich Heine University, Düsseldorf) for power analysis. This analysis was performed on the basis of the findings of a study by Choi et al.[15] Considering a significant difference between two study groups for GCF azurocidin levels at 0.67 effect size (d) and at α=0.05 significance level with a power of 80 % using an independent samples T-test (difference between the means in two unrelated groups) the minimum sample size required was 36 in each group.

All data analyses were performed using a statistical package(SPSSv25.0, IBM, Chicago, IL.) Thedistribution of the clinical and biochemical data was validated by Shapiro Wilk's normality test. Comparisons of clinical and biochemical parameters between the study groups were performed using the independent two samples T- test. Chi-square analysis was used to compare the proportion of genders between groups. The correlations among salivary azurocidin and TNF-α levels and clinical periodontal parameters were determined by Spearman rank correlation analysis. Statistical significance was considered at p <0.05 for all the tests.

RESULTS

The demographic characteristics and whole mouth clinical periodontal parameters are presented in Table 1. There were no statistical differences in terms of age and gender distribution between periodontitis and periodontally healthy groups (p=0.299 for age; p=0.825 for gender distrubition). The mean PD and CAL values of periodontitis patients were significantly higher than those of healthy individuals (p <0.001). PI, GI and BOP (%) scores were also significantly higher in the diseased group compared to the healthy controls (p <0.001).

Azurocidin was detected in all saliva samples. Salivary azurocidin concentrations (ng/ml) of the studied groups were shown Figure 1. Periodontitis group had significantly higher salivary azurocidin levels compared to healthy controls (P =0.006).

Salivary TNF-α concentrations (ng/L) of study groups were demonstrated Figure 2. TNF-α was also detected in all saliva samples. Periodontitis group had significantly higher salivary TNF-α levels compared to healthy controls (P <0.001).

Salivary azurocidin and TNF-α concentrations showed significant positive correlations with all clinical periodontal parameters (P <0.05, Table 2). There was also a positive correlation between azurocidin and TNF-α levels in saliva (r= 0.376, p<0.001, Table 2).

Table 1. The demographic characteristics and whole-mouth clinical periodontal parameters of the study groups.

	Periodontitis (n=40)	Periodontal Health (n=38)	p- value
Demographic variables			
Age (years)	37.75 ± 4.53	36.68 ± 4.46	0.299
Sex (Female / Male)	19/21	19/19	0.825
Periodontal parameters			
PD (mm)	4.43 ± 0.58*	1.66 ± 0.18	<0.001
CAL (mm)	5.39 ± 0.82*	0.02 ± 0.02	<0.001
BOP (%)	82.40 ± 5.58*	1.78 ± 1.09	<0.001
GI	2.34 ± 0.28*	0.03 ± 0.01	<0.001
PI	3.54 ± 0.34*	1.73 ± 0.41	<0.001

All data (except for sex) are given as mean ± SD. *p <0.001, significantly higher than periodontally healthy group.
PD: Probing depth, CAL: Clinical attachment loss, GI: Gingival index, BOP: Bleeding on probing, PI: Plaque index.

Table 2. Correlations of salivary azurocidin and TNF -α levels with clinical periodontal parameters and each others.

Variables		Azurocidin	TNF-α
PD	r	0.261*	0.597**
CAL	r	0.228*	0.553**
BOP (%)	r	0.357**	0.795**
GI	r	0.339**	0.765**
PI	r	0.267*	0.570**
Azurocidin	r		0.376**
TNF-α	r	0.376**	

* Correlation is significant at the 0.05 level. **Correlation is significant at the 0.01 level.
PD: Probing depth, CAL: Clinical attachment loss, BOP: Bleeding on probing,
GI: Gingival index, PI: Plaque index, TNF-α: Tumor necrosis factor-α.

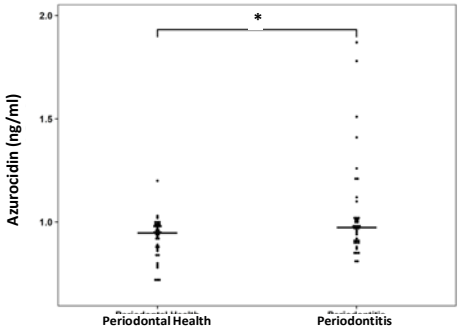

Figure 1: Saliva azurocidin levels (ng/ml) in periodontitis and in periodontal health. Each dot represents an individual sample, and the solid horizontal lines indicate median values. Significant difference at *p <0.05.

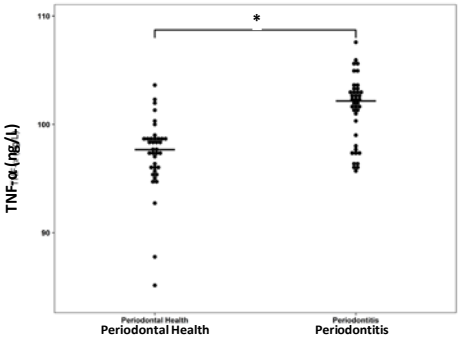

Figure 2: Saliva TNF-α levels (ng/L) in periodontitis and in periodontal health. Each dot represents an individual sample, and the solid horizontal lines indicate median values. Significant difference at *p <0 .05.

DISCUSSION

To the best of our knowledge, the present study demonstrated for the first time the salivary levels of azurocidin in periodontally healthy and periodontal diseased individuals. Generalized stage III or IV, grade C periodontitis patients had higher salivary azurocidin and TNF-α levels compared to periodontally healthy individuals.

There is adequate evidence that a significant portion of the inflammatory destruction in periodontal tissues results from the collateral damage by hyperactive neutrophils or neutrophils present in excessive numbers.[4] They can eliminate pathogens by phagocytosis and intracellular killing through oxidative and proteolytic inflammatory reactions, and by extracellular mechanisms such as degranulation and release of neutrophil granule-derived mediators like neutrophil extracellular traps.[4,8] Azurocidin can be released both at a very early stage and at the late stage of inflammation due to its storage in two subsets of granules.[8,9] Therefore, in the present study, increased salivary azurocidin levels in patients with periodontitis could be attibuted to enhanced neutrophils infiltration and azurocidin release in the inflamed periodontal tissues.

Similar to other neutrophil-derived antimicrobial proteins such as defensins, cathelicidin and lysozyme, azurocidin is also involved in host-pathogen interactions and immunoregulation.[5,9] Due to its cationic properties and hydrophobicity, azurocidin has strong affinity for LPS, a

main component of the cell wall of gram-negative bacteria and neutralizes it during infection.[9,10] Periodontal disease is also a polymicrobial inflammatory disease caused by especially Gram-negative periodontopathic bacteria.[3] In the present study increased salivary azurocidin levels in periodontitis patients may be associated with elevated Gram-negative periodontopathic bacteria count and the strong affinity of azurocidin to LPS. Further mechanistic studies will provide more information about the role of azurocidin in periodontitis.

Although human saliva proteomic analysis helped in the identification of azurocidin[24], there are no data about salivary azurocidin levels in patients with periodontitis. The role of azurocidin in periodontal disease was investigated in GCF studies. Leppilahti et al.[14] have found that GCF azurocidin levels were higher in periodontitis sites relative to healthy sites and those levels showed high diagnostic accuracy ($\geq$ 0.90) for periodontitis. Choi et al.[15] have found that GCF levels of azurocidin are higher in gingivitis and moderate periodontitis compared to severe periodontitis as well as periodontal health. The authors suggested that azurocidin might serve as a promising biomarker for a biomarker for the early detection of inflammatory periodontal destruction. In the same study[15], it has been also demonstrated that azurocidin was expressed at a higher level in gingival tissues of periodontitis patients and inhibited the differentiation of macrophages to osteoclasts. Therefore, the authors speculated that elevated azurocidin levels during gingivitis may have a protective effect on alveolar bone during the early stages of periodontitis. On the other hand, increased alveolar bone loss during later stages of periodontitis could be due to decreased azurocidin levels and lack of its protective effect in severe periodontitis.[15] Guzman et al.[16] reported decreased GCF azurocidin levels after non-surgical periodontal treatment in chronic periodontitis. In agreement with those studies[14-16] increased salivary azurocidin levels in periodontitis and positive association with all clinical periodontal parameters might reflect the potential association of azurocidin with periodontal inflammation.

In addition to its antimicrobial activity, azurocidin also contributes to enhance the LPS-induced induction of proinflammatory cytokines such as TNF-α, IL-6 and IFN-γ from monocytes and macrophages via β2-integrins.[9,11] TNF-α, the well-known marker of periodontal inflammation and alveolar bone resorption[25], has been found increased levels in GCF and saliva of patients with periodontal disease[18]. It causes connective tissue and alveolar bone destruction by enhancing matrix metalloproteinase secretion and osteoclast formation.[25] Similar to previous studies[26,27], in the current study periodontitis patients had elevated salivary TNF-α levels and its positively correlation with azurocidin may provide further support for the previous studies reporting that azurocidin enhanced the LPS-mediated proinflammatory cytokines release from monocytes and macrophages.

The present study had some limitations. Given the cross-sectional nature of the study, causality cannot be established between biomarker concentrations and periodontal status. Another limitation may be the lack of gingivitis group. Gingivitis group might allow us to compare how azurocidin and TNF-α are involved in the destructive process during the periodontal disease progression. Finally, the lack of GCF and serum analyses may be considered as a limitation. The correlations among GCF, serum and salivary biomarker levels might provide further information regarding the role of azurocidin and TNF-α in periodontal inflammation.

CONCLUSIONS

To the best of our knowledge, the present study demonstrated for the first time the salivary levels of azurocidin in patients with peridontitis and also in periodontally healthy individuals. Within the limitations of this study, elevated salivary levels of azurocidin in patients with generalized stage III-IV, grade C periodontitis and its positively correlations with whole-mouth clinical periodontal parameters and TNF-α implies that salivary azurocidin levels may involved in periodontal inflammation and may be useful to discriminate periodontitis to periodontal health. Further investigations with larger sample size are needed to elucidate the potential relationship of salivary azurocidin and TNF-α levels with periodontal inflammation.

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