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ORIGINAL ARTICLE

Salivary osteocalcin levels are decreased in smoker chronic periodontitis patients

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OBJECTIVES: This study was planned to investigate whether smoker chronic periodontitis patients exhibit different salivary concentrations of C-telopeptide pyridinoline cross-links of type I collagen (ICTP) and osteocalcin (OC) compared to the non-smoker counterparts.

METHODS: Whole saliva samples, full-mouth clinical periodontal recordings were obtained from 33 otherwise healthy chronic periodontitis patients and 36 systemically, periodontally healthy control subjects. Chronic periodontitis patients and healthy control subjects were divided into smoker and non-smoker groups according to their self reports. Salivary ICTP, OC levels were determined by Enzyme-linked Immunoassays.

RESULTS: Healthy control groups exhibited significantly lower values in all clinical periodontal measurements ($P < 0.001$). Smoker periodontitis patients revealed similar clinical periodontal index values with non-smoker counterparts ($P > 0.05$). Chronic periodontitis patients exhibited significantly higher salivary OC levels than healthy controls ($P < 0.05$). Smoker periodontitis patients revealed lower salivary OC levels than non-smoker counterparts ($P < 0.001$). Log ICTP levels in non-smoker chronic periodontitis patients were higher than non-smoker controls ($P < 0.05$). Smoker healthy control group revealed higher log ICTP levels than non-smoker counterparts ($P < 0.001$).

CONCLUSIONS: Within the limits of this study, it may be suggested that suppression of salivary osteocalcin level by smoking may at least partly explain the deleterious effects of smoking on periodontal status.

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Keywords: ICTP; osteocalcin; periodontitis; saliva; smoking

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Introduction

Smokers are accepted to be more susceptible to advanced and aggressive forms of periodontitis than non-smokers (Haber *et al*, 1993; Calsina *et al*, 2002). Tobacco smoking modifies the periodontal response to microbial challenge (Barbour *et al*, 1997; Palmer *et al*, 2005). Although, smoker and non-smoker patients exhibit more or less the same periodontal pathogens (Preber *et al*, 1992; Buduneli *et al*, 2005a) smokers also tend to respond less favourably to periodontal treatment (Ah *et al*, 1994; Renvert *et al*, 1998). Smoking was suggested to influence host cytokine levels (Boström *et al*, 1999; Buduneli *et al*, 2005b, 2006). Furthermore, smoking was reported to reduce salivary osteoprotegerin concentrations in untreated and also treated chronic periodontitis patients (Buduneli *et al*, 2008). Chronic periodontitis is a bacterially induced inflammatory disease that has been associated with various systemic diseases and/or conditions such as cardiovascular diseases and preterm low birth weight. Despite that a clear dose-response relationship between periodontitis and smoking was reported (Martinez-Canut *et al*, 1995) the mechanisms by which smoking contributes to the pathogenesis of periodontitis are poorly understood.

Carboxyterminal-telopeptide pyridinoline cross-links of type I collagen (ICTP) is released into the periodontal tissues as a consequence of collagen degradation and alveolar bone resorption (Seibel, 2003). Type I collagen composes 90% of the organic matrix of bone and is the most abundant collagen in osseous tissue (Narayanan and Page, 1983). Studies assessing the role of ICTP levels in gingival crevicular fluid (GCF) or peri-implant crevicular fluid as a diagnostic marker of periodontal disease activity have reported promising results so far (Oringer *et al*, 1998, 2002). ICTP was suggested to predict future bone loss, to correlate with clinical parameters and putative periodontal pathogens and also to reduce following periodontal therapy (Giannobile, 1999).

Osteocalcin (OC) is a calcium-binding protein of bone and the most abundant non-collagenous protein of the mineralized tissue (Lian and Gundberg, 1988). Serum

level of OC is considered as a marker of bone formation (Christenson, 1997). Serum levels of OC were reported to be lower in periodontitis patients compared with healthy subjects suggesting lower osteoblastic activity and bone formation ability (Shi *et al*, 1996).

Saliva is a mirror of the body that could be used to monitor the systemic as well as the oral health status. Whole saliva contains constituents of exocrine glands in the oral cavity and also gingival crevicular fluid (GCF). Saliva is readily available and easily collected without specialized equipment or personnel. Several mediators of chronic inflammation and tissue destruction have been detected in whole saliva of periodontitis patients (Kaufman and Lamster, 2000; Kinane and Chestnutt, 2000; Lamster *et al*, 2003). In addition, as whole saliva represents a pooled sample with contributions from all periodontal sites, analysis of biomarkers in saliva may provide an overall assessment of disease status as opposed to site-specific GCF analysis (Miller *et al*, 2006).

As yet, the relationship between smoking, chronic periodontitis, and salivary, ICTP and osteocalcin concentrations has not been clarified. Our hypothesis was that smoking may increase salivary concentrations of ICTP and decrease that of OC thereby negatively affect clinical periodontal situation in chronic periodontitis patients. Thus, the aim of this study was to investigate whether smoker chronic periodontitis patients exhibit different salivary concentrations of ICTP and OC than the non-smoker counterparts and also to compare the data with periodontally healthy smoker and non-smoker subjects.

Materials and methods

Study population

A total of 69 subjects were included in the present cross-sectional study. Thirty-three untreated chronic periodontitis patients (CP) (8 smokers, 25 non-smokers) initially presenting to the School of Dentistry, Ege University and 36 systemically and periodontally healthy subjects (11 smoker, 25 non-smokers) were recruited between May 2006 and December 2008. Patients with medical disorders such as diabetes mellitus, immunological disorders, hepatitis and those had antibiotic and/or periodontal treatment in the previous 6 months were excluded from the study. The volunteer subjects comprising the healthy control group were drawn from the staff of the Dental School and had no history of periodontal disease, no attachment loss, and no sign of clinical inflammation. The study protocol was approved by the ethics committee of Ege University, School of Medicine. The study was conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki. The study protocol was explained and written informed consent was received from each individual before clinical periodontal examination and saliva sampling. Medical and dental histories were obtained.

Smoking history was recorded basing on the self-reports on a standardized questionnaire. Those who reported smoking ≥10 cigarettes per day for more than 5 years were recruited into the smoker groups while; subjects who claimed to have never smoked were recruited into the non-smoker groups. Subjects who smoked ≥10 cigarettes per day for less than 5 years, and those who smoked <10 cigarettes per day for more than 5 years were excluded in an attempt to make a clear discrimination between smokers and non-smokers.

CP patients were diagnosed in accordance with the clinical criteria stated in the consensus report of the World Workshop in Periodontitis (Mariotti, 1999). All of the CP patients had at least 20 teeth present. The CP group had at least four teeth in each jaw with a probing depth (PD) of ≥5 mm, clinical attachment level (CAL) of ≥4 mm, and ≥50% alveolar bone loss in at least two quadrants. Assessment of the extent and severity of alveolar bone loss was done radiographically. Bitewing radiographs were evaluated for interproximal bone loss from the cemento-enamel junction (CEJ) of the tooth to the bone crest. These patients also had bleeding on probing (BOP) at $>$ 80% of the proximal sites. Moreover, a diagnosis of CP was made if the CAL was commensurate with the amount of supragingival plaque. In total 46 CP patients were examined and 13 were excluded since they did not appeal to the inclusion criteria.

The healthy control group also had at least 20 teeth present, ≥90% of the measured sites exhibited PD <3 mm and CAL ≤1 mm, and no BOP, no radiographic sign of alveolar bone loss (i.e. a distance of <3 mm between the CEJ and bone crest at >95% of the proximal tooth sites).

Saliva sampling

Whole saliva samples were obtained by expectorating into polypropylene tubes prior to clinical measurements and any periodontal intervention in the morning following an overnight fast during which subjects were requested not to drink (except water) or chew gum. The saliva samples were clarified by centrifugation (800 *g*) for 10 min at $+4$°C and aliquoted into 500 μl amounts with H_2O. The samples were immediately frozen and stored at −40°C until the sample collection period was completed and thawed immediately before assays.

Clinical measurements

Subsequent to saliva sampling, clinical periodontal recordings, including dichotomous plaque index (+/−), probing depth (PD), clinical attachment level (CAL), and presence of bleeding on probing (BOP; +/−) were performed at six sites (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual locations) on each tooth present, except the third molars, using a Williams probe. CAL was assessed from the cementoenamel junction to the base of the probable pocket. BOP (deemed positive if it occurred within 15 s after periodontal probing) and visible plaque accumulation were recorded dichotomously by visual examination. All measurements were performed by a single calibrated examiner (ÖÖ).

Measurement of ICTP, OC in saliva samples
The ICTP Radio Immuno Assay (RIA) kit (Orion Diagnostica Oy, Espoo, Finland) and the Osteocalcin Enzyme Linked Immunosorbent Assay (ELISA) development kit (Biosource Europe S.A., Nivelles, Belgium) were used for saliva samples. Manufacturers' guidelines were followed for each assay. Concentrations of ICTP and osteocalcin in the saliva samples were then determined by comparing the average sample optical density readings with the concentrations from the assay standard curve. The lower detection thresholds for the ICTP and osteocalcin assays were 0.4 and 0.08 ng ml^{-1}, respectively. The saliva ICTP and osteocalcin concentrations in each sample were calculated basing on the dilutions.

Statistical analysis
A sample size of 8 for each group was estimated to achieve 90% power to detect a difference of 1.0 between the null hypothesis and the alternative mean.

Following Kolmogorov–Smirnov test performed for normality of distributions of the study groups, the standard deviations of saliva ICTP concentrations were found to be too high. Logarithmic transformation was therefore, performed for the salivary ICTP concentrations of chronic periodontitis and control groups. Saliva osteocalcin and Log ICTP concentrations were analyzed between the chronic periodontitis and healthy controls by Covariance-analyses (ANCOVA). First, ANCOVA was used with group (healthy/CP), sex (male/female), smoking (smoker/non-smoker) and age (as a covariate) factors. Sex and age were treated as confounding factors. Since the main effects and the interactions of these factors with others were not significant, they were then removed from the model. So, we performed 2×2 factorial ANOVA for two factors; group (healthy/CP) and smoking. When the interaction between group and smoking factors were found to be significant, independent sample t test was used for homogenous subgroups (smokers/non-smokers). Bleeding on probing and plaque index measurements were obtained in terms of scores at six sites of each tooth present (all subjects had ≥20 teeth present) and then the full mouth percentages were calculated for bleeding on probing as well as plaque accumulation. Therefore, the possible correlations between the biochemical variables and clinical periodontal measurements were computed by the Pearson correlation coefficient. All tests were performed at $\alpha = 0.05$ significance level. All the statistical calculations were performed using the SPSS version 17.0 statistical software package.

Results

Clinical analyses
Demographic variables and mean values of clinical measurements are outlined in Table 1. The age range of the healthy control group was 35–64 years, whereas that of the CP group was 37–67 years. There were no significant differences between the smoker and non-smoker chronic periodontitis patient groups in terms of clinical periodontal measurements ($P > 0.05$). The healthy control group exhibited significantly lower clinical periodontal measurement values than the chronic periodontitis patients ($P < 0.001$). Both plaque and BOP measurements were analysed by Kolmogorov–Smirnov test for normality of distributions and there were no evidence indicating non-normality of these measurements ($P = 0.14$ and $P = 0.67$, respectively).

Salivary ICTP, OC measurements
In the non-smoker chronic periodontitis group, saliva log ICTP concentrations were significantly higher than that of the non-smoker control group ($P = 0.03$) (Table 2). The saliva log ICTP concentrations were significantly higher in the smoker control group than the non-smoker control group ($P = 0.001$). There were no differences between the smoker and non-smoker chronic periodontitis groups in the saliva log ICTP concentrations ($P > 0.05$). The saliva OC concentrations were significantly higher in the smoker and non-smoker periodontitis groups than the two control groups ($P < 0.0001$). The smoker periodontitis group exhibited significantly lower OC concentration than the non-smoker counterparts ($P < 0.0001$). The smoker healthy control group revealed significantly lower OC concentrations than the non-smoker control group ($P > 0.05$).

Correlation analyses between clinical parameters and analyte concentrations revealed a significant positive correlation between saliva OC concentrations and PD, CAL, BOP and plaque scores ($P < 0.0001$) (data not

Table 1 Demographic variables and clinical periodontal measurements (mean ± s.d.) in the study groups where plasma samples were analysed

	Chronic periodontitis n = *33*		*Controls* n = *36*	
Clinical variable	*Smokers* n = *8*	*Non-smokers* n = *25*	*Smokers* n = *11*	*Non-smokers* n = *25*
Age (years)	42.56 ± 8.63	46.25 ± 8.12	40.53 ± 8.49	44.55 ± 8.06
Female/Male (*n*)	2/6	8/17	5/6	14/11
PD (mm)	3.8 ± 0.2	4.2 ± 1.1	1.6 ± 0.2	1.6 ± 0.2
CAL (mm)	5.7 ± 0.2	4.7 ± 0.6	0.0 ± 0.0	0.0 ± 0.0
Plaque (%)	72.5 ± 25.7	81.0 ± 28.4	7.5 ± 10.0	17.8 ± 10.0
BOP (%)	66.4 ± 20.1	66.5 ± 29.1	9.2 ± 9.3	13.8 ± 8.2

Age and gender distribution was similar in chronic periodontitis and healthy control groups ($P > 0.05$). All clinical periodontal measurements were significantly lower in the healthy control group than the chronic periodontitis group ($P < 0.001$).

Table 2 Salivary log ICTP and osteocalcin concentrations (mean ± s.d.) in the study groups

	Chronic periodontitis n = *33*		*Controls* n = *36*	
Biochemical variable	*Smokers* n = *8*	*Non-smokers* n = *25*	*Smokers* n = *11*	*Non-smokers* n = *25*
Log ICTP (Log ng ml^{-1})	0.66 ± 0.30	0.73 ± 0.46[a]	0.83 ± 0.39[b]	0.43 ± 0.29
Osteocalcin (ng ml^{-1})	4.73 ± 1.69[c,d]	5.32 ± 2.38[d]	2.88 ± 0.82[c]	3.19 ± 1.10

[a]Significantly higher than the non-smoker control group ($P = 0.03$).
[b]Significantly higher than the non-smoker counterparts ($P = 0.001$).
[c]Significantly lower than the non-smoker counterparts ($P < 0.0001$).
[d]Significantly higher than the smoker and non-smoker control groups ($P < 0.05$).

shown). No significant correlations were observed between the gender and analyte concentrations. Saliva OC concentrations also correlated positively with ICTP concentrations ($r = 0.566$, $P < 0.0001$).

Discussion

Adverse relationship between smoking and periodontal diseases has been reported in various cross-sectional studies. Smokers are almost four times more likely to have severe periodontitis than non-smokers (Haber *et al*, 1993). Apart from the direct cigarette smoke-mediated effects, tissue damage mediated by impaired balance of bone turnover markers originating from tobacco smoke and tobacco-induced inflammation may be a potential mechanism. In the present study we investigated possible effects of smoking on saliva ICTP and OC concentrations in chronic periodontitis patients and healthy controls.

Several methods of saliva collection are available, including the collection of unstimulated whole saliva; whole saliva stimulated with, typically, paraffin wax, gum base or citric acid; or the collection of saliva from specific salivary glands. Whole saliva contains gingival crevicular fluid (GCF), immune cells and tissue metabolites (Navazesh, 1993; Kaufman and Lamster, 2000) and reflects most closely the predominant intraoral condition (Edgar, 1992). Stimulation, on the other hand, may increase the flow of GCF and this may result in false increases in the concentration of various contents in the saliva (Chapple *et al*, 1997). Accordingly, we collected expectorated whole saliva, where the degree of stimulation was minimal relative to that obtained when using gum, citric acid, or paraffin wax.

ICTP and osteocalcin levels in peri-implant crevicular fluid samples of dental implants with or without peri-implant bone destruction have been investigated in a recent study (Tümer *et al*, 2008). Significant increases in all clinical periodontal measurements as well as OC levels in peri-implantitis sites compared with the clinically healthy sites were reported. Although a slight increase was detected in ICTP levels, the difference between the diseased and healthy sites was not statistically significant. This finding was confirmed in other studies (Shi *et al*, 1996 Lappin *et al*, 2009) reporting no difference in circulating ICTP concentrations between periodontitis patients and healthy controls. Increased levels of ICTP have been observed in the GCF of periodontitis patients (Giannobile, 1999; Giannobile *et al*, 2003). Lappin *et al* (2009) reported higher plasma concentrations of ICTP in periodontitis groups than the healthy subjects where the differences failed to reach statistical significance. In a recent study (Gürlek *et al*, 2009) similar salivary ICTP levels in smoker, non-smoker and ex-smoker patient groups with similar clinical periodontal findings were detected. There was no clinically healthy control group in that study and the number of teeth present, average probing depths and attachment levels were all similar in the three study groups. Our present findings are in line with this report in that there were no significant differences in saliva ICTP concentrations between the smoker and non-smoker chronic periodontitis patients. It may be suggested that the similarity in clinical periodontal disease parameters may explain the similar salivary ICTP levels obtained in these studies.

In an experimental periodontitis study in rats, significant increases in serum OC levels was suggested as a sign of an increase in bone remodeling and thus inhibition of progression of alveolar bone resorption (Buduneli *et al*, 2005c). Serum OC is presently considered to be a valid marker of bone turnover when resorption and formation are coupled, and a specific marker of bone formation when formation and resorption are uncoupled (Giannobile *et al*, 2003). Lappin *et al* (2009) reported reduced plasma OC levels in type 1 diabetics compared to systemically healthy counterparts suggesting that these patients have a reduction in their intrinsic ability to replace bone, such as that which has been destroyed during "acute bursts" of periodontitis. They speculated that this lower OC may make them more susceptible to progression of this disease. Reduced circulating levels of OC in the presence of periodontitis have been reported before by other researchers (Buduneli *et al*, 2005c; Bullon *et al*, 2005).

Shi *et al* (1996) demonstrated lower serum OC levels in periodontitis patients and this has been confirmed recently by Lappin *et al* (2009) that plasma OC levels were lower in periodontitis patients than healthy individuals. Furthermore, OC concentrations were reported to correlate negatively with the extent of periodontitis (Lappin *et al*, 2009; Yoshihara *et al*, 2009). Our present

study revealed significantly higher saliva OC concentrations in the chronic periodontitis patients than the healthy controls. The opposite findings in the systemic level (serum/plasma) and local level (saliva samples) may be explained by the nature of periodontitis being confined to the periodontal tissues. Thus, the increase in saliva OC concentration in chronic periodontitis patients observed in the present study may indicate an increase in the cellular activities of osteoblasts aiming at repair of the damaged alveolar bone. The positive correlations found in the present study between the saliva OC concentrations and clinical periodontal measurements provide further support for this assumption. Moreover, the present study indicated significantly lower salivary OC concentrations in both healthy and diseased smokers than their non-smoker counterparts. The detrimental effects of smoking may explain these decreases in salivary levels of OC in smokers also indicating a deficiency in tissue response to the injuries in smoker subjects. The differences in patient numbers and/or the possible differences in the disease activity states may explain the differences in findings of the present study and the previous ones.

On the other hand, significantly lower salivary OC concentrations in the smoker patients than the non-smokers as well as the ex-smokers may at least partly explain the mechanisms of negative effects of smoking on periodontal health. The present findings are in line with those of the previous study in that smokers exhibited significantly lower salivary OC levels than the non-smokers. Furthermore, the present chronic periodontitis groups revealed significantly higher salivary OC levels than the healthy controls providing further support for the hypothesis that salivary OC level is closely related with the clinical periodontal situation. In our previous study, the significant negative correlation between salivary OC concentration and years smoked suggests that smoking has deteriorating effects. There was no clinically healthy group in the previous study and the present study provides further data comparing the chronic periodontitis patients with clinically healthy control groups. Yet, one possible limitation of the present study is the rather low patient numbers in the smoker groups.

In conclusion, within the limits of the present study it may be suggested that smokers have reduced salivary OC levels compared to non-smokers possibly playing a role in the increased susceptibility of smoker patients to periodontal tissue destruction. Salivary OC level increases in chronic periodontitis patients compared to the healthy controls. Larger scale and intervention studies are required to better address this issue.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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