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Effects of smoking on clinical parameters and the gingival crevicular fluid levels of IL-6 and TNF-α in patients with chronic periodontitis

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Abstract

Objective: Smoking is an important environmental risk factor for the initiation and progression of periodontal diseases. The aim of this study was to evaluate the effects of smoking on clinical parameters and the gingival crevicular fluid (GCF) contents of the pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) levels in patients with chronic periodontitis.

Material and Methods: The study base consisted of 41 patients including 22 volunteer current smokers with an age range of 32–59 (44.41 ± 7.88) years and 19 volunteer non-smokers with an age range of 36–59 (46.94 ± 6.07) years. The first month after non-surgical periodontal therapy was accepted as the baseline of the study. The clinical parameters including plaque index (PI), gingival index (GI), bleeding on probing (BOP), probing depth (PD), clinical attachment loss (CAL) were recorded and GCF samples were collected for analysis of GCF contents of IL-6 and TNF-α levels. At the 3rd and 6th months, all of these procedures were repeated.

Results: In smokers, only CAL was significantly higher at the 3rd month compared with non-smokers ($p < 0.05$). GI and BOP were higher in non-smokers than smokers in both periods ($p < 0.05$). PI showed increases from the initial to the 6th month in smokers ($p < 0.05$). Although the differences between two groups with regard to IL-6 and TNF-α were not significant ($p > 0.05$), the total amount of TNF-α in GCF decreased from the initial to the 6th month in smokers ($p < 0.05$). There were no significant correlations between the mean total amount of IL-6 and TNF-α in GCF and clinical parameters in both evaluation periods in smokers ($p > 0.05$).

Conclusion: The present study demonstrated that cigarette smoking increases the amount of dental plaque over time in smokers and does not influence GCF contents of IL-6 and TNF-α.

Key words: chronic periodontitis; GCF; IL-6; non-surgical therapy; smoking; TNF-α

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Periodontitis is initiated by specific bacteria and the local host response to these bacteria includes the recruitment of leukocytes and the subsequent release of inflammatory mediators and cytokines such as interleukin (IL)-1, IL-6, IL-8, IL-10, IL-12 and TNF-α, which are thought to play an important role in the pathogenesis of the disease. These increased levels of several cytokines are involved in periodontal tissue destruction (Genco 1992). IL-6 is a multifunctional cytokine, of which biological activities include B-lymphocyte differentiation, T-lymphocyte proliferation and stimulation of immunoglobulin (Ig) secretion by B-lymphocytes, stimulation of acute-phase protein synthesis and complement cascade activation (Hirano et al. 1990). Of particular significance is the ability of IL-6 to induce bone resorption, both by itself and in conjunction with other bone-resorbing agents (Ishimi et al. 1990).

TNF-α is also a monocyte-derived protein that has a wide range of pro-inflammatory and immunomodulatory effects on a number of different cell populations. TNF-α can stimulate fibroblasts, including gingival fibroblasts, to produce collagenase (Meikle et al. 1989), an enzyme implicated in the tissue destruction of periodontal disease, and to stimulate bone resorption (Bertolini et al. 1986). For example, TNF-α activates monocytes and stimulates the production of IL-1β, platelet activating factor and prostaglandins (Decker 2000). Monocyte stimulation by lipopolysaccharide enhances the production of TNF-α, which has also been shown to induce collagenase release and bone resorption in vivo (Meikle et al. 1989).

Smoking is one of the major environmental risk factors for periodontal diseases (Feldman et al. 1983, Bergström & Eliasson 1987a, b, Bergström 1989, Genco & Löe 1993, Bergström & Preber 1994, Grossi et al. 1994, 1995). However, there is no consensus about the mechanisms of smoking as a risk factor. Smoking does not seem to influence the subgingival colonization of some important periodontal pathogens as most previous studies suggest that smoking and non-smoking patients largely exhibit the same microflora (Preber et al. 1992, Stoltenberg et al. 1993, Boström et al. 1998a, b). This suggests as a possible alternative that smoking interferes with the immune response of the host. Tobacco components may also modify the production of cytokines or inflammatory mediators. Nicotine, one of the most deleterious products of cigarette, has been shown to increase the release of IL-6 by cultured murine osteoblasts (El-Ghorab et al. 1997). Smokers have been reported to have increased gingival crevicular fluid (GCF) levels of TNF-α (Boström et al. 1998a). Such alterations in host response may affect the reparative and regenerative potential of the periodontium in tobacco smokers. Since many studies were cross-sectional and the other factors like plaque could not be eliminated, the effect of smoking alone was not evaluated.

The aim of the present study was to evaluate the influence of tobacco smoking on clinical parameters and the GCF contents of IL-6 and TNF-α in patients with chronic periodontitis at the 3rd and 6th months after non-surgical periodontal therapy.

Material and Methods

Selection of patients

The study population included 41 patients, 21 men and 20 women, in the age range of 32–59 (45.59 ± 7.13) years. The patients had moderate-to-severe periodontal disease as evidenced by multiple sites with a probing depth (PD) of 5 mm or more and bone loss by radiographs. All participants were in principle periodontally untreated and had not previously received surgical therapy and were drawn from the patients with chronic periodontitis at the Department of Periodontology. They did not have any systemic disease or take antibiotics for the past 6 months.

Patients were classified as either current smokers [S(+)], i.e. regular daily smoke 20 cigarettes (22 patients), or non-smokers [S(−)], i.e. who had never smoked tobacco (19 patients). All smokers were cigarette smokers. The mean age of current smokers and non-smokers was 44.41 ± 7.88 and 46.94 ± 6.07 years, respectively. The age differences between smoking groups were not statistically significant ($p > 0.05$).

All participants received the primary phase of non-surgical treatment including oral hygiene instruction, scaling and root planning. The first month after non-surgical periodontal therapy was accepted as the baseline of the study. At the 3rd and 6th months, clinical recordings and sampling procedures were repeated.

Clinical recordings

Prior to crevicular fluid collection, supragingival plaque was scored using plaque index (PI) (Silness & Löe 1964). Gingival inflammation was scored following crevicular fluid collection using gingival index (GI) (Löe & Silness 1963). Bleeding on probing (BOP) was measured dichotomously (Ainomo & Bay 1975). PD and clinical attachment loss (CAL) measures were obtained from sample sites (mesial or distal midpoints) of teeth using a conventional periodontal probe (Hu-Friedy, Chicago, IL, USA). The probe was directed parallel to the long axis of the tooth. CAL measurements were made from the cemento-enamel junction to the bottom of the sulcus. All clinical data were recorded by one examiner (E. O. E.).

Crevicular fluid sampling

After supragingival plaque was removed from each tooth, the individual tooth site was gently air-dried and isolated with cotton rolls. A saliva ejector was used to avoid salivary contamination of the samples. Each GCF sample was collected with paper strips (Periopaper, Amityville, NY, USA) from randomly selected 4 sites of each patient with 5 mm or more PD. The paper strips were consecutively inserted into the crevice at the mesial or distal midpoints until mild resistance was felt. The strips were left in situ 30 s and then transferred, for volume determination, to the chair-side located Periotron 8000 (Oraflow Inc., Plainview, NY, USA), which was calibrated using known volumes of phosphate-buffered saline (PBS). Four strips of each patient were immediately placed in a labeled tube containing 500 μl PBS and transported to the laboratory. Following 10 s vortexing and 20 min shaking, the strips were removed and the eluates centrifuged for 5 min at 5800 *g* to remove plaque and cellular elements. The samples were stored at -80°C for subsequent assays.

Cytokine assay

Levels of IL-6 and TNF-α in samples were determined by using an appropriate commercial ELISA kit (Immunotech, Marseilles, France); 300 μl of eluted sample was assayed according to the kit's instructions. These are ''sandwich'' enzyme immunoassays. Samples and standards are incubated in microtiter plate wells, coated with the first monoclonal anti-IL-6 antibody, in the presence of a second anti-IL-6 monoclonal antibody linked to acetylcholinesterase for IL-6 assay and coated with the first monoclonal anti-TNF-α antibody, in the presence of a second anti-TNF-α monoclonal antibody linked to alkaline phosphatase for the TNF-α assay. For both of the cytokines after incubation, the wells are washed and bound enzymatic activity is measured by adding a chromogenic substrate. The intensity of the color is proportional to the concentration of IL-6 and TNF-α in the sample or standard. The results were read using a microplate reader at 405 nm wavelength. Concentrations of the cytokines in each 300 μl sample were determined by generation of a standard curve for comparison. Concentrations of the cytokines were corrected for GCF volume and were defined as pg/μl. The total amounts of cytokines were expressed as pg/4 sites.

Statistical analysis

Data were expressed as means and standard deviations. The statistical significance of differences between groups was tested according to univariate analysis of variance. Baseline measurements of parameters, age and gender were taken as covariate factors to eliminate the effects of individual differences between subjects. Therefore, analysis of covariance was used to analyze the differences in GCF cytokine levels and clinical parameters between S(+) and S(−) groups at the 3rd and 6th months. The intra-group measurements over time were analyzed by using Friedman two-way ANOVA, including Bonferroni-adjusted Wilcoxon signed ranks test ($p = 0.017$) post hoc multiple comparison procedure when significant associations were observed. Simple pairwise correlations were calculated according to the rank correlation of Spearman (r_s). The null hypothesis was rejected at $p < 0.05$.

Results

Clinical characteristics

The clinical characteristics of this study at the initial, 3rd and 6th months are shown in Table 1. Although we applied non-surgical periodontal therapy to match the periodontal conditions of the patients, there were differences among initial measurements. The confounding factors are taken as covariates for homogenizing these measurements.

When the clinical parameters were compared between groups, in the S(+) group, only CAL was significantly higher compared with S(−) at the 3rd month ($p < 0.05$). GI and BOP were significantly higher in the S(−) group compared with the S(+) in both evaluation periods ($p < 0.05$).

Once comparisons of clinical parameters were evaluated in each group, in the S(+) group, PI showed increases from the initial to the 6th month ($p < 0.05$). In the S(−) group, GI and BOP showed an increase from the 3rd to 6th month ($p < 0.05$). Therefore, a significance was obtained between the initial and 6th months ($p < 0.05$). In each group, from the initial to the 3rd month a significant decrease or increase was not seen.

GCF sample levels of IL-6 and TNF-α

The concentration and total amount measurements of GCF levels of IL-6 and TNF-α and intra-group comparisons of these cytokines are shown in Table 2. The detection limit for both of these assays was the same (0.05 pg/μl). Samples with levels below the detection limit were set to 0 and analyzed in this way. In both periods, there was no significant difference between groups in the concentration and total amounts of GCF IL-6 and TNF-α, of which initial values were used as covariates ($p > 0.05$).

In the S(+) group, the concentration of GCF levels of IL-6 and TNF-α decreased from the initial to the 6th month ($p < 0.05$). The total amount of TNF-α in GCF decreased from the initial and 3rd months to the 6th month ($p < 0.05$). In the S(−) group, there were no significant differences in these cytokine levels ($p > 0.05$).

Correlations

Correlations between the mean total amount of IL-6 and TNF-α levels in GCF and clinical parameters are shown in Table 3. There were no significant correlations between the mean total amount of IL-6 in GCF and clinical parameters in the S(+) group ($p > 0.05$). In the S(−) group, correlations between the mean total amount of IL-6 in GCF and initial PD values ($p < 0.05$) were found to be negative, and positive between the mean total amount of IL-6 in GCF and GI and BOP values of the 3rd month ($p < 0.05$).

There were no significant correlations between the mean total amount of TNF-α in GCF and clinical parameters in all evaluation periods of both groups ($p > 0.05$).

Discussion

Although bacteria are the primary etiologic factors in periodontal disease, the patient's host response is a determinant of disease susceptibility, and smoking also interferes with the immune response of the host. In this study, the relation between smoking and the levels of GCF IL-6 and TNF-α, which play an important role in the pathogenesis of periodontal diseases and immune response, was evaluated and it was shown that there was no effect of smoking on these two cytokines of patients with chronic periodontitis to whom non-surgical periodontal therapy was applied.

The presence of excessive amount of subgingival and supragingival plaque makes the evaluation of the effect of smoking on periodontal health extremely difficult. Therefore, in the studies of smoking and periodontal health, the effect of oral hygiene must be controlled and higher oral hygiene must be provided. In this study, non-surgical periodontal therapy was performed to match the amount of dental plaque and degree of gingival inflammation of patients with chronic periodontitis who regularly smoke and never smoke. Because of this the first month after periodontal therapy was accepted as the baseline of the study.

Table 1. Mean values of clinical parameters at the initial, 3rd and 6th months in sampling sites (mean ± SD)

Parameters	Initial values		3rd month values		6th month values	
	S(+) ($n = 22$)	S(−) ($n = 19$)	S(+) ($n = 22$)	S(−) ($n = 19$)	S(+) ($n = 22$)	S(−) ($n = 19$)
PI	1.56 ± 0.37	1.33 ± 0.31	1.68 ± 0.40	1.68 ± 0.18	1.90 ± 0.30†	1.84 ± 0.24
GI	1.48 ± 0.40	1.59 ± 0.34	1.29 ± 0.26	1.59 ± 0.22*	1.40 ± 0.28	1.89 ± 0.17*,†,‡
BOP	0.48 ± 0.40	0.59 ± 0.34	0.29 ± 0.26	0.59 ± 0.22*	0.40 ± 0.28	0.88 ± 0.15*,†,‡
PD	5.28 ± 0.24	5.21 ± 0.29	5.35 ± 0.19	5.27 ± 0.18	5.33 ± 0.28	5.17 ± 0.19
CAL	5.31 ± 0.78	4.54 ± 0.49	5.10 ± 0.85*	4.65 ± 0.52	5.17 ± 0.72	4.52 ± 0.58

PI = plaque index; GI = gingival index; BOP = bleeding on probing; PD = probing depth; CAL = clinical attachment loss.
$^{*}p < 0.05$ according to S(+) or S(−) (initial values were used as covariates).
$^{\dagger}p < 0.05$ according to initial value (Friedman- and Bonferroni-adjusted Wilcoxon signed ranks test).
$^{\ddagger}p < 0.05$ according to the 3rd month value (Friedman- and Bonferroni-adjusted Wilcoxon signed ranks test).

Table 2. Mean values of GCF IL-6 and TNF-α at the initial, 3rd and 6th months in sampling sites (mean ± SD)

	Parameters	Initial values*		3rd month values		6th month values	
		S(+) ($n = 22$)	S(−) ($n = 19$)	S(+) ($n = 22$)	S(−) ($n = 19$)	S(+) ($n = 22$)	S(−) ($n = 19$)
concentration (pg/μl)	IL-6	1.07 ± 1.32	0.57 ± 0.75	1.03 ± 1.32	0.92 ± 1.40	0.50 ± 0.71†	0.36 ± 0.42
	TNF-α	0.31 ± 0.39	0.51 ± 0.81	0.34 ± 0.47	0.49 ± 0.64	0.13 ± 0.16†	0.27 ± 0.41
total amount (pg/4)	IL-6	0.32 ± 0.38	0.18 ± 0.23	0.32 ± 0.35	0.21 ± 0.26	0.20 ± 0.19	0.11 ± 0.08
	TNF-α	0.09 ± 0.11	0.13 ± 0.16	0.11 ± 0.13	0.13 ± 0.16	0.05 ± 0.05†,‡	0.10 ± 0.11

GCF = gingival crevicular fluid; IL-6 = interleukin 6; TNF-α = tumor necrosis factor alpha.
*In statistical comparisons, the initial values were used as covariates.
†$p < 0.05$ according to the initial value (Friedman- and Bonferroni-adjusted Wilcoxon signed ranks test).
‡$p < 0.05$ according to the 3rd month value (Friedman- and Bonferroni-adjusted Wilcoxon signed ranks test).

Table 3. Correlations between the total amount (pg/4 sites) of IL-6 and TNF-α in GCF and clinical parameters in S(+) and S(−)

	Month	PI		GI		BOP		PD		CAL	
		S(+) ($n = 22$)	S(−) ($n = 19$)	S(+) ($n = 22$)	S(−) ($n = 19$)	S(+) ($n = 22$)	S(−) ($n = 19$)	S(+) ($n = 22$)	S(−) ($n = 19$)	S(+) ($n = 22$)	S(−) ($n = 19$)
IL-6	0	−0.189	0.173	−0.220	0.118	−0.220	0.118	−0.167	−0.516*	−0.017	0.261
	3	−0.026	−0.018	−0.158	0.470*	−0.158	0.470*	0.380	−0.099	0.380	0.090
	6	−0.306	0.069	−0.283	−0.191	−0.283	−0.145	0.102	−0.351	0.372	0.454
TNF-α	0	−0.068	−0.044	−0.352	−0.385	−0.352	−0.385	−0.104	−0.141	0.015	−0.539
	3	−0.119	−0.240	−0.099	−0.073	−0.099	−0.073	0.107	0.061	0.102	0.017
	6	0.210	0.009	−0.109	0.001	−0.109	−0.103	0.203	−0.076	0.103	0.180

GCF = gingival crevicular fluid; IL-6 = interleukin 6; TNF-α = tumor necrosis factor alpha.
*The correlation at $p < 0.05$ level (Spearman rank correlation coefficients).

Cross-sectional studies searching the effect of smoking on clinical parameters suggest that non-smokers have higher GI and BOP values than smokers (Feldman et al. 1983, Bergström 1989, Axelsson et al. 1998). However, there are studies that show no significant difference between smokers and non-smokers (van der Weijden et al. 2001) and smokers have higher values than non-smokers (Haber et al. 1993). Pucher et al. (1997) reported that GI and BOP values were similar in smokers and non-smokers 9 months after periodontal therapy. In this study, GI and BOP values were found to be higher in non-smokers than smokers in both evaluation periods. The reason for this may be associated with the suppressive effect of smoking on blood vessels of marginal gingiva. In addition, in our study, increased mean values of GI and BOP from the baseline to the 6th month may depend on poor oral hygiene in the two groups. In smokers, the failure of gingivitis signs to become ''adequately expressed'' may be due to a suppressive effect of smoking on inflammation and it continues for a long time. Danielsen et al. (1990) have found that the amount of gingival vessels in smokers was half of that of the non-smokers. Although there was no difference in plaque amounts between smokers and non-smokers, the higher GI scores of non-smokers dependent on this decreased gingival bleeding in smokers has been explained as being due to nicotine, which causes vasoconstriction of blood vessels such as in the forearm, skin and hands. However, the effects of nicotine has been disputed; some claim that the blood flow is reduced (Clarke & Carey 1985) and others claim it is significantly increased (Baab & Öberg 1987) or unchanged (Meekin et al. 2000).

In the literature, there are several studies that show that PD values were higher in smokers than in non-smokers (Feldman et al. 1983, Bergström & Eliasson 1987b, Stoltenberg et al. 1993, Linden & Mullally 1994, Zambon et al. 1996, Bergström et al. 2000, Machuca et al. 2000, van der Weijden et al. 2001). A significant positive correlation has been shown between smoking and CAL (Grossi et al. 1995, Zambon et al. 1996, Axelsson et al. 1998, Albandar et al. 2000, Machuca et al. 2000, van der Weijden et al. 2001). The reason for increased PD and CAL levels in smokers may be dependent on accumulation of dental plaque and poor oral hygiene (Preber et al. 1980, Ismail et al. 1983, Zambon et al. 1996). However, when homogen groups were examined, deleterious effects of smoking on periodontium resulted not only from plaque amount and poor oral hygiene but also from the effect of direct tissue destruction of smoking (Bergström & Eliasson 1987a, b). Preber & Bergström (1985) and Kaldahl et al. (1996) found that the mean values of PD were higher in smokers than non-smokers after non-surgical periodontal therapy. Surprisingly, in this study mean values of CAL were higher in the S(+) group at the 3rd month; we could not find any significant difference between groups at the 6th month. Pucher et al. (1997) could not find any difference in PD and CAL scores between smokers and non-smokers 9 months after initial periodontal therapy. Similarly, in the present study, no difference was detected in the mean values of PD for a 6-month period. The design of the study differed from literature; since the first month after non-surgical periodontal therapy was accepted as the baseline of the study, the effect of dental plaque was removed.

Some cross-sectional studies interpreted the effect of cigarette smoking on the periodontium to be indirect and due to inadequate levels of oral hygiene and increased plaque accumulation among smokers relative to non-smokers (Preber et al. 1980, Linden & Mullally 1994, Zambon et al. 1996). This increase due to smoking can alter the environment,

increase bacterial adherence and affect immune response. Adversely, the decreased plaque levels in smokers due to smoking can act as an anti-plaque agent and change the contents of saliva (Feldman et al. 1983, Bergström et al. 2000, Machuca et al. 2000). Boström et al. (1998a) suggested that there was no difference between smokers and non-smokers in plaque levels after surgical periodontal therapy. In this study, PI levels of both groups were the same after non-surgical periodontal therapy in all evaluation periods. This result supports the concept that cigarette smoking does not have a direct effect on dental plaque. However, when intra-group comparisons were evaluated, increase of PI levels from baseline to the 6th month in the S(+) group may be dependent on altered environmental factors in the long term by smoking.

GCF volume is influenced by many factors such as flow rate (Challacombe et al. 1980), gingival trauma (Curtis et al. 1988) and repeat sampling (Lamster et al. 1989). It is logical to expect that with some cytokines, total amounts in gingival fluid will correlate better with disease than cytokine concentrations. Saliva contamination is a potential problem when sampling GCF with filter paper strips and this could clearly influence the results expressed as cytokine concentration. The total amounts of cytokines are more associated with disease activity than concentration (Nakashima et al. 1994). Therefore, the comparisons of cytokines between groups, the changes of cytokines intragroups and the correlations between cytokines and clinical parameters were evaluated and discussed according to the total amount in our study. There are many ways of collecting GCF and some of them are paper strips, capillary tubes, paper cones and gingival washing. We preferred to use paper strips as some of the authors (Rossomando et al. 1990, Geivelis et al. 1993, Heasman et al. 1993, Reinhardt et al. 1993, Lee et al. 1995, Atilla & Kütükçüler 1998, Ataoğlu et al. 2002). There is no commonly accepted method for the depth of insertion, time of sampling or the most appropriate expression and computation of cytokine levels (i.e. total amount versus concentration). Presently, GCF collection is usually made by paper strips and 30 s time of sampling is generally preferred (Rossomando et al. 1990, Reinhardt et al. 1994, Atilla & Kütükçüler 1998, Ataoğlu et al. 2002).

There are limited studies about the effect of smoking on GCF IL-6 levels. Boström et al. (1999) suggested that there was no significant difference between smokers and non-smokers in the IL-6 levels. Similarly, we found no difference between two groups and no correlation with clinical parameters at the 3rd and 6th months in the S(+) group. Geivelis et al. (1993) reported that the IL-6 level was below detection, suggesting that IL-6 was absent or present in low concentrations in GCF in about 30% of the patients. In this study, in about 10% of the patients, the IL-6 level was below detection.

Boström et al. (1998a, b, 1999) have observed significantly increased levels of TNF-α in GCF of both current and former smokers in treated and untreated patients with periodontal disease. On the other hand, we could not find any difference between the two groups in the level of TNF-α for a 6-month period. These conflicting results may be due to different sampling methods. Although Boström et al. (1998a, b, 1999) have used the gingival crevice lavage method, we have used paper strips. In this study, in about 40% of the patients the TNF-α level was below detection and we did not observe any significant correlation between periodontal parameters and TNF-α in GCF in smoking and non-smoking patients. These findings support the idea that the local production of inflammatory mediators differs from site to site and subject to subject, and levels of inflammatory mediators in periodontitis patients may be influenced by multiple factors such as the local bacterial composition (Masada et al. 1990). In the S(+) group, TNF-α in GCF reduced from the initial and 3rd months to the 6th month; the reason for this could not be understood, perhaps it may be due to increasing PD values, although this increase was not significantly different. Rossomando et al. (1990) have found TNF-α most frequently in sites with shallow pockets and they concluded that TNF may be found in sites before clinically observable disease and therefore may prove to be a suitable indicator for periodontal disease.

Finally, although cigarette smoking does not affect the degree of gingival inflammation, it increases the amount of dental plaque over time in smokers. Moreover, this study reveals that cigarette smoking does not have an effect on the levels of IL-6 and TNF-α in GCF after non-surgical periodontal therapy.

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