Original Article

Serum, saliva, and GCF concentration of RANKL and osteoprotegerin in smokers versus nonsmokers with chronic periodontitis

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Abstract

Background: The role of host response in periodontitis pathogenesis is confirmed, and it is well established that immune response plays a major role in the alveolar bone destruction. In the investigation of these responses, the role of receptor activator of the nuclear factor-kB ligand (RANKL)-osteoprotegerin (OPG) system is the most promising. Smoking can affect the RANKL-OPG system in a manner that will further enhance bone loss in periodontitis. The aim of this study is to assess the serum, saliva, and gingival crevicular fluid (GCF) concentration of RANKL and OPG in smoker versus nonsmoker untreated chronic periodontitis (CP) patients.

Materials and Methods: Thirty-nine subjects were included in the present cross-sectional study: 29 systemically healthy CP male patients (15 smokers, 14 nonsmokers) and 10 systemically and periodontally healthy nonsmoker male subjects. Serum, GCF, and whole saliva samples were obtained from the subjects. The enzyme-linked immunosorbent assay (ELISA) kits were used for assaying the concentrations of RANKL and OPG in the samples. The one-way analysis of variance (ANOVA) test and the least significant difference (LSD) *post hoc* test were utilized to compare differences between the groups.

Results: RANKL and OPG concentrations in saliva, serum, and GCF did not show any significant difference among all groups ($P > 0.05$). Salivary RANKL/OPG ratios were significantly higher in the nonsmoker CP group than in the healthy control group ($P > 0.05$) but they were not statistically significant among smoker periodontitis patients.

Conclusions: The salivary RANKL/OPG ratio was higher in nonsmokers with periodontitis in comparison with smoker periodontitis patients.

Key Words: Gingival crevicular fluid, OPG, periodontitis, RANKL, saliva, serum, smoking

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INTRODUCTION

To date, the role of host response in periodontitis pathogenesis has been confirmed,[1] and alveolar bone destruction is one of the main diagnostic features of periodontitis. Also, it is well established that immune response plays a major role in this alveolar bone destruction.[2,3] The close relationship between

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the immune system and bone metabolism has been studied in osteoimmunology, an interdisciplinary field of study on the interactions of immune skeletal cells with their common regulatory cytokines and other molecules.[2,4] In the investigation of these interactions, the roles of three novel members of the tumor necrosis factor (TNF)-ligand and receptor superfamilies show the most promise.[3,5]

The activation of osteoclasts to initiate bone destruction is signaled by receptor activator of the nuclear factor-kB Ligand (RANKL), receptor activator of nuclear factor kB (RANK), and osteoprotegerin (OPG). RANKL attaches to RANK, a receptor on the cell surfaces of osteoclasts and osteoclast precursors, which stimulates the proliferation and differentiation of cells to form the osteoclast phenotype.[6-8] Conversely OPG, a soluble decoy receptor produced by osteoclasts and many other cells, modifies the effects of RANKL by inhibiting RANKL/RANK interaction.[9] Bone loss occurs as a result of an increased RANKL/OPG ratio, and the RANKL/OPG ratio has been found to increase because of either an increase in RANK or a decrease in OPG, or both. This ratio is increased at the sites of active periodontal disease and correlates with disease severity[10-12] as well. However, RANKL and OPG can be detected in gingival tissue, gingival crevicular fluid (GCF), saliva, and serum. It can prove reliable information on the state of periodontal disease activity.[3,5,13-15]

Since smoking is the most important environmental risk factor in the development of periodontal disease,[16-18] many studies have addressed the effect of smoking on the RANKL/OPG ratio in periodontitis. In whole saliva, smokers with untreated periodontitis had significantly lower OPG concentrations than untreated nonsmokers with periodontitis.[19] Saliva RANKL levels appeared to be higher in the smokers, but the difference was not statistically significant. The same results were observed in serum, GCF, and the periodontal tissue of smokers with periodontitis on maintenance (supportive therapy).These studies also show higher RANKL/OPG ratios in maintenance groups.[20,21] Different results, however, have been reported. RANKL and OPG concentrations and their ratios have not been found to have statistically significant differences in the serum and GCF of smokers versus. nonsmokers with untreated periodontitis.[22-24] However, some authors believe that smoking can indeed affect the RANKL-OPG system in a manner that would further enhance bone loss in periodontitis.[3,5]

The current literature regarding the considerable variation between studies in the reported levels of RANKL, OPG, and RANKL/OPG ratios in saliva, serum, and GCF.[5] Also claims of the critical effect of smoking on the RANKL-OPG system are still controversial. To our knowledge, there is no comprehensive report to evaluate the RANKL and OPG concentrations in serum, saliva, and GCF all together in patients with periodontal disease. Therefore, the aim of this study was to assess the serum, saliva, and GCF concentration of RANKL and OPG in smoker versus nonsmoker untreated chronic periodontitis (CP) patients.

MATERIALS AND METHODS

Thirty-nine individuals were included in the present cross-sectional study. Twenty-nine systemically healthy with CP male patients (15 smokers, 14 nonsmokers) seeking dental treatment at the School of Dentistry, Isfahan Medical University and 10 systemically and periodontally healthy nonsmoker male subjects were invited to participate in this study. This study was approved by the Human Ethics Research Committee of Isfahan Medical University. The subjects were informed about the study protocol, and the patients were all in good health and had not received any antibiotic treatment or nonsteroidal anti-inflammatory drugs (NSAIDs) during the last 3 months. All CP patients had at least 16 teeth present and two single-rooted teeth with a probing depth (PD) of 4 mm and a clinical attachment level (CAL) of 3 mm. Since it was difficult to find female smokers, all subjects recruited were male.

Smoking history was collected by means of self-reporting following a standardized questionnaire.[25] Those who reported smoking10 cigarettes per day for more than 5 years were recruited into the smoker group. The lifetime smoking exposure of smoker patients were expressed as pack years. One pack year is defined as 20 manufactured cigarettes (one pack) smoked per day for 1 year.

Saliva sampling

Whole saliva samples were obtained in the morning following an overnight fast and all the subjects were required to not drink anything except water. The spitting method was used where the subject was asked to accumulate saliva in the floor of the mouth and then spit into a polypropylene tube.[26] The saliva samples were clarified by centrifugation (1500 g) for 15 min and aliquoted into 0.3 ml portions. The samples were stored at -70°C until the sample collection period was completed.

Serum sampling

A blood sample was obtained from the antecubital vein in the morning after an overnight fast. The

venous blood samples were collected by a standard venipuncture method and the serum was separated from blood by centrifugation at 1500 g for 15 min. The serum samples were then stored at -70°C until the sample collecting period was completed.

GCF sampling

In periodontitis subjects, GCF samples were obtained from the interproximal aspect of a singlerooted tooth with PD of at least 4 mm and CAL of 3 mm. In the healthy group, GCF samples were collected from the interproximal aspect of single-rooted teeth exhibiting PD up to 3mm without bleeding on probing. The GCF was sampled by using the method of Offenbacher[27] with a slight modification. Supragingival plaque was removed by sterile curettes, and cotton rolls were used for drying and isolating the selected sites. A sterile PerioPaper strip (Oraflow Inc., Plainview, NY, USA) was gently inserted into the periodontal pocket until mild resistance was felt and the strip was kept in the selected site for 30 s. Mechanical trauma was avoided and the strips with traces of blood were discarded. The strips were placed in a polypropylene tube containing 175µl phosphate buffered saline (PBS). The tubes were shaken gently for 1 min and the samples were left at 4°C for 3 h. Then the tubes were centrifuged at 4000 g and 4°C for 5 min and finally, were stored at -70°C for subsequent assays.

Clinical examination

The day before sampling, clinical periodontal recordings, including PD, CAL,[28] Plaque Index (PI),[29] and bleeding on probing (BOP) (modified Sulcus Bleeding Index)[30] were performed at six sites (mesiobuccal, mid-buccal, distobuccal, mesiolingual, mid-lingual, and distolingual locations) on each tooth present, except the third molars, using a Williams probe (Hu-Friedy, Chicago, IL, USA).

Enzyme immunoassay

The human RANKL enzyme-linked immunosorbent assay (ELISA) development kit (Peprotech EC, London, UK), and the human OPG instant ELISA kit (eBioscience, Vienna, Austria) were used for assaying the concentration of these proteins in the serum, saliva, and GCF samples according to the manufacturers' recommendation. The concentration of RANKL and OPG in each of the samples was then determined by comparing the average sample optical density readings with the concentrations from the assay standard curve. Data were reported as concentrations of cytokine in picograms per milliliter (pg/ml).

Statistical analysis

Statistical analyses were performed using the one-way analysis of variance (ANOVA) test, and the least significant difference (LSD) *post hoc* test to compare differences between groups. Differences were considered statistically significant at $P < 0.05$. The correlations between RANKL and OPG concentrations and RANKL/OPG ratios with clinical parameters were found by performing Spearman's correlation analysis. Also, Pearson correlation analysis was used to investigate correlations between GCF, saliva, and serum RANKL and OPG levels.

RESULTS

Clinical characteristics of study groups

The demographic and clinical data are shown in Table 1. The healthy control group showed significantly lower clinical periodontal measurement values than the CP patients ($P < 0.001$). The mean of pack years of the smoker group was 13.76.

GCF, saliva, and serum RANKL and OPG measurements

OPG concentrations in saliva, serum, and GCF did not show significant differences among all groups [Table 2]. Salivary RANKL concentrations were significantly higher in the nonsmoker CP group than in the smoker CP group. Also nonsmokers had significantly higher salivary RANKL/OPG ratios than the smokers. Both CP groups had significantly lower serum RANKL concentrations than the healthy controls ($P < 0.05$).

Table 1: Demographic and clinical characteristics of study groups (mean±SD)

Groups	Healthy control	Nonsmokers	Smokers
Age (years)	30±2.06	48.4±9.84	38.33±9.39*
TL (n)	-	4.71±3.98	4±3.27
PD (mm)	1.41±0.20	2.92±0.62	2.41±0.34*
CAL (mm)	-	3.65±0.72	2.78±1.06
PI	0.87±0.12	1.54±0.23	1.32±0.21
BOP (%)	18±5.17	67±18.13	47.2±13.92*

*Smokers significantly different from the nonsmokers ($P<0.05$)

Table 2: RANKL and OPG concentrations (mean±SD) and RANKL/OPG ratios in study groups

Groups	Healthy control	Non smokers	Smokers
RANKL (pg/ml)			
Serum	28.58±24.62[1]	13.57±11.25	12.83±10.69
Saliva	13.33±6.02	20.04±8.8[2]	12.61±6.49
GCF	8.61±5.44	8.08±4.68	8±4.2
OPG (pg/ml)			
Serum	92.13±29.09	97.39±24.9	83.98±18.63
Saliva	80.25±26.75	64.31±19.16	71.86±21.83
GCF	75.91±20.92	69.55±23.6	72.53±20.93
RANKL: OPG ratio			
Serum	0.28±18	0.16±0.12	0.15±0.13
Saliva	0.16±0.11	0.33±0.18[2]	0.17±0.12
GCF	0.12±0.07	0.12±0.07	0.11±0.05

[1]Healthy controls significantly different from the smoker and nonsmoker groups ($P<0.05$). [2]Nonsmokers significantly different from the smokers ($P<0.05$)

Correlations between GCF, saliva, and serum RANKL and OPG levels

In all groups, there were significant positive correlations between RANKL concentration and RANKL/OPG ratio in serum, saliva, and GCF samples ($P < 0.01$).In nonsmoker patients, the salivary RANKL/OPG ratio was positively correlated with the RANKL/OPG ratio in GCF ($P < 0.05$). In healthy controls, the salivary RANKL/OPG ratio had a significant positive correlation with the serum RANKL/OPG ratio ($P < 0.01$).

Correlation of clinical parameters with RANKL and OPG

In the nonsmoker patient group, the RANKL concentration ($P < 0.05$) and RANKL/OPG ratio ($P < 0.01$) in saliva showed a statistically significant negative correlation with telomere length (TL). In smoker patients, PI was negatively correlated with serum RANKL and OPG concentrations ($P < 0.05$) and their ratios ($P < 0.01$). In this group the RANKL/OPG ratio in GCF showed a significant negative correlation with age and pack years ($P < 0.05$).

DISCUSSION

Smoking is the most important environmental risk factor in the development of periodontal disease,[16-18] but claims of the critical effect of smoking on the RANKL-OPG system are still controversial. The present study explored the effect of smoking on the RANKL-OPG system in the serum, saliva, and GCF of patients with periodontitis. In the molecular context, the attachment of RANKL to its receptor (RANK) ends in the differentiation of progenitor monocytes to mature osteoclasts,[8] but OPG competes with RANKL to bond with RANK and inhibits osteoclastic activity and maturation.[9] Thus an increase in RANKL/OPG ratio indicates the possibility of osteoclastic alveolar bone resorption.

In the present study, the mean OPG concentration in saliva, serum, and GCF did not show significant differences among all groups. Salivary RANKL concentrations were significantly higher in the nonsmoker CP group than in the smoker CP group. Also nonsmokers had significantly greater concentrations of Salivary RANKL/OPG ratios than the smokers. Both CP groups had significantly lower serum RANKL concentrations than the healthy controls.

Previous studies had not shown significant differences between the salivary OPG levels of periodontal patients and healthy controls.[19,31-33] Only in Ramsier's study,[34] was the salivary OPG level in periodontitis patients significantly lower than in the gingivitis patients and the healthy control groups. In the present study, the salivary OPG levels in both CP groups were lower than the healthy controls, although the differences were not statistically significant. OPG inhibits osteoclastic activity, and by decreasing OPG level, alveolar bone loss can occur in CP groups.[9] In the present study, in contrast with Buduneli *et al*., a higher level of saliva OPG concentration but a lower level of RANKL and RANKL/OPG ratio in smoker group was observed compared to nonsmoker patients. Buduneli *et al*. assessed the concentration of OPG and RANKL in both patients with periodontitis on maintenance phase and patients with untreated periodontitis. The effects of nicotine on osteoblasts's OPG production have been proved,[35] so they assessed the salivary cotinine level (nicotine's major metabolite) and patients with higher than 100 ng/ml cotinine were included in smoker periodontitis groups. In their study, the higher RANKL/OPG ratio might be due to the higher level of nicotine metabolites in smokers compared to nonsmokers.

In this study, the lower concentration of OPG in smoker serum samples might illustrate that cigarettes can decrease the OPG level and consequently have an impact on bone resorption. However, this difference was not statistically significant. In Lappin's study lower levels of OPG and RANKL were observed in smokers, and this result was statistically significant for OPG.[20] Although the effect of smoking on the OPG levels is still unclear, in one *in vitro* study, nicotine has been shown to reduce the OPG production by osteoblasts.[35] In contrast with the present study, Lappin *et al*. surveyed the patients with periodontitis in the maintenance phase, but in that study the results were not compared with healthy controls. In the Ozcaka *et al*. study the serum OPG and RANKL concentrations did not show significant differences between smoker and nonsmoker patients with untreated periodontitis and nonsmoker healthy controls.[23] Although in the present study OPG concentrations in serum have not shown significant differences among all groups, against our expectation both CP groups had significantly lower serum RANKL concentrations than the healthy controls. Various levels of sub-clinical inflammation among the healthy subjects or differences in the sensitivity of ELISA kits might be the reason of differences.

Like the present results, previous studies didn't show statistical differences between GCF RANKL and OPG level of smoker and nonsmoker patients with untreated periodontitis.[22,24] Tang *et al*. suggest that a minimum threshold of lifetime exposure to cigarette smoking is needed before significant changes can start showing in OPG levels and in the

RANKL/OPG ratio.[22] By considering pack years, they found significant differences between the high pack years group and the never smoker group. However, the present results did not confirm those findings. A negative correlation was found between the RANKL/OPG ratio in GCF and pack years among smoker group. In contrast with previous studies, the present results have shown greater GCF RANKL/OPG ratios in healthy controls than in patients with periodontitis. The episodic pattern of periodontal disease maybe the probable reason as Vernal *et al*. reported that the total amount of GCF RANKL level was significantly higher in patients than in controls, whereas active sites revealed significantly higher levels than inactive counterparts.[11]

This study showed that as the age increased the amount of RANKL/OPG ratio decreased in nonsmoker's GCF; which was similar to the findings of the Tang GCF study and to the Ozcaka serum results. This may indicate that the pace of alveolar bone loss in younger periodontitis patients. It can also suggest a sign of decreased osteoclastic activity in older patients. In the nonsmoker patients group, the RANKL concentration and the RANKL/OPG ratio in saliva showed a negative correlation with tooth loss. This confirmed the correlation between periodontal disease and the higher RANKL/OPG ratio because when the tooth loss happened and the periodontal infection decreased in the oral cavity, the RANKL/OPG ratio decreased in saliva. Also, it may show the validity of salivary samples over other biological fluid for assessing the RANKL/OPG ratio in periodontitis patients.

In previous studies, a significant correlation between periodontitis and RANKL/OPG ratios was obtained with changing OPG concentrations.[19,20,23,36] In the present study, significant positive correlations were found between RANKL/OPG ratios and changing of RANKL concentration in serum, saliva, and GCF samples in all three groups. This may confirm the role of RANKL as the principal biomarker for increasing the RANKL/OPG ratio toward the progress of alveolar bone loss. Based on the present results, the salivary RANKL/OPG ratio was positively correlated with the RANKL/OPG ratio in the GCF of nonsmoker patients and a significant positive correlation with the serum RANKL/OPG ratio in healthy controls. Whole saliva is easy to collect and can manifest host-derived products (e.g., serum-derived products, GCF, host enzymes) that have been suggested as potential diagnostic markers for periodontal disease susceptibility.

CONCLUSION

The salivary RANKL/OPG ratio was higher in nonsmokers with periodontitis in comparison with smoker periodontitis patients. It may be concluded that smoking does not affect bone resorption via the RANKL-OPG system.

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