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Araştırma Makalesi / Research Article

DETERMINATION of the ANTI-INFLAMMATORY CYTOKINES in SMOKING INDIVIDUALS with PERIODONTITIS

SİGARA İÇEN PERİODONTİTİSLİ BİREYLERDE ANTİ-INFLAMATUVAR SİTOKİNLERİN BELİRLENMESİ

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ABSTRACT

Periodontitis, a chronic condition impacting the supportive tissues of teeth, manifests when the host's immune response becomes imbalanced with periodontal pathogens. Smoking emerges as a significant risk factor in both initiation and progression of periodontitis. During inflammatory process, a conspicuous upregulation of both proinflammatory and regulatory cytokines is unavoidable. Resolution of inflammation holds significance in the context of periodontitis, predominantly orchestrated by immune cells generating interleukin (IL)-4 and IL-10. Objective of this study was to evaluate the local and systemic involvement of IL-4 and -10 in individuals with severe periodontitis who are smokers. 60 individuals with Stage III Grade C periodontitis were recruited, comprising both smokers and non-smokers, along with an additional 60 periodontally healthy individuals who were divided into smoking and non-smoking categories. Comprehensive periodontal indices were documented for all participants, and both venous blood and gingival crevicular fluid (GCF) samples were obtained for cytokine level analysis. Levels of IL-4 and -10 in GCF and serum samples were determined using a multiplex immunoassay. Statistical evaluation involved the use of Mann-Whitney U, Student-t, and Chi-square tests. In our investigation, all periodontal indices among individuals with periodontitis exhibited a statistically significant elevation compared to those of their healthy counterparts ($p<0.05$). Levels of IL-4 and -10 in GCF of non-smoking and smoking individuals with periodontitis were notably lower than those in the GCF of healthy individuals ($p<0.05$). Smoking individuals with periodontitis had the lowest GCF IL-4 levels than other groups ($p<0.05$). Serum concentrations of IL-4 and -10 in periodontitis patients who were smoking exhibited a reduction compared to all other groups ($p<0.05$). It can be inferred that the onset and advancement of periodontal inflammation might be attributed to a deficiency or inadequate response of anti-inflammatory. Smoking could potentially act as a co-suppressor for IL-4 and -10.

Keywords: Interleukin, Gingival crevicular fluid, Plasma, Serum, Periodontitis, Nicotine

ÖZET

Periodontitis, dişleri destekleyen dokuları etkileyen kronik bir hastalık olup periodontal patojenlere karşı konak bağışık yanıt dengesizliklerinde ortaya çıkar. Sigara, periodontitisin başlangıcında ve ilerlemesinde önemli bir risk faktörüdür. İnflamatuvar süreç sırasında, sitokinlerin belirgin bir şekilde artışı kaçınılmaz olup inflamasyonun çözünmesi periodontitiste önem taşımaktadır. İnflamasyonun çözülmesinde başlıca rol alan sitokinler interlökin (IL)-4 ve IL-10'dur. Bu çalışmanın amacı, şiddetli periodontitisli, sigara içen bireylerde IL-4 ve -10'un lokal ve sistemik katılımını değerlendirmekti. Bu amaç doğrultusunda, sigara içen ve içmeyen olarak ayrılmış Evre III Derece C periodontitisli 60 birey ile yine sigara içen ve içmeyen olarak ayrılmış periodontal olarak sağlıklı 60 birey çalışmaya dahil edildi. Periodontal indekslerin kaydını takiben IL-4 ve

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IL-10 düzeylerinin serumda ve diş eti oluğu sıvısındaki (DOS) analizi için tüm katılımcılardan venöz kan ve DOS örnekleri alındı. Sitokişn değerleri multipleks immunoassay yöntemi ile belirlendi. İstatistiksel değerlendirme Mann-Whitney U, Student-t ve Ki-kare testleri kullanılarak yapıldı. Araştırmamızda, periodontitisli bireyler arasında tüm periodontal indekslerin, sağlıklı bireylere oranla istatistiksel olarak önemli ölçüde yüksek olduğu bulundu ($p<0.05$). Sigara içmeyen ve içen periodontitisli bireylerin DOS IL-4 ve -10 düzeyleri, sağlıklı bireylerinkinden belirgin olarak daha düşüktü ($p<0.05$). Sigara içen periodontitisli bireylerin DOS IL-4 düzeyleri ise diğer tüm gruplardan daha düşük bulundu ($p<0.05$). Serum IL-4 ve -10 konsantrasyonları sigara ice periodontitisli bireylerde, diğer tüm gruplara kıyasla düşüktü ($p<0.05$). Sonuç olarak, periodontal inflamasyonun başlaması ve ilerlemesi, anti-inflamatuarın yetersiz yanıtına veya eksikliğine bağlanabilir. Sigara, potansiyel olarak IL-4 ve -10 için baskılayıcı bir işlev görebilir.

Anahtar Kelimeler: İnterlökin, Diş eti oluğu sıvısı, Plazma, Serum, Periodontitis, Nikotin

INTRODUCTION

Periodontitis is defined as a bacterial infection causing tissue damage and tooth loss. In the pathogenesis of periodontal disease, besides the mechanisms through which pathogenic microorganisms cause damage, environmental and genetic risk factors also play a significant role (Beck et al., 2000; Kinane and Chestnutt, 2000; Salvi et al., 1997). The part of smoking as a risk factor in periodontitis is thoroughly established (Gonçalves et al., 2011). Smoking seems to alter the composition, encourage the colonization of crucial periodontal pathogens, and impact bacterial aggregation, rather than affecting the rate and quantity of plaque accumulation (Bunæs et al., 2017a). It is conceivable that numerous mechanisms contributing to tissue degradation in periodontitis among tobacco smokers could differ significantly from those in non-smokers (Palmer et al., 2005).

Immune mechanisms triggered by host-bacteria interaction lead to the abundant release of inflammatory mediators, initiating tissue destruction(Okada and Murakami, 1998). Among these inflammatory mediators are pro-inflammatory cytokines, which play a significant role in the pathogenesis of periodontitis and serve as immune-regulatory agents derived from T lymphocytes (Gemmell et al., 1997; Kusumoto et al., 2004; Okada and Murakami, 1998). It is believed that the cytokine response plays a critical role in the pathogenesis of periodontal disease. A proper cytokine response against microbial plaque is accepted to be associated with a protective immune balance and a stable periodontal disease condition. Conversely, an inappropriate response alters the immune balance in a way that increases tissue destruction, leading to the progression of periodontal disease (Seymour and Gemmell, 2001). According to the Al-Hamoudi et al. the reduced expression of anti-inflammatory cytokines in the gingivocrevicular fluid (GCF) might have worsened clinic and radiographic periodontal inflammatory parameters(Al-Hamoudi et al., 2020).

Numerous studies have shown that anti-inflammatory cytokines, such as IL-4 and IL-10, have the ability to suppress the production of pro-inflammatory cytokines from effector cells like macrophages(Giannopoulou et al., 2003a; Kinney et al., 2014; Ozer Yucel, 2015; Stolf et al., 2023). IL-4 effectively suppresses macrophage function by inhibiting the secretion of IL-1β, tumor necrosis factor-α (TNF-α), and IL-6. Additionally, it hinders the secretion of prostaglandin (PGE2) by human monocytes, thereby contributing to bone resorption. Moreover, the localized deficiency of IL-4 in diseased periodontal tissues has been linked to the activity and progression of periodontal disease (Kamma et al., 2004). IL-10 is a potent anti-inflammatory cytokine that suppresses both immunoproliferative and inflammatory responses. It has the ability to downregulate the synthesis of proinflammatory cytokines and chemokines, including IL-1, IL-6, and TNF-α. Additionally, IL-10 can suppress the synthesis of nitric oxide, gelatinase, and collagenase. Specific neutralization of IL-10 leads to an upregulation in the synthesis of IL-1 and TNF-α. Therefore, IL-10 is also considered a crucial regulator of bone homeostasis in both homeostatic and inflammatory conditions(Borbour et al., 1997; Zhang et al., 2014).

In our hypothesis, we postulated that the clinical parameters and concentrations of anti-inflammatory cytokines in both serum and gingival crevicular fluid (GCF) would deteriorate in the presence of smoking, irrespective of the severity of periodontitis. Consequently, the objective of this study is to assess the levels of IL-

4 and IL-10 in both serum and GCF among individuals with Stage III Grade C periodontitis who are smokers, and non-smokers and compare them with periodontally healthy individuals in both smoker, and non-smoker categories.

MATERIALS and METHODS

The study comprised 120 participants in total, who were referred to the periodontology department of Istanbul University faculty of dentistry between the years 2008-2010. Based on the retrieved data, four study groups were created according to the 2018 Periodontal disease classification (Papapanou et al., 2018a). Group I consisted of 30 periodontally healthy non-smokers, Group II consisted of 30 periodontally healthy smokers, Group III consisted of 30 non-smokers with Stage III periodontitis, and Group IV consisted of 30 smokers with Stage III periodontitis. The study excluded individuals with systemic health conditions, pregnant or lactating individuals, those on long-term medication, individuals who have taken antibiotics for any health issues in the past three months, as well as individuals who have undergone radiotherapy or chemotherapy. The criteria for oral evaluation in our study encompass a minimum of 14 teeth, absence of recent periodontal treatment, absence of periodontal surgical treatment, non-usage of removable dentures, absence of ongoing orthodontic treatment, and lack of parafunctional habits. The study's inclusion criteria do not take into account the daily quantity of cigarettes consumed (a minimum of 10 for smokers and no smoking history for non-smokers) or the duration of smoking (at least five years for smokers).

All the participants underwent examination by a trained researcher (ECU), who used a standardized periodontal probe (UNC-15, Hu Friedy, IL, USA) to measure and record the plaque index (PI), gingival index (GI), probing pocket depths (PPD), clinical attachment levels (CAL), and bleeding on probing (BOP) values. The clinical measures were obtained from six specific locations surrounding each tooth, using the mesial and distal lines as reference points: mesiobuccal, buccal midpoint, distobuccal, mesiopalatal/lingual, palatal/lingual midpoint, and distopalatal/lingual.

The GCF samples were obtained from two tooth areas with a depth of 5 mm or greater, utilizing standardized paper filters. The control group involved collecting samples from both the front and posterior teeth, using uniform paper filters. The weights of the standardized paper strips were measured and recorded using a precision scale before obtaining GCF samples, while they were still inside the Eppendorf tubes. Upon collection, the samples were subsequently transferred into Eppendorf tubes, re-weighed, and the reported weights were documented. The weight discrepancy between the two measurements was subsequently converted to microliters (μl), and all samples were preserved at a temperature of -80°C until biochemical investigations were carried out.

In order to acquire plasma samples, a volume of 9 ml of venous blood was drawn from each person. Subsequently, the blood was subjected to centrifugation to separate the serum part, which was then preserved at a temperature of -80°C until it was ready for biochemical tests. The samples were analyzed for cytokine levels using cytokine multiplex immunoassay kits on a Luminex 100TM instrument, following the manufacturer's protocols.

The data were analyzed utilizing the SPSS 21 computer software (SPSS, IBM Inc. Chicago, IL, USA). The Student t-test was used to compare quantitative data with a normal distribution between two groups. If the parameters did not follow a normal distribution, the Mann-Whitney U test was employed to compare two groups. The qualitative data was compared using both the Chi-square test and Fisher's Exact Chi-square test. Spearman's rho correlation analysis was employed to investigate the links between parameters that had a non-normal distribution. The significance level was set at $p<0.05$.

RESULTS

Table 1 presents the participants' demographic characteristics and smoking status as recorded in the study. The participants' clinical periodontal index measurement values are displayed in Table 2. The plaque index (PI), gingival index (GI), probing pocket depths (PPD), and clinical attachment levels (CAL) values were observed to be significantly higher in Group III compared to Group I ($p=0.001$).

TABLE 1: Demographic data and the smoking status of the study population

	Group I (n=30) *Mean ± sd*	*Group II* (n=30) *Mean ± sd*	*Group III* (n=30) *Mean ± sd*	*Group IV* (n=30) *Mean ± sd*
Age	38.24 ± 6.42	35.32 ± 2.38	39.10 ± 4.63	38.18 ± 6.57
Smoking (cigarette/day)	-	15.50 ± 2.81	-	16.46 ± 2.30

(**Group I:** Periodontally healthy non-smoker individuals; **Group II:** Periodontally healthy smoker individuals; **Group III:** Non-smoker individuals with stage III grade C periodontitis; **Group IV**: Smoker individuals with stage III grade C periodontitis).

TABLE 2: Comparison of the clinical periodontal index scores of the study groups.

		Group I (n=30) n (%)	*Group II* (n=30) n (%)	*Group III* (n=30) n (%)	*Group I* (n=30) n (%)	*p* Group I vs II	Group III vs IV	Group I vs III	Group II vs IV
PI	+	9 (30.0)	6 (20)	24 (80)	27 (90)	0.565	1.000	0.001*	0.001*
	-	21 (70)	24 (80)	6 (20)	3 (10)				
GI	**0**	27 (90)	27 (90)	0 (0)	0 (0)	0.274	0.001*	0.001*	0.002*
	1	0 (0)	3 (10)	6 (20)	18 (60)				
	2	3 (10)	0 (0)	24 (80)	12 (40)				
PPD	**1-3 mm**	30 (100)	30 (100)	0 (0)	0 (0)	-	0.059	0.001*	0.001*
	5-6 mm	0 (0)	0 (0)	21 (70)	15 (50)				
	≥ 7 mm	0 (0)	0 (0)	9 (30)	15 (50)				
BOP	+	0 (0)	0 (0)	27 (90)	24 (80)	-	0.344	0.001*	0.001*
	-	30 (100)	30 (100)	3 (10)	6 (20)				
CAL		1.21 ± 0.81	0.82 ± 0.25	7.20 ± 0.40	6.95 ± 0.90	0.388	0.082	0.001*	0.001*

p<0.05 accepted as statistically significant. * Indicates the statistically significant difference between the groups. A significant difference was observed between the periodontally healthy and periodontitis subjects in terms of all measured periodontal index. As expected, the index scores of the periodontitis patients were higher. (**PI:** Plaque index; **GI:** Gingival index; **PPD:** Probing pocket depth; **BOP:** Bleeding on probing; **CAL:** Clinical attachment level; **Group I:** Periodontally healthy non-smoker individuals; **Group II:** Periodontally healthy smoker individuals; **Group III:** Non-smoker individuals with stage III grade C periodontitis; **Group IV**: Smoker individuals with stage III grade C periodontitis)

The levels of IL-4 and -10 in the GCF of Group III were notably lower than those in the GCF of Group I (p<0.001, p<0.001, respectively). The levels of IL-4 and -10 GCF in Group IV were found to be significantly less compared to Group II (p=0.038, p<0.001; respectively). Group III exhibited higher IL-4 and -10 concentration in the GCF compared to Group IV (p=0.034, p=0.040; respectively). Furthermore, Group I showed higher amounts of IL-4 and -10 in GCF samples than the Group II (p=0.042, p=0.030; respectively).

A significant difference in the levels of IL-10 among the study groups was not detected, however, Group I had the highest concentration of IL-4 (p=0.035, p=0.047, p=0.024, p=0,048; respectively) (Table 3).

TABLE 3: Gingival crevicular fluid (GCF) levels and plasma concentrations of interleukin (IL)-4 and -10.

		Group I (n=30) Mean ± sd	*Group II* (n=30) Mean ± sd	*Group III* (n=30) Mean ± sd	*Group IV* (n=30) Mean ± sd	*p* Group I vs II	Group III vs IV	Group I vs III	Group II vs IV
GCF	**IL-4 (pg/µl)**	1.22 ± 0.19	1.03 ± 0.93	0.98 ± 0.06	0.80 ± 0.05	0.042*	0.034*	0.001*	0.038*
	IL-10 (pg/µl)	1.29 ± 0.75	1.08 ± 0.37	0.08 ± 0.03	0.04 ± 0.08	0.030*	0.040*	0.001*	0.001*
SERUM	**IL-4 (pg/µl)**	5.23 ± 0.66	4.59 ± 0.84	4.06 ± 0.43	3.69 ± 0.51	0.035*	0.047*	0.024*	0.048*
	IL-10 (pg/µl)	4.31 ± 0.36	4.94 ± 0.72	4.56 ± 0.43	4.97 ± 0.97	0.456	0.428	0.478	0.429

p<0.05 accepted as statistically significant. * Indicates the statistically significant difference between the groups. Smoker individuals had an increased secretion of IL-4 and IL-10 in comparison to those of their counterparts. Periodontitis patients had elevated levels of IL-4 and IL-10 in comparison to individuals who had healthy periodontal conditions. However, plasma levels of IL-10 showed no difference between the groups. (**Group I:** Periodontally healthy non-smoker individuals; **Group II:** Periodontally healthy smoker individuals; **Group III:** Non-smoker individuals with stage III grade C periodontitis; **Group IV**: Smoker individuals with stage III grade C periodontitis).

DISCUSSION

Multiple investigations have been carried out to determine the amounts and functions of pro-inflammatory and anti-inflammatory cytokines in periodontitis, specifically in the gingiva crevicular fluid (GCF) and serum (Almehmadi and Alghamdi, 2018; Kinney et al., 2014; Robati et al., 2011a). In 2017, the categorization of periodontal disorders underwent revision, resulting in the adoption of a multi-dimensional classification system for these diseases. Furthermore, the declining influence of risk factors, such as smoking and diabetes, on the deterioration of periodontal tissue has been incorporated (Papapanou et al., 2018b). Interleukin (IL)-4 and -10 are well known for their anti-inflammatory properties and their involvement in the development of periodontal disease (Gemmell et al., 1997; Seymour et al., 2009).

The current study specifically focused on the new categorization of periodontal disease and examined the levels of IL-4 and IL-10 in the gingival crevicular fluid (GCF) and serum of persons belonging to different groups: Group I consisted of periodontally healthy nonsmokers, Group II consisted of periodontally healthy individuals who smoke, Group III consisted of nonsmokers with Stage III periodontitis, and Group IV consisted of smokers with Stage III periodontitis and distinct disparities among the research groups have been recognized.

Our data shows that the levels of IL-4 in the gingival crevicular fluid (GCF) of healthy individuals who do not smoke are higher than

those of non-smoking patients with Stage III periodontitis. In addition, upon comparing the levels of IL-4 in the gingival crevicular fluid of Stage III periodontitis patients who smoke and those who do not smoke, we noted a similar pattern with the findings of the healthy individuals. Consistent with our research findings, Pradeep et al., observed a decline in the average level of IL-4 in gingival crevicular fluid (GCF) when periodontal health transitioned to disease (Pradeep et al., 2008). Giannopoulou et al., shown that IL-4 levels were elevated in the periodontally healthy group, while being significantly reduced in the periodontal disease group (Giannopoulou et al., 2003a). These findings align with the research conducted by Kabashima et al., which indicated the absence of IL-4 in GCF from highly inflamed areas (Kabashima et al., 1996).

This study also demonstrated that the levels of IL-10 in the GCF were reduced when individuals who were periodontally healthy engaged in smoking. Furthermore, our findings indicate that smoking significantly inhibits the secretion of IL-10 in individuals with periodontitis. Casarin et al., found that aggressive periodontitis has reduced levels of IL-10 production, which aligns with our own findings (Casarin et al., 2010). Contrary to our results, the study conducted by Al-Ghurabi did not observe any variations in the levels of IL-10 in the gingival crevicular fluid (GCF) between patients with chronic periodontitis and healthy individuals, as well as between smokers and non-smokers (Al-Ghurabi, 2013). The discrepancy in the findings between Al-Ghurabi's study and ours could potentially be attributed to differences in the criteria used for selecting patients. Our study included individuals with Stage III grade C periodontitis, which can be categorized as severe or aggressive. In contrast, Al-Ghurabi included patients with chronic periodontitis.

A further component of this investigation is assessing the serum levels of IL-4 and IL-10 in each of the study groups. Non-smoker individuals with healthy periodontal conditions had elevated levels of plasma IL-4 and IL-10 compared to smokers. However, there was no statistically significant difference observed in serum IL-10 levels between patients with non-smoking Stage III Grade C periodontitis and those who were smokers. Additionally, neither smokers nor non-smokers with optimal periodontal health exhibited this difference. There is a considerable amount of research in the existing literature that investigates the correlation between plasma levels of IL-4 and IL-10 and periodontal disease. Nevertheless, like our investigation, many studies yield varying results (Chen et al., 2015; Mattuella et al., 2013; Mendes Duarte et al., n.d.; Moretti et al., 2015; Robati et al., 2011b). Based on the existing data, it appears that the primary discrepancy in the studies is to the collection of samples, the techniques used for testing, and the presence of genetically distinct populations. Therefore, it is necessary to conduct a cytokine assay utilizing more sophisticated techniques on a local tissue sample or gingival crevicular fluid (GCF) in order to confirm these findings.

Smoking, which is known to have an impact on the severity of periodontal disease and serum cytokine levels, was deemed a confounding factor (Passoja et al., 2010). The current investigation found correlations between smoking and the levels of IL-4 and IL-10 in the GCF, as well as the plasma levels of IL-4. However, no correlation was detected between smoking and the plasma level of IL-10. This aligns with the findings of Giannopoulou et al., who examined the levels of IL-4 in GCF in patients with gingivitis and periodontitis in relation to smoking (Giannopoulou et al., 2003b). Furthermore, it was shown that the levels of inflammatory cytokines did not show significant changes in smoker periodontitis patients, even after undergoing initial periodontal treatment, which provides strong evidence for the suppressive effect of smoking over the cytokine expression (Bunæs et al., 2017b). However, after six months following the initial periodontal therapy, the levels of both IL-4 and IL-10 in the GCF showed a tendency to increase compared to their baseline levels. The greater proportion of IL-4 and IL-10 in the GCF may be attributed to the immunoinflammatory hyperactivity pattern commonly observed in smokers (da Silva et al., 2022).

Cytokines play a pivotal role in modulating the immune response, particularly in the context of periodontitis, where inflammation is a central feature. Among these cytokines, IL-4 and IL-10 are of particular interest due to their anti-inflammatory properties. In smoking periodontitis patients, determining the levels of IL-4 and IL-10 is crucial as smoking has been shown to dysregulate cytokine production, exacerbating the inflammatory response in periodontal tissues. Monitoring these cytokines allows for a deeper understanding of the immune

dynamics in smokers with periodontitis and aids in risk assessment and treatment planning. Moreover, establishing parallelism between clinical parameters, such as probing depth and clinical attachment loss, and anti-inflammatory cytokine levels provides valuable insights into disease severity and progression (da Silva et al., 2022; Martins et al., 2019; Miranda et al., 2020; Taiete et al., 2019). However, Studies involving individuals with severe periodontitis typically focus on identifying predictive factors for long-term surveillance, particularly tooth loss or disease recurrence, rather than short-term therapy outcomes. Understanding the intricate relationship between clinical characteristics and immunopathological patterns is essential for accurately evaluating the risk of periodontitis in a population, given the complexity of its etiology. Therefore, integrative approach enables clinicians to tailor interventions effectively, targeting not only the clinical manifestations but also the underlying immunopathological mechanisms driving periodontal disease in smokers. Additionally, analyzing IL-4 and IL-10 levels in the GCF offers valuable insights into the immune response within the periodontal tissues. Elevated levels of IL-4 and IL-10 indicate an anti-inflammatory environment, potentially indicative of disease resolution or stability. Integrating these diagnostic approaches allows for a comprehensive understanding of periodontal health status, facilitating personalized treatment planning and monitoring of therapeutic outcomes.

One potential disadvantage of our study is that we only assessed the absolute amounts of anti-inflammatory cytokines. Conversely, our study excels in assessing the patterns of anti-inflammatory cytokines both locally and systemically when exposed to a significant risk factor, smoking.

CONCLUSION

In conclusion, the findings of this study support the initial hypothesis, as heavy smokers exhibited a diminished inflammatory response, as seen by decreased expression of gingival crevicular fluid indicators. This reduction was particularly notable for proinflammatory markers and chemokines. This could be a crucial advancement in understanding the development of the disease and devising targeted treatments and preventive measures. Further investigations should be specifically planned to examine the collective influence of diabetes mellitus and smoking on the progression of periodontitis, focusing on the correlation with cytokine levels.

ETHICAL APPROVAL

The study design was approved by the Istanbul University Faculty of Medicine Ethics Committee and granted by the Istanbul University Scientific Projects (1217).

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