

Full Length Research Paper

The effect of chronic periodontitis on serum levels of matrix metalloproteinase-2 (MMP-2), tissue inhibitor of metalloproteinase-1 (TIMP-1), interleukin-12 (IL-12) and granulocyte–macrophage colony-stimulating factor (GM-CSF)

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A complex network of chemokines and pro- and anti-inflammatory mediators is involved in the initiation and progression of chronic periodontitis. Matrix metalloproteinases (MMPs), the main enzymes responsible for matrix degradation, are important for periodontal tissue destruction, but their activity can be inhibited by tissue inhibitors of metalloproteinases (TIMPs). Interleukin-12 (IL-12) and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been shown to be involved in inflammatory and autoimmune diseases. Until now, no studies have reported on serum levels of MMP-2 and GM-CSF in chronic periodontitis patients and periodontally healthy subjects. Therefore, the aim of the present study was to determine the serum levels of MMP-2, TIMP-1, IL-12 and GM-CSF in chronic periodontitis patients, compared with periodontally healthy subjects. The test group of the study comprised 40 chronic periodontitis patients, whereas the control group included 108 periodontally healthy individuals. Blood samples were collected from all participants and examined using enzyme-linked immunosorbent assay (ELISA) analysis. Clinical periodontal parameters (bleeding on probing, clinical attachment level and probing pocket depth) and the serum levels of MMP-2, TIMP-1, IL-12 and GM-CSF were statistically significantly higher in the test group than in the control group. These results may indicate that MMP-2, TIMP-1, IL-12 and GM-CSF could be involved in the initiation and progression of chronic periodontitis.

Key words: Chronic periodontitis, cytokines, matrix metalloproteinase-2 (MMP-2), tissue inhibitor of metalloproteinase-1 (TIMP-1), interleukin-12 (IL-12), granulocyte–macrophage colony-stimulating factor (GM-CSF).

INTRODUCTION

Periodontitis is a multifactorial infection characterized by a destructive inflammatory process affecting tooth-supporting tissues and resulting in periodontal pocket

formation and alveolar bone resorption, which might eventually lead to tooth loss (Armitage, 1999; Cazalis et al., 2009). The chronic form of periodontitis, termed chronic periodontitis, is the most prevalent disease type (Armitage, 1999). Although, periodontal bacteria are the main causative agents inducing the initiation of periodontitis, subsequent progression and disease severity are also determined by the host immune response (Kornman

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et al., 2000). The continuous challenge of host immune and resident cells by periodontopathogens and their virulence factors results in a complex network of pro- and anti-inflammatory cytokines acting in the inflamed periodontal tissues (Okada and Murakami, 1998). These host mediators directly or indirectly participate in periodontal tissue destruction and particularly in bone resorption (Birkedal-Hansen, 1993a).

The degradation of periodontal tissues can be mainly mediated by matrix metalloproteinases (MMPs), which comprise a family of zinc-dependent endopeptidases (including collagenases, gelatinases, stromelysins and membrane-bound proteinases) promoting the degradation of various extracellular matrix proteins (Birkedal-Hansen, 1995). However, MMPs can be inhibited by a family of naturally occurring specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), which are essential in maintaining a dynamic equilibrium in connective tissue metabolism (Brew et al., 2000; Spinale, 2002).

Matrix metalloproteinase-2 (MMP-2) (also known as gelatinase A or type IV collagenase) is a 72 kDa enzyme in humans encoded by the MMP2 gene (Devarajan et al., 1992). MMP-2 is responsible for the breakdown of type IV collagen of the extracellular matrix, which is a major structural component of a typical basement membrane (Uitto et al., 2003). In addition, MMP-2 is also able to cleave native type I collagen, which is the abundant component of gingival connective tissue matrix (Aimes and Quigley, 1995). Matrix metalloproteinase-2 has been immunolocalized in fibroblasts and macrophages, as well as in epithelial cells of gingival tissues in periodontitis-affected patients (Meikle et al., 1994). Elevated levels of matrix metalloproteinase-2 have also been detected in gingival crevicular fluid (Ingman et al., 1996; Soell et al., 2002), peri-implant sulcular fluid (Ma et al., 2003) and gingival tissues (Séguier et al., 2001) of periodontitis / peri-implantitis patients.

Tissue inhibitor of metalloproteinase-1 (TIMP-1) can precisely regulate MMP activation and function and inhibit MMP-1, 2, 3, 8, 9 and 13 (Goldberg et al., 1992) by binding to active sites on the MMP molecules.

Interleukin-12 (IL-12) is comprised of two disulphide-linked protein subunits (p35 and p40), which are encoded by two different genes, IL-12A and IL-12B, respectively, (Trinchieri, 1995). In humans, IL-12 is mainly produced by monocytes, macrophages, neutrophils (Trinchieri, 1997) and other cell types with antigen-presenting properties. Since IL-12 has been identified as a natural killer-stimulating factor enhancing cytolytic activity and interferon- γ (IFN- γ) production, a positive feedback effect between IL-12 and IFN- γ would promote the activities of macrophages in the inflamed tissue, increasing their phagocytic and bacteriocidal activity (Duffield, 2003). Although, these proinflammatory effects of IL-12 are of primary importance in the innate resistance of the organism to infectious agents, they may also lead to patho-

logical effects (Trinchieri, 2003; Lee et al., 1998). The cytokines form complex networks in periodontitis lesions and that their overlapping and redundant effects should be taken into account when considering the pathology of inflammatory periodontal disease. Expression of IL-12 p40 mRNA in the periodontal lesion may be associated with disease entity (Yamazaki et al., 1997).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a glycosylated polypeptide cytokine or growth factor with multiple biological activities. (de Queiroz et al., 2008). GM-CSF is a multilineage hematopoietic cytokine, stimulating the proliferation and differentiation of hematopoietic cells into various cell lines, such as neutrophils, monocytes/macrophages, eosinophil and megakaryocytic lineages (Shi et al., 2006; Ottmann et al., 1989). The recognition that differentiated granulocytes and macrophages maintain their responsiveness to granulocyte-macrophage colony-stimulating factor and respond with enhanced functional activity underscores that the actions of GM-CSF are not limited to replenishing the hematopoietic compartment (Gamble et al., 1985; Hamilton et al., 1980; Handman and Burgess, 1979). Additionally, GM-CSF is involved in the inflammation and immune response (Gamble et al., 1985; Hamilton et al., 1980; Handman and Burgess, 1979). Stimulation of granulocyte-macrophage colony-stimulating factor production by gingival keratinocytes might cause accumulation and activation of neutrophils in the epithelium and may be involved in the initiation and development of inflammation in periodontal tissues (Sugiyama et al., 2002).

Some previous studies have reported, increased serum levels of MMP-2 (Trombone et al., 2009), TIMP-1 (Buduneli et al., 2010) and IL-12 (Renvert et al., 2009; Kobayashi et al., 2010) in periodontitis patients but till date, few studies have reported combination study of serum MMP-2, TIMP-1, IL-12 and GM-CSF levels in periodontal health and disease.

Therefore, the aim of the present study was to investigate the serum levels of MMP-2, TIMP-1, interleukin-12 and granulocyte-macrophage colony-stimulating factor in chronic periodontitis patients, compared with periodontally healthy subjects.

MATERIALS AND METHODS

Study population

Blood sampling was performed firstly in Hong Kong Red Cross station for subjects in the control group. This group included 108 periodontally healthy subjects (47 females and 61 males; mean age: 36.5 ± 3.48 years; Table 1) and the test group comprised 40 patients (16 females and 24 males; age range: 18 to 74 years; mean age: 49.5 ± 9.78 years; Table 1) with moderate to advanced chronic periodontitis, defined according to the 1999 International Workshop for a Classification of Periodontal Diseases and Conditions (Armitage, 1999). Subjects in the control group were selected from Hong Kong Red Cross between September 2005 and

Table 1. Baseline demographic and periodontal clinical data for the control and test group and their statistical comparison.

Parameter	Control group (N=108)	Test group (N=40)	p value
Age (mean \pm SD; years)	36.5 \pm 3.48	49.5 \pm 9.78	---
Sex	47 females and 61 males	16 females and 24 males	---
Supragingival/subgingival calculus (mean \pm SD; %)	14.21 \pm 0.50	63.45 \pm 22.02	0.0008*
Full-mouth bleeding on probing (mean \pm SD; %)	12.14 \pm 4.65	78.28 \pm 18.05	0.0006*
Gingival recession (mean \pm SD; %)	5 \pm 1.25	32.14 \pm 3.7	0.001*
Probing pocket depth (mean \pm SD; mm)	2.0 \pm 0.33	5.6 \pm 2.15	0.0004*
Clinical attachment loss (mean \pm SD; mm)	0	5.9 \pm 2.45	0.001*
Tooth mobility (class)	I	II	---

SD = Standard deviation; *p < 0.001: highly statistically significant difference between the control and test group.

February 2007, whereas patients in the test group were recruited from West China Hospital of Stomatology, Sichuan University, China. Informed consent was obtained from all participants and the study protocol was approved by the Sichuan University Ethics Committee, China. All participants were determined to be free from the following: systemic or chronic disease, past and current smoking habit, swelling of the lymph nodes, temporal mandibular joint disease and soft tissue abnormalities.

Preparation of control and patient blood samples

Blood sampling was performed in Hong Kong Red Cross for subjects in the control group and in West China Hospital of Stomatology, Sichuan University, China for patients in the test group.

Ten milliliters of blood was collected from the antecubital fossa of each participant by venipuncture, using a 20-gauge needle with lithium heparin tubes (Vacuette, Austria) and immediately transferred to the Hospital laboratory. The blood sample was allowed to clot at room temperature and after 1 h, serum was separated from blood by centrifuging for 10 min at 1500 revolutions per minute (rpm) and plasma was removed for subsequent enzyme-linked immunosorbent assay (ELISA) analysis.

Baseline examination of study participants

After the blood samples were drawn, 148 subjects underwent a general and full mouth periodontal examination at Keenlink Dental Clinic, Hong Kong and Hospital of Stomatology, Sichuan University. The diagnosis of chronic periodontitis was made following the criteria defined by the American Academy of Periodontology in 1999 (Armitage, 1999). An intra-oral examination of periodontal conditions for 108 represented control cases including supragingival/subgingival calculus, gingival recession, bleeding on probing (BOP), probing depth (PD), clinical attachment loss (CAL) and tooth mobility was performed. The subjects were regarded as healthy when they were found to present no signs of periodontal disease with the absence of gingival recession, clinical attachment loss and no sites with probing depth >3 mm. If subjects presented with probing depth >5 mm, CAL>4 mm some degree of gingival recession and tooth mobility they were said to be chronic periodontitis patients (Armitage, 1999).

ELISA analysis

Enzyme-linked immunosorbent assay (ELISA) was performed using the serum plasma samples and following the manufacturer's

instructions from commercially available ELISA kits for MMP-2 (MMP-2 ELISA kit, Calbiochem, Germany), TIMP-1 (TIMP-1 ELISA kit, Calbiochem, Germany), IL-12 (IL-12 ELISA kit, Diaclone, France) and GM-CSF (GM-CSF ELISA kit, Diaclone, France). The normal ranges of biomarker were 1.56 to 50 ng/ml for MMP-2, 0.05 to 1.6 ng/ml for TIMP-1 and the limit of detection was 20 pg/ml for IL-12 and 4.4 pg/ml for GM-CSF. The procedures were as follows: 50/100 μ l of the standard group solutions and serum/plasma of each subject were pipeted into a 96-well plate provided. The plate was incubated for 2 to 3 h at 350 rpm and washed with washing buffer three times. Subsequently, the wells were dried and 200 μ l of substrate tetramethylbenzidine was added into each well for 20 min in the dark at room temperature. The plates were read at 450 nm wavelength using a microplate reader (Universal Microplate Reader, Sunrise, TECAN, Austria). The levels of MMP-2, TIMP-1, IL-12 and GM-CSF in the samples were obtained by comparison with the standard curve generated from standards supplied by the manufacturer.

Statistical analysis

The clinical parameters and continuous variables were expressed as mean value \pm standard deviation, based on the subject as the statistical unit. Statistical comparisons of the serum levels of MMP-2, TIMP-1, IL-12 and GM-CSF between the test and control group were performed using the independent-Student's t-test. Differences between the test and control group were considered to be statistically significant, when the p value was <0.05. The statistical analysis was performed using commercially available software (Statistical Package for the Social Sciences, version 16.0 for Windows, SPSS Inc., Chicago, IL, USA).

RESULTS

At the baseline of the study, the incidences of supragingival/subgingival calculus were 14.21 \pm 0.50% and 63.45 \pm 22.02% in the control and test group, respectively (Table 1). Percent full-mouth BOP was 12.14 \pm 4.65% and 78.28 \pm 18.05% for the control and test group, respectively (Table 1). There was no occurrence of gingival recession and CAL in the control group, while 32.14 \pm 3.7% patients accounted for gingival recession and the mean CAL was 5.9 \pm 2.45 mm in the periodontitis group (Table 1). The mean PD was 2.0 \pm 0.33 mm in the control group versus 5.6 \pm 2.15 mm in the test group

Table 2. Serum levels of MMP-2, TIMP-1, IL-12 and GM-CSF in the control and test group and their statistical comparison.

Parameter	Control group (N=108)	Test group (N=40)	p value
MMP-2 (mean \pm SD; pg/ml)	89.84 \pm 48.32	1463.60 \pm 518.23	0.0001*
TIMP-1 (mean \pm SD; ng/ml)	22.57 \pm 9.62	110.12 \pm 35.45	0.0001*
IL-12 (mean \pm SD; pg/ml)	68.46 \pm 13.47	166.21 \pm 24.36	0.0001*
GM-CSF (mean \pm SD; pg/ml)	91.31 \pm 10.41	244.45 \pm 90.25	0.0001*

SD = Standard deviation; *p < 0.001: highly statistically significant difference between the control and test group.

(Table 1). Tooth mobility class I was observed in the control group and this was within physiological movement, whereas tooth mobility class was II in the test group (Jill, 2008; Table 1). Highly statistically significant differences in the percentage of supragingival/subgingival calculus, percent full-mouth BOP, percentage of gingival recession, PD and CAL were revealed between the two groups at the baseline of the study ($p < 0.001$; Table 1). Table 2 shows the serum levels of MMP-2, TIMP-1, IL-12 and GM-CSF in the control and test group and their statistical comparison. Highly statistically significant differences in the serum levels of MMP-2, TIMP-1, IL-12 and GM-CSF were demonstrated between the two groups ($p < 0.001$; Table 2).

DISCUSSION

Periodontitis could be induced by various specific and non-specific micro-organisms through different mechanisms: (1) the pathogens may directly release proteolytic enzymes that degrade periodontal structures without the intervention of host cells; (2) pathogen products such as toxins, enzymes and lipopolysaccharide may trigger immune cells for the expression of degradative enzymes, such as cytokines and inflammatory mediators (Epelman et al., 2008; Fernández et al., 2005); (3) the pathogens may stimulate an immune response resulting in release of cytokines from lymphocytes and macrophages that activate one or more degradative pathways (García et al., 2001; Quiroga et al., 2004). The inflammation in periodontal tissues is maintained by a network of chemokines and pro- and anti-inflammatory mediators with opposed and/or shared biological activities (García et al., 2001; Quiroga et al., 2004).

In our research, serum levels of MMP-2, TIMP-1, IL-12 and GM-CSF were all significantly higher in chronic periodontitis patients than in periodontally healthy subjects. These findings may indicate that MMP-2, TIMP-1, IL-12 and GM-CSF might play potential roles in periodontal tissue destruction.

Since MMPs are the main enzymes responsible for extracellular collagen matrix degradation (Miller et al., 2006), normal tissues do not store MMPs and constitutive expression is minimal. MMPs are transcriptionally regulated by growth factors, cytokines and extracellular matrix

components (Shapiro and Senior, 1999). Increased expression of MMPs in diseased periodontal tissues seems to be the consensus in the literature and is thought to account for the destruction of both soft and hard periodontal tissues (Birkedal-Hansen, 1993b; Ingman et al., 1996). MMP-2 has the unique ability to breakdown type IV collagen. During the progression of periodontal destruction, the expression of MMP-2 could be induced by pro-inflammatory cytokines, such as IL-1 and IL-6, which are produced by neutrophils present in the exudative zone of the granulomas (Márton and Kiss, 2000) to degrade the extracellular matrix. The results of previous studies on the expression of MMP-2 in periodontitis are controversial. MMP-2 was expressed in the acute phase of apical periodontitis, while being absent in chronic periodontitis (Corotti et al., 2009). Nevertheless, in another research, the differences in the levels of MMP-2 in acute and chronic periodontitis were not significant, suggesting that MMP-2 was not specifically involved in the acute phase of periodontal destruction (Garlet et al., 2004). Our results are in agreement with the earlier-mentioned finding that levels of MMP-2 were elevated, suggesting a potential contribution of MMP-2 to chronic periodontitis lesions. However, further research is needed to elaborate on the specific mechanisms of action of MMP-2 in the periodontal tissues and the mechanisms by which the expression of MMP-2 is mediated by other cytokines or chemokines.

Matrix metalloproteinase-2 (MMP-2) is responsible for the breakdown of type IV collagen of the extracellular matrix, which is a major structural component of a typical basement membrane (Uitto et al., 2003). However, type IV collagen does not exist in the internal basal lamina, which is the gingival basement membrane facing the tooth surface (in the area adjacent to the junctional epithelium) (Sawada et al., 1990; Hormia et al., 1992). For this reason, the internal basal lamina, which is the main basement membrane that could be important for the progression of periodontitis, is not considered to be a "true" (a typical) basement membrane nowadays (Hormia et al., 2001). From this aspect, the role of matrix metalloproteinase-2 in the progression of periodontitis could be questioned.

The activation of MMPs could be regulated by tissue inhibitors of metalloproteinases (TIMPs); each member of TIMPs is capable of inhibiting almost any member of the

MMP family in a non-specific manner (Baker et al., 2002). Physiologically, the relative expressions of MMPs and TIMPs are in a state of dynamic equilibrium and therefore, the extracellular matrix is remodeled in a highly regulated fashion. However, in many disease processes the levels of MMPs are elevated without a concomitant increase in the levels of TIMPs, thereby resulting in an imbalance in the aforementioned dynamic equilibrium, causing tissue destruction, such as periodontal tissue destruction (Golub et al., 2001). Certain studies have demonstrated a decrease in the levels of TIMPs in diseased periodontal tissues, suggesting that an imbalance in the levels of TIMPs/MMPs results in periodontal tissue destruction (Alexander and Damoulis, 1994; Soell et al., 2002). For example, TIMP-1 can inhibit MMP-1, -2, -3, -8, -9 and -13 (Goldberg et al., 1992) and TIMP-2 can bind preferentially to MMP-2 and inhibit its activity, but can also inhibit the activity of MMP-1, -3, -7 and -9 (Umenishi et al., 1991). Imbalances in the MMPs/TIMPs system (that is, lower levels of TIMPs and/or higher levels of MMPs) are also involved in the pathogenesis of several other diseases, including rheumatoid arthritis (Katrib et al., 2003; Yoshihara et al., 2000), which share several features with periodontitis, including the chronic nature of the inflammatory reaction and tissue destruction activity.

In the present study, TIMP-1 serum levels were increased in chronic periodontitis patients, in contrast to previous studies detecting an increase in the expression of tissue inhibitors of metalloproteinases in diseased periodontal tissues (Alexander and Damoulis, 1994; Haerian et al., 1995). The up-regulation of TIMP-1 may reflect an attempt to maintain the tissue homeostasis, in view of the increased expression of MMP-2, but the increase in TIMP-1 expression may not be sufficient to compensate for the up-regulation of MMP-2; such an imbalance could result in periodontal destruction. In the present study, although serum levels of TIMP-1 increased as the serum levels of MMP-2 elevated, it appears possible that other inhibitors of MMP-2 decreased, such as TIMP-2 (Umenishi et al., 1991), in order to counterbalance the upregulation of MMP-2 expression.

As the periodontal pathogens are involved in the progression of periodontitis, they may stimulate an immune response resulting in a release of cytokines from lymphocytes and macrophages that activate one or more degradative pathways to exacerbate the pathological process (Taubman et al., 2005; Teng et al., 2000). The results of the present study revealed that, the serum levels of IL-12 were statistically significantly higher in chronic periodontitis patients than in periodontally healthy subjects. As IL-12 is regarded as a pro-inflammatory cytokine with immunoregulatory functions, it may be greatly involved in chronic periodontitis (Trinchieri, 1995). IL-12 could potently induce IFN- γ gene transcription and synergize with other inducers (for example, IL-2, antigen stimulation mitogens, etc.) of IFN- γ production (Chan et

al., 1992) primarily from natural killer cells and also T lymphocytes. IFN- γ induced by IL-12 acts not only as an activator of macrophages in the inflamed tissue, increasing their phagocytic and bacteriocidal activity, but also increases the ability of macrophages to produce IL-12 in a powerful positive feedback loop (Kubin et al., 1994). Not only as an inflammatory mediator, IL-12 can also initiate host immune response within the periodontal tissues. This activity of IL-12 is mediated through its ability to induce generation of T helper type 1 (Th1) cells, producing IFN- γ and IL-2 and favoring cell-mediated immunity and macrophage activation, while inhibiting the generation of Th2 cells, producing IL-4, IL-5 and IL-10 and favoring humoral immunity. In humans, the major effect of IL-12 is to stimulate IFN- γ production by Th1 cells and regulate the transition from an early innate immune response to an adaptive immune response. The results of the present study showed increasing serum levels of IL-12 in chronic periodontitis patients, suggesting that IL-12 could play a potential role in the progression of periodontal inflammation by inducing a Th1 response. Several studies have attempted to determine the IL-12 profile in periodontal disease. A previous study demonstrated that, the total amount of IL-12 in gingival crevicular fluid was significantly higher in chronic periodontitis than in gingivitis and healthy subjects, while significantly lower concentrations of IL-12 were revealed in chronic periodontitis patients, compared with healthy individuals (Tsai et al., 2005). The results of this study and also the findings reported in the present study indicate that, IL-12 could be related to the pathogenesis of inflammatory periodontal disease.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) was originally described as a potent stimulator of growth and differentiation of granulocyte and macrophage precursor cells *in vitro* (Young, et al., 1995; Baldwin, 1992). Subsequent studies revealed that, it also stimulates proliferation and activation of mature immune cells, as well as of antigen-presenting dendritic cells to increase their functional capacity in combating infections (Young, et al., 1995; Baldwin, 1992). Apart from a hemopoietic regulator, GM-CSF has now been recognized as a key activator of innate immunity and as such, is involved in chronic stages of inflammatory and autoimmune diseases, in which macrophages, granulocytes, neutrophils, eosinophils and dendritic cells can contribute to tissue damage and disease progression (Hamilton, 2002). When periodontal pathogens invade into the periodontal tissues, they may stimulate an immune response, releasing cytokines from lymphocytes and macrophages. Although, the immune response could eliminate the pathogens to some extent, it could lead to tissue destruction as well. As revealed in the present study, GM-CSF was significantly higher in chronic periodontitis patients than in periodontally healthy subjects. This finding may indicate the involvement of GM-CSF in the progression of chronic periodontitis. The

essential roles of granulocyte-macrophage colony-stimulating factor in chronic inflammatory and autoimmune diseases have been confirmed in several studies. In a previous research, GM-CSF induced production of type 1 pro-inflammatory cytokines (IL-12, IFN- γ , tumor necrosis factor- α) by human peripheral blood mononuclear cells, T lymphocytes and antigen-presenting cells, while type 2 cytokines (IL-10 and IL-4) are down-regulated by GM-CSF (Eksioglu et al., 2007). These findings seem to be in agreement with our results that both GM-CSF and IL-12 serum levels were increased in chronic periodontitis patients.

In conclusion, in the present research, the serum levels of MMP-2, TIMP-1, IL-12 and GM-CSF were all significantly higher in chronic periodontitis patients than in periodontally healthy subjects, suggesting an involvement of these molecules in the initiation and progression of chronic periodontitis.

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Abbreviations

TIMPs, Tissue inhibitors of metalloproteinases; **IL-12**, interleukin-12; **GM-CSF**, granulocyte-macrophage colony-stimulating factor; **ELISA**, enzyme-linked immunosorbent assay; **MMP-2**, matrix metalloproteinase-2; **IFN- γ** , interferon- γ ; **BOP**, bleeding on probing; **PD**, probing depth; **CAL**, clinical attachment loss.

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