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INTERLEUKIN-1, INTERLEUKIN-6 AND TUMOR NECROSIS FACTOR ALPHA IN PERIODONTITIS: SMOKERS VS. NONSMOKERS

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Key Message: There is no consent on the influence of smoking on GCF levels of different cytokines in literature.

A B S T R A C T

Background and Aim: Cigarette smoking is considered as an important environmental risk factor for the initiation and progression of periodontal diseases. This study aimed to evaluate the effects of smoking on two clinical parameters, Plaque Index and Bleeding Index and also, gingival crevicular fluid (GCF) contents of three proinflammatory cytokines, interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) level in patients with adult generalized moderate periodontitis. **Materials and method:** This analytical study was performed on fifty six 20 to 45 year-old male patients with adult generalized moderate periodontitis, including 28 smokers and 28 non-smokers. After recording concerned clinical parameters, GCF samples were collected using paper points, from four teeth with 4 mm or more probing depth per patient, selected randomly in any of the four quadrants. Then cytokines levels were determined by ELISA method. **Results:** In smokers and non smokers, there were no significant differences with regard to IL-1, IL-6 and TNF-α level in GCF. In addition, significant difference in Plaque Index and Bleeding Index was not observed. **Discussion and Conclusion:** The present study did not reveal any difference between concerned parameters in smokers and non smokers. These findings and conflicts may be due to differences in quantity of samples, sampling methods, statistical populations and rate of smoking in smokers.

INTRODUCTION

Graves suggested gingivitis is associated with IL-1 a, TNF. Delima showed periodontitis is retarded by antagonist and inhibition of IL-13

Smoking is a periodontitis factor.4 Boström found differences of IL-6 and TNF-alpha in smokers,5 with no influence on IL-1beta ,IL-1ra .6 Rawlinson Found, differences of IL-1beta ,IL-1ra .7 Erdemir showed smoking increases dental plaque and does not influence IL-6 ,TNF-alpha.8 Petropoulos *et al* concluded smoking decreases IL-1alpha in GCF .9

There is no consent on the influence of smoking on GCF levels of different cytokines in literature. So, we aimed to study GCF levels of Il-1, Il-6 and TNFα ..

Subjects and Method

Study design

Case-control, descriptive design based on a simple randomized sampling approach and laboratory findings.

Study population

This study included fifty six healthy 20 to 45 year-old males with adult moderate generalized periodontitis no history of periodontal affecting medication and no medications at the time of study. Half of the cases were smokers at the time of study meaning they had smoked more than 100 cigarettes and had not quit at the time of study. The other half was non-smokers without the pervious habit. Patients were selected based on the routine standard examinations to detect moderate generalized periodontitis. All diagnostic examinations were carried out by one blind periodontologist. This uniformity in the study population was considered to further prevent bias.

Index measurements

Plaque index

Silness and Loe index (GI) was used to describe the clinical severity of gingival inflammation as well as its location. The plaque scores were as follows: Zero for plaque-free surface, One for a narrow line of plaque on the margin of free gingiva, Two for the presence of more plaque on the lateral margins of the tooth (no plaque on the interproximal area) which is visible, and Three for the abundant plaque both on the lateral margin and the interproximal area of the teeth.6

Bleeding index

Barnet bleeding index (BI) was used to determine the

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bleeding status of the study sites.Buccal and lingual surfaces were examined using a periodontal probe (Williams Probe; Hu-Friedy Manufacturing Inc., Chicago, IL, USA). The bleeding scores were as follows: Zero for no bleeding on probing, one for bleeding 30 seconds after probing, two for bleeding immediately on probing, and three for spontaneous bleeding.6The tip of the periodontal probe was inserted up to one to two millimeters into the distobuccal sulcus and moved in a walking pattern toward the mesiobuccal. The mean score of the two probed surfaces was considered.

Sampling sites

At the beginning, A tooth with at least one pocket of four mm or more in depth was randomly selected per quadrant for each patient. Inclusion criteria were as a two to three millimeter of attachment loss on probing and the presence of bleeding on probing (BOP) (Williams Probe; Hu-Friedy Manufacturing Inc., Chicago, IL, USA). Bleeding on probing was indicated as a clinical sign of an active pathologic condition. The total number of study sites was then 224, which means 112 for each of the two study groups.

GCF sampling

Through another visit at least 24 hours later to prevent blood contamination, plaque was removed and tongue and cheek retracted using cotton roles. Free gingiva was then gently dried of saliva with mild air flow. Next, GCF was collected by placing a no. 25 paper point till the state of saturation which occurred usually within 30 seconds. Blood contaminated samples were repeated within another half an hour and non-contaminated samples transferred to test tubes using a sterile forceps. Each test tube contained four samples of each individual. Blind coding was done and samples were then stored in -70 cm refrigerator.

Laboratory procedures

Tubes were then left in the room temperature. Paper points were then immersed into the PBS and undergone ELISA test.

PBS preparation

To prepare phosphate buffer solution (PBS), 40 mg NaCl, 13.5 g Na_2HPO_4, and 1.77 g NaH_2PO_4 were mixed in 5 ml distilled water and *PH* was measured. A *PH* of 7.2 to 7.4 was considered acceptable because the studied cytokines would not denatured within this *PH* range and preserve their spatial structure. The solution was used to rarefy antibodies and samples and also to irrigate inside the wells.

Elisa

In-House Enzyme-Linked Immunosorbent Assay (ELISA) was used to determine the GCF levels of the studied cytokines. First, 100 µl of the specific antibodies of each cytokine (Diaclone, Amsterdam), was applied to 96-well formatted micro plate (*Nunk*, Roskilde, *Denmark*) well using a pipette. After 16 hours of incubation in a 2 to 4 ºC, wells were irrigated with PBS. The empty cells of the micro plate were blocked and rarefied samples were added to each cell and stored in 37 ºC for 30 minutes. After irrigation, second layer antibody which was conjugated to HRP enzyme was added. Half an hour later, chromo gene substrate was added. Cut-off solution was added after 15 minutes. The density of each solution was then determined by a wavelength of 450 nm and based on the standard samples.

Stat work

Data were transferred to SPSS v.15 (SPSS Inc; Chicago, Ill) and analyzed for significant values using ANOVA and *t*-test.

RESULTS

In smokers and non smokers, there were no significant differences with regard to IL-1, IL-6 and TNF-α level in GCF.

Table 1 Summary of the study outcomes for each study group. No significant findings were noted.

	smokers	Non-smokers	total	p-value
Age(yrs)	35.89±7.60	37.43±7.60	36.66±7.57	>0.05
Pocket depth(mm)	5.00±0.79	5.13±0.64	5.06±0.72	>0.05
IL-1(pg/ml)	263.73±180.37	257.74±201.78	260.74±189.65	>0.05
TNF-α(pg/ml)	301.78±212.09	442.91±342.00	372.34±290.81	>0.05
IL-6(pg/ml)	101.72±225.62	82.53±202.45	92.129±212.617	>0.05
Plaque index	1.78	1.75	1.76	>0.05
Bleeding index	1.71	1.82	1.76	>0.05

In addition, significant difference in Plaque Index and Bleeding Index was not observed

DISCUSSION

Erdemir *et al* in 2004[8] studied the effects of smoking on clinical parameters and the gingival crevicular fluid levels of IL-6 and TNF-α in the patients with chronic periodontitis. The study consisted of 41 patients including 22 current smokers, of 32-59 years and 19 ,36-59 years non-smokers 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. All procedures were repeated at the 3rd and 6th months. They found that in smokers, only CAL was significantly higher at the 3rd month compared with non-smokers. We considered BOP as the inclusion criteria of the sampling sites to assure the inclusion of active sites of involvement only. GI and BOP were higher in non-smokers than smokers in both periods. PI showed increases from the initial to the 6th month in smokers. Although the differences between two groups with regard to IL-6 and TNF-α were not significant, the total amount of TNF-α in GCF decreased from the initial to the 6th month in smokers. 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. They showed an increased plaque accumulation rate with cigarette smoking which is not supported by our findings.

Consistent with the findings of our study, no relation between smoking and GCF levels of IL_6 and TNF-α was found. Unfortunately under the circumstances of the present study, it was not possible to track patients longitudinally. Both studies lack the useful outcomes of the treated/maintained active sites in terms of GCF levels of the studied cytokines.

Table 2 Plaque index, frequency of each score and the smoking . N: number of the patients. "% same score": the percent with the same plaque index . "% same group" means the percent of the within the same group

Scores		Smokers	Non-smokers	Total
1	N	10	11	21
	% same score	47.6	52.4	100
	% same group	35.7	39.3	37.5
	% total	17.9	19.6	37.5
2	N	15	12	27
	% same score	55.6	44.4	100
	% same group	53.6	42.9	48.2
	% total	21.4	26.8	48.2
3	N	3	5	8
	% same score	37.5	62.5	100
	% same group	10.7	17.9	14.3
	% total	5.4	8.9	14.3
t	N	28	28	56
	% same score	50	50	100
	% same group	100	100	100
	% total	50	50	100

According to the studies of Boström *et al* in 1999,[5] GCF levels of IL is similar in smokers and non-smokers. GCF levels of TNF-α, however, showed a significant increase which is not supported by the outcomes of the present study. They studied 108 patients including 45 current smokers, 28 former smokers and 35 non-smokers. This classification was not considered in our study. The median GCF sample levels of IL-6 and TNF-α were 5.0 pg/ml and 61.0 pg/ml, respectively, for current smokers, 13.0 pg/ml and 51.0 pg/ml, respectively, for former smokers, and 10.0 pg/ml and 12.0 pg/ml, respectively, for non-smokers. The differences between smoking groups with regard to IL-6 were not significant. This might suggest that the IL-6 content was not influenced by smoking. In contrast, the TNF-alpha content was significantly increased in current smokers as compared to non-smokers confirming similar previous observations which do not support our findings. The results of Boström *et al* in patients with moderate to severe periodontal disease may indicate different mediator functions of IL-6 and TNF-α in response to smoking. We have just considered chronic moderate cases of periodontitis. This may suggest that different cytokines may possess their main role in different disease stages.

Boström *et al* conducted another study within a year (2000)[6] and analyzed the GCF levels of IgA, IgG, albumin and total protein in 22 smokers and 18 non-smokers in the age range 32-86 years. Samples of GCF were collected from two diseased sites in each patient by an aspiration method. IL-1beta, IL-1ra, IgA and IgG were determined with immunoelectrophoresis. They suggested that smoking does not influence the GCF cytokines, supragingival plaque and pocket depth. Bleeding was the only variable in their new study which was significantly decreased in smokers. The probing depth and frequency of diseased sites and supragingival plaque did not differ between smokers and non-smokers consistent with their findings. However, gingival bleeding was significantly decreased in smokers. IL-1beta was detected in GCF of 95% of both smokers and non-smokers and IL-1ra in all patients. The GCF level of IL-1ra was approximately 1,000 times as much as that of IL-1beta. The GCF levels of IL-1beta and IL-1ra were high in comparison with those of TNF-alpha and IL-6 determined by the same method in their earlier studies.

The studies of Giannopoulou *et al* in 2003, showed a significant relation between smoking and GCF levels of IL_4 and IL_8.[10] They claimed that smoking interferes with cytokine production. Twenty-two healthy subjects, 10 smokers and 12 non-smokers, participated in their study. PI, GI, probing pocket depth (PPD) and BOP were assessed , GCF was collected and total amounts of IL-1beta, IL-4 and IL-8 were determined by ELISA. Both smokers and non-smokers showed an increase in PI, GI and BOP scores during the experiment. Although no differences were noted with regard to PI at day 10, the GI and BOP were significantly less pronounced in smokers than non-smokers. Non-smokers showed higher total amounts of IL-4 but lower amounts of IL-8 than smokers, throughout the experiment.

Table 3 Bleeding index according to the frequency of each score , smoking. N: number of the patients. "% same score": the percent of the patients with the same plaque index. "% same group": the percent within the same study group

Scores		Smokers	Non-smokers	Total
0	N	1	1	2
	% same score	50.0	50.0	100
	% same group	3.6	3.6	3.6
	% total	1.8	1.8	3.6
1	N	3	7	10
	% same score	30.0	70.0	100
	% same group	10.7	25.0	17.9
	% total	5.4	12.5	17.9
2	N	24	19	43
	% same score	55.8	44.2	100
	% same group	85.7	67.9	76.8
	% total	42.9	33.9	76.8
3	N	1	0	1
	% same score	100	0	100
	% same group	3.6	0	3.6
	% total	1.8	0	1.8
total	N	28	28	56
	% same score	50	50	100
	% same group	100	100	100
	% total	50	50	100

Total amounts of IL-1beta and IL-8 increased significantly during plaque accumulation in both groups. IL-4 remained stable for the smoker group and decreased for the non-smoker group. In another study the same year they found GCF levels of IL_1, IL_4, IL_6, and IL_8 are directly related to the pocket depth and also GCF levels of IL4, IL_6, and IL_8 is significantly related to the smoking.

Calsina G *et al* in 2002[11] studied the effect of smoking on the periodontium and found that despite a higher gingival

recession and plaque depth in smokers, plaque index is not significantly different between them and non-smokers. Bergstrom J and Bostrom J in 2001[12] reported an increased sulcular bleeding on probing both in smokers with periodontal disease and smokers without. The increase, however, was more significant in the periodontal compromised patients.

Villar C and Martorelli in 2003 studied the impact of smoking on the thickness of marginal gingiva and claimed cigarette smoking masquerades the signs of plaque-induced inflammation, including bleeding, redness and swelling, by the mechanism of thickening and further keratinization of the marginal gingival and also vasoconstriction of the local capillaries.

Controversial findings of these studies could be due to the low number of study population in some researches. In our study, for instance, some differences may turn out to be significant if a higher number of cases were included.

Variation in sampling methods could be another reason leading to the inconsistencies. For example if the cytokine levels were measured in the volume unit, the relevant results could be different to the present study. Age range, gender differences, culture, overall hygiene, variation in oral normal flora and other factors could also alter the outcomes. We also believe that the patterns of smoking need to be considered as an important factor in similar studies.

Keelen D. Tymkiw *et al* in 2012[14], compared 22 chemokines and cytokines in GCF from 42 persons with chronic periodontitis (22 smokers and 20 non smokers),and 12 periodontally healthy control subjects. Four diseased and 2 healthy sites were selected .Compared to healthy control subjects ,GCF in subjects with chronic periodontitis contained significantly higher amounts of IL-1α,IL-1b,IL-6,IL-12.Smokers displayed decreased amounts of IL-1α,IL-6,IL-12.So,in this study ,periodontitis subjects had significantly elevated cytokine and chemokine profiles .Smokers exhibited a decrease in pro-inflammatory cytokines and chemokines .

This reflects the immunosuppressant effects of smoking which may contribute to an enhanced susceptibility to periodontitis.

In the study of Sukumaran Anil *et al*, in 2013[1,]90 participants (30 periodontally healthy individuals, 30 non-smoking individuals with periodontitis, and30 smokers with periodontitis)formed the study group. Serum and GCF samples were collected and MCP-1 levels were estimated.MCP -1 levels in serum and GCF were found to be highest in smokers with periodontitis, followed by the peridontitis group, and then by the healthy controls. It can be concluded that the high levels of both serum and GCF MCP -1 found in smokers could explain the severity of periodontitis in smokers.

In 2013[16]Maliji *et al*, studied the effect of smoking on INF-γ and IL-10 cytokines level in GCF of patients with chronic periodontitis. They showed that IL-10 and INF-γ levels in the GCF of smokers were higher than nonsmokers, however not significantly.

CONCLUSION

1. GCF levels of IL_1, IL_6, and TNF_α is not significantly different between smokers and non-smokers with adult moderate generalized periodontitis.
2. Bleeding and plaque indices are not significantly different between smokers and non-smokers with adult moderate generalized periodontitis.

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