Effect of Non-Surgical Therapy on Receptor Activator Level of Salivary Nuclear Factor Kappa B Ligand and Osteoprotegerin in Chronic Periodontitis: A Prospective Cohort Study

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Introduction: The receptor activator of NF-kB ligand (RANKL) and osteoprotegerin (OPG) are molecules that play a significant role in bone resorption. The aim of this study was to evaluate the effect of periodontal therapy on the salivary levels of RANKL, OPG, and their relative ratio in patients with chronic periodontitis. **Materials and Methods:** This prospective cohort study was conducted and twenty-five patients with chronic periodontitis and 25 volunteers with healthy periodontium were recruited in this study. All the patients received scaling and root planing (SRP) combined with oral hygiene instructions (OHI). Five mL unstimulated saliva sample was collected from all individuals at baseline and four weeks after periodontal therapy. Salivary RANKL and OPG concentrations were determined by enzyme -linked immunosorbent assays (ELISA). Demographic and clinical vari¬ables were compared between the groups using Fisher's exact test. Comparisons of indices and analyses between the test and control groups were performed by independent t-test and Mann-Whitney U test. The correlations between the salivary and periodontal parameters were evaluated by Spearman correlation coefficient test. **Results:** SRP improved all examined clinical parameters, and the RANKL concentration and sRANKL/OPG ratio decreased significantly in the saliva after the treatment (p=0.012 and p=0.011). However, no significant change was found for OPG (p=0.840). **Conclusion:** Our observations indicated that non-surgical periodontal treatment significantly reduced the RANKL and sRANKL/OPG levels. Salivary levels of RANKL and sRANKL/OPG reflected the disease response to therapy, which is suitable for monitoring the results of periodontal treatment.

Keywords: Chronic periodontitis; Osteoprotegerin; Root Planing; RANK Ligand; Saliva

Introduction

Periodontal disease is an inflammatory condition characterized by the interplay between the periodontal pathogens and the inflammatory–immune system, which causes the destruction of the connective tissue attachment. Development of chronic inflammation, progressive degradation of connective tissues, and resorption of alveolar bone contribute to the clinical characters of the periodontal diseases. Clinical parameters are routinely used for diagnosis of periodontal diseases, but increasing the amounts of circulating biological molecules have been detected in the gingival crevicular fluid (GCF) and whole saliva of the patients with periodontal disease (1-7).

Interleukin (IL)-1b, C reactive protein, human macrophage inflammatory protein (MIP)-1a, matrix metalloproteinase (MMP)-8 and -9, osteoprotegerin (OPG), tumor necrosis factor (TNF)-a, receptor activator of nuclear factor-kB ligand (RANKL), and pyridinoline cross-linked carboxyterminal telopeptide of type I collagen (ICTP) have the potential to be used as biomarkers to diagnose the presence and severity of periodontitis (7-18). Recently, it has been reported that the formation and activity of osteoclasts are regulated by three members of the large family of Tumor Necrosis Factor and

Tumor Necrosis Factor Receptor: Receptor Activator of Nuclear Factor (RANK), RANK Ligand (RANKL), and Osteoprotegerin (OPG) (19). RANKL stimulates bone resorption through attachment to RANK in the membrane of the osteoclasts. In contrast, OPG is a soluble decoy receptor for RANKL, interferes with RANK to connect with RANKL, and prevents the maturation and activation of osteoclasts and their precursors (5, 20). The regulatory axis of RANK / RANKL / OPG also causes inflammatory bone destruction induced by pro-inflammatory cytokines such as PGE2, IL-1B, IL6, IL-1, TNF-α (20, 21).

Studies have shown that patients with periodontitis have a significantly higher RANKL/OPG ratio in their gingival fluid, indicating the role of RANKL and OPG in alveolar bone degradation in periodontitis (22, 23). In periodontics, the common clinical criteria are often inadequate to determine the areas of active disease and response to treatment or to measure the extent or potential progression of the disease in the future. Compared with GCF, the use of saliva as a diagnostic liquid for the analysis of oral-related diseases is convenient and non-invasive. Saliva collection is easy and there is enough saliva (24). However, it cannot be a site-specific examination and can be used only for the whole mouth follow-up (25, 26). Quantitative studies have examined the profile of salivary biomarkers in periodontal patients (27, 28). There are very few studies on the ratio of protective and pathogenic biomarkers in the healthy people and patients or their comparison before and after periodontal treatment. It should also be noted that there is no study on the effect of periodontal treatments on the quantitative changes of RANKL / OPG ratio in saliva before and after periodontal treatment.

Therefore, this study aimed to evaluate the quantitative changes of RANKL/ OPG ratio in patients with chronic periodontitis in response to non-surgical periodontal treatments and compare it with healthy subjects. In this way, it can be assuming that salivary biomarkers can be used for periodontal diagnosis and follow-up.

Materials and Methods

Participants

Fifty patients were included in this longitudinal, case-control, clinical study from among 200 subjects visiting the Department of Periodontics, School of Dentistry, Qazvin University of Medical Sciences from March 2012 to April 2013. They were diagnosed with chronic periodontitis based on the criteria defined by the American Academy of Periodontology (29, 30). As a part of the study protocol, personal, medical, and dental history questionnaires and written consent were obtained from all the subjects, and the study protocol was clearly explained to each patient. The ethical clearance for this study was approved by the Ethical Committee of Qazvin University of Medical Sciences (No. 576).

The inclusion criteria consisted of age over 18 years, good general health, and a minimum of 18 erupted teeth. The exclusion criteria were history of periodontal therapy in the past two years, alcoholism and smoking; liver, kidney, or salivary gland dysfunction; infectious diseases, inflammatory bowel disease; rheumatoid arthritis, granulomatous diseases; and diabetes, organ transplant, or cancer therapy. Pregnancy or lactation, use of glucocorticoids, cyclooxygenase inhibitors, bisphosphonates, antibiotics or immunosuppressant medications within the last six months, need for antibiotics for infective endocarditis prophylaxis during the dental procedures, symptoms of acute illness (e.g., fever, sore throat, body aches, and diarrhea), orthodontic appliances or presence of an oral mucosal inflammatory condition (e.g., aphthous, lichen planus, leukoplakia, and oral cancer) and any other conditions that could alter the course of periodontal disease were other exclusion criteria.

Clinical evaluation

For each subject, a full-mouth periodontal probing and charting was performed. Periodontal health measures, including BOP, PPD, CAL, PI, and periapical radiographs were assessed by an expert periodontist at 4 locations per tooth using a probe of University of North Carolina. The selected patients had to have a diagnosis of generalized moderate to severe chronic periodontitis and at least five teeth with CAL ≥3 mm and PPD>5 mm distributed among at least two quadrants with a minimum of two affected teeth in each quadrant.

Saliva collection

After patient selection, unstimulated whole expectorated saliva (5 mL) was collected from each subject between 10 AM and 12 PM according to the modified version of the method described by Navazesh [31]. The patients were asked not to eat anything one hour before saliva sampling and then to swallow the saliva before allowing it to drain passively for five minutes over the lower lip into a sterile tube while seated in an upright position. The collected saliva was immediately placed on ice prior to

freezing at –80°C. The samples were defrosted and analyzed within six months of collection.

The participants with periodontitis received scaling and root planing (SRP) (hand instrumentation of the teeth to remove dental plaque and/or calculus, ultrasonic scaling therapy) and oral hygiene instructions (OHI). Two weeks after periodontal treatment, supra gingival prophylaxis was performed, and patient's drug use, hygienic condition, and plaque control were checked. Four weeks after periodontal treatment, the mentioned clinical periodontal indicators were reassessed, and 5ml saliva samples were prepared by unstimulated method.

Biomarker analysis

Each saliva sample was pipetted into a clean microcap tube and clarified by centrifugation at 10,000 g for 5 minutes. The supernatant was transferred to clean microcap tubes and used immediately for enzyme-linked immunosorbent assay (ELISA). The concentrations of sRANKL and OPG were determined by a human sRANKL ELISA Kit (Bioassay Technology Laboratory, Shanghai, China) and a human OPG ELISA Kit (Bioassay Technology Laboratory) according to the manufacturer's directions. The results of the sRANKL and OPG assays were expressed as pg/mL for the concentrations. All of the laboratory tests were performed in the immunology department of Qazvin University of Medical Sciences.

Statistical analysis

Statistical analysis was performed by IBM SPSS ver. 18.0 (IBM Co., Armonk, NY, USA). Demographic and clinical variables were compared between the groups using Fisher's exact test. Comparisons of indices and analyses between the test and control groups were performed by independent t-test and Mann-Whitney U test. The correlations between the salivary and periodontal parameters were evaluated by Spearman correlation coefficient test. *P*-value <0.05 was considered statistically significant.

Results

A total of 50 participants, 25 healthy subjects (11 females and 14 males aged 24 to 50 years) and 25 patients with chronic periodontitis (25 females and 25 males aged 22 to 62 years), were recruited in this study. There were no significant differences between the two groups in terms of gender and age. The demographic and clinical characteristics and pre-treatment ELISA findings of the control and test groups are shown in Table 1 and Table 2. As expected, the mean periodontal indices were significantly higher in the periodontitis group before periodontal treatment (PI (*P*=0.000), PPD (*P*=0.01), CAL (*P*=0.001), and BI (*P*=0.000).

All of periodontal indices reduced significantly in the test group after treatment (PI (*P*=0.000), PPD (*P*=0.010), and BI (*P*=0.000) except CAL, which reduced insignificantly after treatment (*P*=0.480) (Table 3).

As shown in table 4, comparison of salivary levels of sRANKL and sRANKL/OPG in the test group before and after periodontal treatment showed a significant decrease (*P*=0.012 and *P*=0.011). However, no significant difference was observed in salivary OPG concentration before and after treatment (*P*=0.840). Moreover, PI and CAL had a positively direct relationship with sRANKL and sRANKL/OPG. Also, there was no significant difference between the treated patients and healthy subjects in salivary levels of sRANKL and sRANKL/OPG (*P*≤0.05).

Table 1. Demographic and clinical characteristics of subjects before treatment

	Healthy subjects (n=25)	Periodontitis patients (n=25)	*P*-value
Age (year)	24–50	22–62	-
Sex (female/male), n (%)	11/14 (44/56)	14/11 (56/44)	0.1540
Probing pocket depth (mm)	1.50±0.27	4.10±0.59	0.0005*
Plaque index	0.45±0.43	2.23±0.62	0.0005*
Clinical attachment level (mm)	0	3.57±0.93	0.0010*
Bleeding index	0.05±0.07	0.78±0.26	0.0005*

sRANKL: Receptor Activator Level of Salivary Nuclear Factor Kappa B Ligand, OPG: Osteoprotegrin, *: significant results

Table 2. Enzyme-linked immunosorbent assay of salivary biomarkers in healthy subjects and chronic periodontitis before treatment

	Healthy subjects (n=25)	Periodontitis patients (n=25)	*P*-value
sRANKL (pg/mL)	207±83	266±48	0.0040*
OPG (pg/mL), median±IQR	2.1±1.0	2.20±0.78	0.4550
sRANKL/OPG	91.5±29.7	120±27	0.0010*

sRANKL: Receptor Activator Level of Salivary Nuclear Factor Kappa B Ligand, OPG: Osteoprotegrin, *: significant results

Table 3. Comparison of clinical characteristics in the test group before and after treatment

	Patients before treatment	Patients after treatment	*P*-value
Probing pocket depth (mm)	4.10±0.59	2.50±0.32	0.010*
Plaque index	2.23±0.62	0.95±0.35	0.0005*
Clinical attachment level (mm)	3.57±0.93	3.15±0.85	0.480
Bleeding index	0.78±0.26	0.06±0.15	0.0005*

*: significant results

Table 4. Comparison of sRANKL, OPG, and sRANKL/OPG in the test group before and after treatment

	Patients before treatment	Patients after treatment	*P*-value
sRANKL (pg/mL)	266±48	216±106	0.012*
OPG (pg/mL)	0.78±2.2	2.6±1.1	0.840
sRANKL/OPG	120±27	96.5±25.5	0.011*

sRANKL: Receptor Activator Level of Salivary Nuclear Factor Kappa B Ligand, OPG: Osteoprotegrin, *: significant results

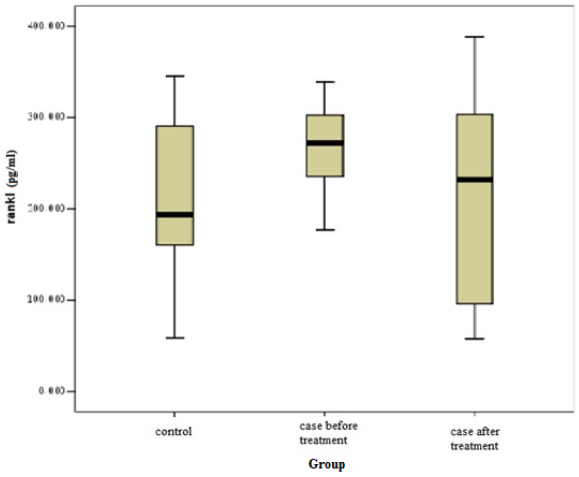

Figure 1. A box plot showing the salivary level of soluble receptor activator of nuclear factor kappa B ligand (sRANKL). The results of this study showed that sRANKL was significantly different between the healthyand patient groups before the treatment, but there was no significant difference between the healthy controls and the patients after treatment.

The salivary levels of sRANKL, OPG and sRANKL/OPG in the entire study subjects before and after treatment are shown in figures 1–3. The salivary levels of sRANKL and sRANKL/OPG were significantly different between the test and control groups before periodontal treatment (*P*=0.004 and *P*=0.001). For the OPG concentrations, however, no significant differences were found (*P*=0.455).

Discussion

Although periodontal bacteria are the main causes of periodontitis, the progress and severity of the disease are determined by the host immune responses (24). Since periodontitis is one of the most common oral diseases, it can be considered an important health problem affecting the quality of life. As a result, timely diagnosis, treatment, and follow-up of this disease are important. Periodontitis is usually diagnosed and rechecked by measuring the clinical indicators and

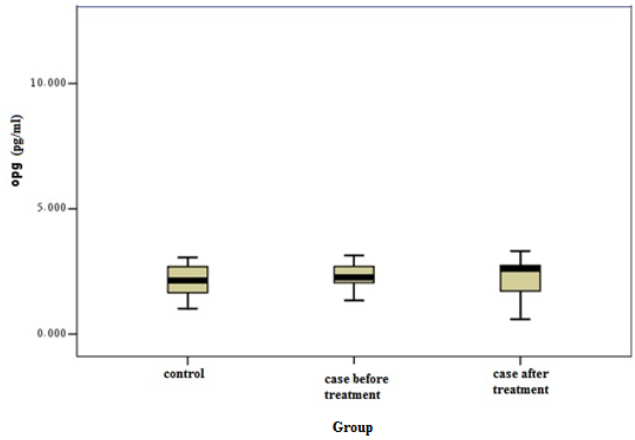

Figure 2. A box plot showing the salivary level of osteoprotegerin (OPG). The results of this study showed no significant difference between the healthy and test groups before and after the treatment.

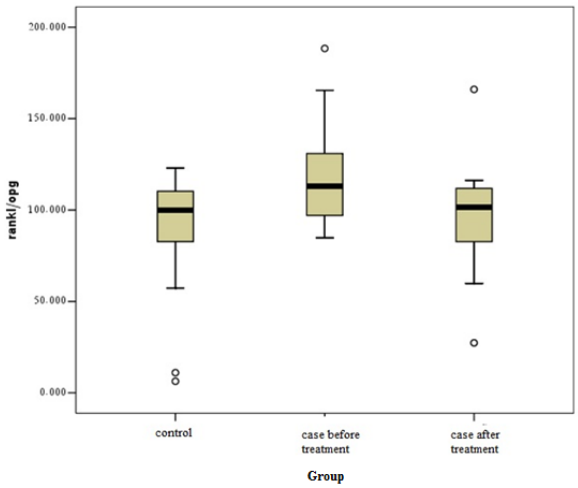

Figure 3. A box plot showing the salivary soluble receptor activator of nuclear factor kappa B ligand/osteoprotegerin (sRANKL/OPG) ratio. The results of this study indicated that the salivary sRANKL/OPG ratio was significantly different between the healthy and patient groups before the treatment, but there was no significant difference between the two healthy and the patients after treatment.

radiographic findings, which are not sufficient to determine the extent of disease activity, the ability to develop in the future, and the response to treatment (23). Biomarkers in oral fluids are able to provide additional information about the disease condition in addition to the clinical parameters. Various biomarkers related to the formation, analysis, and reconstruction of bone have been found in the saliva and gingival fluid. Quantitative and qualitative changes in these biomarkers are useful as a method of diagnosis, and combination of two or more biomarkers can provide a more accurate evaluation of the periodontal disease (27). Of course, these methods can never replace clinical examinations (32, 33).

In periodontitis, which is an inflammatory disease with bone resorption, the pathophysiologic process of bone destruction can be interpreted through osteo-immunology. TNF family proteins and TNF receptors such as RANK, RANKL, and OPG are essential factors involved in bone resorption and reconstruction. The RANK / RANKL / OPG system plays an important role in the production and activation of osteoclasts as well as regulation of bone resorption (20, 21). Patients with periodontitis have been shown to have a significantly higher RANKL/OPG ratio in their gingival fluid, suggesting the role of RANKL and OPG in alveolar bone degradation (22, 23, 34-37).

In the present study, we examined changes in the salivary concentrations of RANKL, OPG, and RANKL/OPG ratio in response to periodontal treatment in patients with chronic periodontitis. Regarding the results of periodontal treatment which indicated significant changes in clinical indexes, including BI, PI, and PPD, the concentration of RANKL and RANKL / OPG decreased significantly, while there were no significant changes in the salivary concentrations of OPG. Also, based on the results of this study, CAL and PI were positively correlated with RANKL concentrations and RANKL/OPG ratio.

Most similar studies including RANKL and OPG reviews in periodontal patients, healthy subjects, and in response to treatment were on the gingival crevicular fluid.

According to the results of a study by Bunduneli *et al.,* that examined the changes in concentrations of OPG and RANKL four weeks after periodontal treatment, all clinical parameters decreased significantly and the concentration of OPG in the groups was reduced after SRP, but there was no difference in RANKL level. In this study, contrary to expectations, despite the success of the treatment, OPG was decreased (38).

In 2011, Bostanci *et al.,* performed a study to investigate the changes of OPG, RANKL, and RANKL/OPG ratio in gingival fluid in response to periodontal treatment. Despite improvement in clinical symptoms, the RANKL/OPG ratio did not change, and there was no correlation between this ratio and clinical parameters, which contradicted the results of this study.

So far, few studies have considered the role of these biomarkers in saliva (39).

In 2008, Frodge *et al.,* examined the tumor necrosis factor-alpha (TNF-a), C-telopeptide pyridinoline cross-links of type 1 collagen (ICTP), and RANKL in the saliva associated with bone

remodeling and destruction of periodontal tissues. They reported that RANKL levels in 81% of the samples were lower than the levelsfound and measured in our study in all specimens. This can be due to the technical challenges of sampling, methodological variations in immunoassay analysis, and differences in the extent, severity, and activity of the disease (15). In a study by Sexton *et al.,* in 2011 that examined changes in the salivary biomarkers such as OPG in response to periodontal treatment after 16 and 28 weeks, as in the present study, all clinical parameters except CAL were improved. OPG also decreased in these patients as a result of treatment, while this change was not seen in our study. In our study, as expected, the salivary OPG was increased although it was not significant (20).

Like the findings of the studies performed on the gingival fluid, the results of this study and other studies on salivary RANKL and OPG indicated that the concentration of this biomarker was not steady in the healthy samples and patients in response to treatment.

Conclusion

The present study is the only study that investigated the changes of RANKL/OPG ratio in saliva as a result of periodontal treatment, which showed a decrease in those responding to the treatment. But in order to be able to use this ratio to determine the response rates to periodontal treatments, we need to confirm this result by conducting more studies on a higher number of samples and in different populations.

Conflict of Interest: 'None declared'.

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