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EVALUATION OF SALIVARY MATRIX METALLO PROTEINASE-13 AND TISSUE INHIBITOR OF METALLOPROTEINASE-1 LEVELS IN PATIENTS WITH CHRONIC PERIODONTITIS

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Abstract

Background: Periodontitis is an infectious disease caused by specific microorganisms that cause inflammation and damage to the supporting tissues of the teeth. Increased Matrix Metalloproteinase (MMP) and decreased tissue inhibitors of metalloproteinase (TIMP) levels cause degradation of connective tissue collagen and alveolar bone. MMP-13 is an enzyme involved in periodontal tissue damage. TIMP-1 is known as inhibitor of MMP. **Objective**: The objective of this study was to show the comparison of MMP-13 levels and TIMP-1 levels in the saliva of patients with chronic periodontitis and healthy patients. **Research Methods**: The study samples were selected from patients who came for treatment at Periodontic Installation Universitas Sumatera Utara (USU). The selected patients were 16 patients with a diagnosis of chronic periodontitis and 16 healthy patients. MMP-13 and TIMP-1 level in saliva were tested using ELISA technique. **Results**: The results showed that salivary MMP-13 levels in chronic periodontitis patients were higher than those of in healthy patients and salivary TIMP-1 levels in chronic periodontitis patients were lower than those of in healthy patients with statistically significant difference($p < 0.05$). There was a positive correlation between clinical parameters and salivary MMP-13 levels in patients with chronic periodontitis and healthy patients but it was not statistically significant ($p > 0.05$). There was a negative correlation between clinical parameters and TIMP-1 levels of saliva in patients with chronic periodontitis and healthy patients but it was not statistically significant ($p > 0.05$). **Conclusions**: MMP-13 levels was higher and TIMP-1 levels was lower in chronic periodontitis patients compared to those in healthy patients.

Keywords: Chronic periodontitis; MMP-13; TIMP-1; saliva

1. Introduction

Periodontitis is an infectious disease caused by specific microorganisms that cause inflammation and damage to the supporting tissues of the teeth. Inflammation in the early stages, which begins with untreated gingivitis, can invade the tissue beneath the tooth structure and will form periodontal pockets, breakdown of the periodontal ligaments leading to loss of clinical attachment and resorption of alveolar bone and ultimately leading to tooth loss.[1,2] Several studies have shown that 50% of all age groups in United States population suffer from gingival inflammation with a prevalence of periodontitis at 14%.[3] Based on 2013 Basic Health Research Data (Rikesdas), Indonesians who experienced oral health problems were 25.9%, among these dental and mouth problems were caries and periodontal disease. [4]

Periodontitis is a chronic inflammatory disease caused by dental plaque biofilms and dysregulation of immune response.[5] Periodontal pathogens such as Aggregatibacter actinomycetemcomitans and

Porphyromonas gingivalis, can cause activation and overexpression of matrix metalloproteinases.[2] In periodontal disease, the synthesis and secretion of Matrix Metalloproteinase (MMP) is dysregulated and the neutrophil levels increase. [6]

Matrix Metalloproteinase (MMP) is a family of dependent Zn2 + endopeptidase that can damage extracellular matrix and basement membrane.[7] MMP forms an important protein group that is involved not only in the degradation of the protein matrix in periodontitis, but also in normal conditions and healing processes. Periodontal damage is the result of an imbalance between matrix metalloproterinase and its inhibitors. Increased matrix metalloproteinases and decreased tissue inhibitors from matrix metalloproteinase levels cause degradation of connective tissue collagen and alveolar bone. The level of specific biomarkers in saliva can be a useful diagnostic and therapeutic tool in periodontitis. [8]

Expression of MMP-13 was first discovered in breast cancer. MMP-13 is involved in inflammatory diseases such as rheumatoid arthritis and osteoarthritis associated with bone resorption and destruction. MMP-13 is the third and final collagenase found. MMP-13 is the second highest enzyme after MMP-8 involved in periodontal tissue damage. Several studies have shown that high levels of MMP-13 are found in gingival crevicular fluid and saliva of patients with periodontitis.[7,9]

Tissue Inhibitor of Metalloproteinase (TIMP) functions to regulate MMP activity. Although the main function of TIMP is to inhibit MMP, TIMP can also be involved in transportation and stabilization of MMP. The imbalance between MMP and TIMP is considered as a trigger of degradation of extracellular matrix, basement membrane and alveolar bone which will eventually initiate periodontal disease.[9] TIMP-1 is known as an inhibitor of matrix metalloproteinase. TIMP-1 not only inhibits collagenase, but also inhibits gelatinase and proteoglycan. [11]

Biomarkers found as diagnostics in saliva potential can be identified by comparing composition of the protein in the disease to that of in healthy individuals; it will describe specific pathologic condition. [12] In some studies, the ability to apply salivary diagnostics can be used as a specific marker to detect periodontitis. Several enzymes deriving from host and bacteria, cytokines, and biomarkers of alveolar bone changes have been analyzed from saliva in periodontitis patients and compared with those of in healthy patients. [10]

Some clinicians state that the procedure to examine changes in clinical parameters in periodontitis patients is a time-consuming one requiring an examination that can inform the clinician about process of developing periodontal disease. Saliva has been used for years to identify several biomarkers. Salivary examination procedures have several advantages, in which they are non-invasive procedures, reasonably priced and able to provide an idea of overall condition of oral cavity. [6]

Saliva plays an important role in determination and development of periodontal disease. Saliva can be the key and is important fluid for diagnosis of periodontal disease. Periodontal inflammatory mediators and tissue damaging molecules were detected in gingival tissue, gingival crevicular fluid, and saliva from patients suffering from periodontitis. In the diagnosis of periodontal disease, the benefit of saliva is that it reflects the activity of all periodontal areas and can therefore provide an indication of the overall oral disease status.

Experimental study suggested that the most important pathway involving Matrix Metalloproteinase (MMP) was found in gingival crevicular fluid, saliva and biopsy specimens in inflamed periodontal tissue. [6,8]

Previous research by Hernandez et al. regarding MMP-13 and TIMP-1 levels in the gingival crevicular fluid of patients with chronic periodontitis, showed that there were an increase in MMP-13 expression and a decrease in TIMP-1 levels.[2] Other studies also demonstrated that MMP-13 was associated with soft and hard tissue damage during the development of chronic periodontitis. [13] The objective of this study was to evaluate salivary MMP-13 levels and TIMP-1 levels in chronic periodontitis patients.

2. Materials and Methods

The study samples were selected from patients who visited for treatment at the Periodontics Installation of the Dental and Oral Hospital of Universitas Sumatera Utara. The selected patients were divided into 2 groups: 16 patients with a diagnosis of chronic periodontitis and 16 healthy patients. Inclusion criteria were patients who had at least 15 teeth, aged 25 - 55 years, had not performed periodontal treatment in the last 3 months, were willing to undergo an examination and were willing to sign an informed consent. Subjects of chronic periodontitis were patients who had lost clinical attachment ≥ 3 mm while healthy patient subjects were patients who did not have clinical attachment loss. The exclusion criteria were patients who had a history of systemic disease, were pregnant and lactated, were smokers, were alcoholics, took vitamins, antibiotics and anti-inflammatory drugs in the past month, and used mouthwash regularly.

2.1 Saliva Collection Process

Saliva collection by draining method was performed by instructing subjects to collect the saliva on floor of their mouths as much as ± 2 ml and to drain it on saliva pots. Before samples were taken, the patients were asked to rinse their mouths with water. Saliva samples were then put into a cooling box and then taken to Integrated Laboratory in Faculty of Medicine USU to be stored in a freezer (-80°C) before an examination was carried out.

2.2 Assesstment of Matrix Metalloproteinase-13 and Tissue Inhibitor of Metalloproteinase-1 Level

Saliva was removed from the refrigerator and left at room temperature then transferred to an Eppendorf tube of 10 µL using a micropipette. After that, centrifugation was carried out at 3000 rpm at 4^{o}C for 20 minutes to separate debris on the saliva contained in tube wall.

The reagent preparation was done by diluting 30 mL of wash buffer concentrate into 750 mL wash buffer using distilled water. Preparation of a standard solution was preceded by standard liquid centrifugation at 10000 mg for 1 minute then 1 ml of reference standards, and the sample diluents were added. Next, dilution of reference standards to various concentrations was determined. Furthermore, the dilution of Biotinylated Detection Ab (1: 100) and the Concentrated HRP Conjugate dilution (1: 100) was made.

The inspection procedure began by adding 100 µL standard solution or sample to each well and incubating at 37^{o}C for 90 minutes. The liquid was removed from each well without washing, then 100 µL Biotinylated Detection Ab working solution was added to each well and covered with a plate sealer. The liquid was mixed slowly then incubated at 37^{o}C for 1 hour. The liquid was aspired and emptied from each well then 350 µL wash buffer was added to each well. After 1-2 minutes the liquid was aspirated again and the back of the well was patted to dry. This step was repeated up to 3 times.

100 µL HRP Conjugate working solution was added to each well. A plate sealer was covered again and incubated for 30 minutes at 37^{o}C. Each well was re-aspirated and the washing process was repeated for 5 times. After that, 90 µL Substrate Reagent was added to each well, covered again with a plate sealer and incubated for 15 minutes at 37^{o}C. The plate was protected from light with aluminum foil. To each well was added 50 µL Stop Solution. Then the optical density (OD) value of each well was determined using a microplate reader with a wavelength of 450 nm.

3. Result

This study was conducted to observe differences in salivary MMP-13 levels and TIMP-1 levels among chronic periodontitis patients and healthy patients, also to observe the correlation between MMP-13 and TIMP-1 with clinical parameters of periodontal disease. Subject groups were selected based on inclusion and exclusion criteria. The subjects of chronic periodontitis and healthy patients were patients who visited for treatment at the Periodontics Installation in Dental and Oral Hospital of Universitas Sumatera Utara i.e. 16 patients with chronic periodontitis patients and 16 healthy patients.

3.1 Characteristics of Study Subjects

Characteristics of the study subjects are presented in table 1 including age, sex, frequency of tooth brushing, and scaling experience.

Table 1. Characteristics of research subjects

Characteristics	Chronic Periodontitis n (%)	Healthy patient n (%)
Age		
Mean ± SD	(49.06 ± 7.35)	(32.94 ± 5.28)
Sex [n (%)]		
Male	7 (43.8)	6 (37.5)
Female	9 (56.3)	10 (62.5)
Frequency of tooth brushing [n (%)]		
0 – 1	5 (31.3)	4 (25.0)
≥ 2	11 (68.8)	12 (75.0)
Scaling experience [n (%)]		
No	12 (75.0)	3 (18.8)
Yes	4 (25.0)	13 (81.3)

Table 1 shows the average age of the chronic periodontitis group i.e. 49.06 ± 7.35 years old, while the mean age group of healthy patients was 32.94 ± 5.28 years old. Sex variations in the study subjects were almost evenly distributed in both chronic periodontitis group and healthy patient group. All subjects brushed

their teeth regularly every day and almost all subjects brushed their teeth with a frequency of more than 2 times a day. Most of the subjects of chronic periodontitis had never been treated with scaling while the majority of healthy patients ever had scaling

3.2 Differences in Clinical Parameters Between Groups

The results of differences in clinical parameters between both groups are illustrated in Table 2 to Table 4.

Table 2. Independent T-Test results on pocket depth, attachment loss, and papillary bleeding index and Mann-Whitney U test results on the gingival index between chronic periodontitis subjects and healthy patients.

Clinical Parameters	Chronic Periodontitis (Mean ± SD)	Healthy patient (Mean ± SD)	*P*
Pocket depth	4.20 ± 0.92	1.48 ± 0.19	0.00*
Attachment loss	5.31 ± 0.96	0.00 ± 0.00	0.00*
Gingival index	2,06 ± 0,64	0,47 ± 0,23	0.00*
Papillary bleeding index	75.31 ± 10.39	29.44 ± 4.14	0.00*

Independent T-Test (pocket depth, attachment loss, and papillary bleeding index)

* significant p <0.05

Mann Whitney U Test (gingival index)

* significant p <0.05

Table 2 illustrates that pocket depth, attachment loss, gingival index and papillary bleeding index in the chronic periodontitis group were significantly higher (p <0.05) than those in healthy patient group.

Table 3. Salivary MMP-13 levels in chronic periodontitis patients and healthy patients

Variable	Chronic Periodontitis (Mean ± SD)	Healthy patient (Mean ± SD)	*P*
Salivary MMP-13 level (ng/ml)	0,85 ± 0,30	0,48 ± 0,35	0.001*

Mann Whitney U Test

* significant p <0.05

Mann-Whitney U test results demonstrated that salivary MMP-13 levels in the chronic periodontitis group were significantly higher than those in the healthy patients group (p <0.05).

Table 4. Salivary TIMP-1 levels in chronic periodontitis patients and healthy patients

Variable	Chronic Periodontitis (Mean ± SD)	Healthy patient (Mean ± SD)	*P*
Salivary TIMP-1 level (ng/ml)	265.75 ± 129.95	356.06 ± 72.39	0.021*

Independent T-Test

* significant p <0.05

Independent T-Test results showed that salivary TIMP-1 levels in the chronic periodontitis group were significantly lower than those in the healthy patients group ($p < 0.05$).

3.3 Correlation between clinical parameters and MMP-13 levels

The correlation test between clinical parameters with salivary MMP-13 levels and TIMP-1 levels in chronic periodontitis patients and healthy patients is shown in Table 5 and 6.

Table 5. Correlation test between clinical parameters and salivary MMP-13 levels in chronic periodontitis patients and healthy patients.

Clinical Parameters	Salivary MMP-13 levels			
	Chronic Periodontitis		Healthy patient	
	R	*p*	*r*	*p*
Pocket depth	0.150	0.578	0.007	0.981
Attachment loss	0.488	0.055	-	-
Gingival index	0.079	0.770	0.468	0.067
Papillary bleeding index	0.013	0.963	0.444	0.085

Spearman Test, 2-tailed chronic periodontitis patients: (MMP-13 → gingival index); healthy patients: (MMP-13 → pocket depth, attachment loss, gingival index and papillary bleeding index)

* significant $p < 0.05$

Pearson test, 2-tailed (MMP-13 → pocket depth, attachment loss, and papillary bleeding index)

* significant $p < 0.05$

Table 5 shows that there was a very weak positive correlation between the salivary MMP-13 in chronic periodontitis patients and pocket depth, gingival index and papillary bleeding index; and a weak correlation between salivary MMP-13 and attachment loss yet it was not statistically significant. ($p > 0.05$).

Table 5 shows that in the healthy patient group was a moderate positive correlation between salivary MMP-13 and gingival index and papillary bleeding index yet it was not statistically significant. ($p > 0.05$).

Table 6. Correlation test between clinical parameters and salivary TIMP-1 levels in chronic periodontitis patients and healthy patients.

Clinical Parameters	Salivary TIMP-1 levels			
	Chronic Periodontitis		Healthy patient	
	R	*p*	*r*	*p*
Pocket depth	-0.52	0.03	-0.02	0.93
Attachment loss	-0.27	0.31	-	-
Gingival index	-0.20	0.44	-0.02	0.93
Papillary bleeding index	-0.14	0.58	-0.18	0.48

Pearson test, 2-tailed chronic periodontitis patients: (TIMP-1 → pocket depth, attachment loss, and papillary bleeding index); healthy patients: (TIMP-1 → pocket depth, attachment loss, gingival index and papillary bleeding index)

* significant $p < 0.05$

Spearman Test, 2-tailed chronic periodontitis patients: (TIMP-1 → gingival index);

* significant p <0.05

Table 6 shows that there was a moderate negative correlation in chronic periodontitis patients between salivary TIMP-1 and pocket depth, a weak negative correlation between salivary TIMP-1 with attachment loss and gingival index, a very weak negative correlation between salivary TIMP-1 and papillary bleeding index yet they were not statistically significant except for the correlation between salivary TIMP-1 and pocket depth. (p> 0.05)

Table 6 shows that in the healthy patient group was a very weak negative correlation between salivary TIMP-1 and pocket depth, gingival index, as well as papillary bleeding index, but it was not statistically significant. (p> 0.05)

4. Discussion

This study demonstrated that the salivary MMP-13 levels in the chronic periodontitis group were higher than those in the healthy patient group, and the difference was statistically significant. These results were in accordance with the study of Gursoy, et al who examined the degradation of type I collagen and its correlation with salivary metalloproteinase of chronic periodontitis patients in 230 subjects divided into 3 groups namely generalized chronic periodontitis group (84), localized chronic periodontitis group (65), group localized chronic periodontitis (65), and a control group of healthy patients (81). Gursoy found saliva levels of MMP-8, MMP-9, and MMP-13 to be higher in the localized chronic periodontitis group compared to those in the control group in healthy patients and it is statistically significant. [10]

Other studies conducted by Rios, et al studied the role of proteolytic MMP-13 during the development of chronic periodontitis in 32 subjects who were divided into 2 groups namely the chronic periodontitis group (21) and the control group of healthy patients (11). Rios discovered that there was an increase in MMP-13 in the gingival crevicular fluid among patients with chronic periodontitis during development of the disease compared to healthy patients. Other studies conducted by Hernandez, et al on MMP-13 expression in periodontal disease activity in 21 subjects who were periodontitis patients divided into active and inactive groups. The study also showed that the levels of MMP-13 gingival crevicular fluid on active side of patients with chronic periodontitis were higher than those on the inactive side. An important role was significantly played by MMP-13 in the initiation and development of bone resorption. MMP-13 can degrade type I, II, and III collagen. MMP-13 also produced a specific microenvironment against degradation of organic components of bone by activation of MMP-9. [2,13]

This study demonstrated that salivary MMP-13 levels were positively correlated with levels of clinical parameters (pocket depth, attachment level, gingival index and papillary bleeding index) in patients with chronic periodontitis and in healthy patients. This is in accordance with research results of Gonzalves, et al who found that MMP-13 levels were correlated with the average percentage of pocket depth. This study indicated that MMP-1, MMP-8, MMP-9, MMP-12 and MMP-13 levels were associated with periodontal disease

process and discovered decreases in MMP-8, MMP-9, and MMP-13 levels after treatment of periodontal disease. [14]

Other studies by Kiili, et al on molecular shape and levels of gingival crevicular fluid, as well as MMP-8 and MMP-13 gingival tissue in 12 patients with chronic periodontitis showed the percentage of MMP-8 and MMP-13 levels that was correlated significantly with the gingival index and papillary bleeding index. [15]

This study found that the salivary TIMP-1 levels in the chronic periodontitis group were lower than those in the healthy patient group and the difference was statistically significant. These results were consistent with the study of Fenol, et al who examined the comparison of salivary TIMP-1 levels in patients with periodontal disease involvement and healthy patients as well as responses to non-surgical periodontal therapy in 52 subjects divided into 2 groups namely chronic periodontitis group (27) , and a healthy patient control group (25). Phenol found low salivary TIMP-1 levels in the chronic periodontitis group with a value of 89.7 ± 43.0 compared to those in healthy patients with a value of 129.7 ± 53.4 and this difference was statistically significant.[8]

Other studies conducted by Popat, et al showed MMP-1 and TIMP-1 levels of gingival crevicular fluid in healthy and diseased patients in 50 subjects who were divided into 2 groups: the generalized chronic periodontitis group (30) and the healthy patient control group (20). Popat found that TIMP-1 levels in gingival crevicular fluid in chronic periodontitis patients were lower than those in healthy patients and TIMP-1 levels in periodontitis patients increased after scaling and root planing treatment and this difference was statistically significant. Pozo, et al reported that TIMP-1 reduced levels were associated with high collagenase and gelatinase activity in periodontitis patients compared to those in control group. A decrease in TIMP-1 levels in periodontitis patients could be resulted from TIMP-1 degradation by neutrophils or TIMP-1 inactivation by neutrophils followed by oxidant release. [16,17]

This study showed that salivary TIMP-1 levels were negatively correlated with levels of clinical parameters (pocket depth, attachment level, gingival index and papillary bleeding index) in patients with chronic periodontitis as well as in healthy patients. This is in accordance with a research by Popat, et al. which found that TIMP-1 levels of gingival crevicular fluid were negatively correlated with gingival index, plaque index, pocket depth and clinical attachment loss, whereas its correlation was statistically significant. [16]

Researches have continued to develop tools to detect periodontitis. At present, the diagnosis of periodontal disease depends mainly on clinical and radiographic parameters. This is useful to detect evidence of past disease, or to verify periodontal health, but it only provides limited information about patients and areas at risk for future periodontal damage. Saliva and blood serum contain the same protein and RNA, which is why saliva is considered a "mirror for body". Diagnosis of the disease using blood serum has been shown to be effective. This procedure is quite invasive, expensive and takes a long time to get diagnostic results. In an examination of periodontal disease, many researchers focus primarily on gingival fluid biomarkers that provide information about disease status locally. However, this shows an approach that is difficult to use as a clinical application. Salivary biomarkers appear to be easier to collect and include non-invasive procedures for the

detection of periodontal disease. The development of salivary protein biomarkers for periodontitis requires a multidisciplinary approach including researchers in biology, biotechnology, as well as technicians and clinicians departments. The development of periodontitis clinical management using salivary biomarkers will provide a paradigm for the application of diagnostic procedures using saliva in the field of clinical dentistry.[18,19,20]

5. Conclusion

Salivary MMP-13 levels in patients with chronic periodontitis were higher than those in healthy patients whereas salivary TIMP-1 levels in patients with chronic periodontitis were lower than those in healthy patients.

There was a very weak positive correlation between salivary MMP-13 in patients with chronic periodontitis and pocket depth, gingival index as well as papillary bleeding index and a weak correlation between salivary MMP-13 and attachment loss. There was a moderate positive correlation between salivary MMP-13 in healthy patients and gingival index as well as papillary bleeding index. There was a moderate negative correlation between salivary TIMP-1 in patients with chronic periodontitis and pocket depth, a weak negative correlation between salivary TIMP-1 and attachment loss as well as gingival index, a very weak negative correlation between salivary TIMP-1 and papillary bleeding index. There was a very weak negative correlation between salivary TIMP-1 in healthy patients and pocket depth, gingival index, as well as papillary bleeding index.

Further research is needed regarding MMP-13 and TIMP-1 levels in serum and gingival samples of chronic periodontitis patients. It is necessary to examine clinical parameters and levels of salivary MMP-13 and TIMP-1 in patients with chronic periodontitis after periodontal treatment.

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