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Investigating salivary matrix metalloproteinase-2 and matrix metalloproteinase-9 activity in fixed orthodontic-induced gingival enlargement

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ABSTRACT

Background: Gingival enlargement (GE) is a common clinical observation among orthodontic patients, yet its underlying causes remain unclear. This study aims to investigate the potential involvement of salivary matrix metalloproteinase (MMP)-2 and MMP-9 activity in orthodontic-induced GE.

Materials and Methods: In this case–control study, we enrolled 50 subjects, including 25 individuals with GE and 25 without. The participants, aged 10–35 years, were in the 4th or 5th month of their orthodontic treatment. Comprehensive clinical assessments, encompassing plaque index, gingival index, and GE score were performed, and saliva samples were subjected to gelatin zymography to assess enzyme activity. Statistical analysis, including the Chi-square test for age distribution, independent samples *t*-test for age comparison between study groups, Mann–Whitney *U* test for MMP activity comparison, and Wilcoxon signed–rank test for comparison of data from the 4th to 5th months of treatment, was performed using SPSS version 23.0, with a significance level set at 0.05.

Results: MMP-2 activity was undetectable in the zymograms. In the 4th month of treatment, MMP-9 activity was more prominent in the case group, though this disparity did not reach statistical significance in the 5th month. Furthermore, MMP-9 activity did not exhibit a correlation with the GE score.

Conclusion: The activity of MMP-9 in the saliva of orthodontic patients with GE increases during the 4th month of treatment, but no correlation exists with the degree of GE.

Key Words: Fixed orthodontic appliances, gingival overgrowth, matrix metalloproteinase-2, matrix metalloproteinase-9

INTRODUCTION

Gingival enlargement (GE) stands as a common complication in patients undergoing fixed orthodontic appliance therapy.[1] Although the etiology of GE has been explored in the literature, the precise pathophysiological mechanisms underlying GE induced by orthodontic treatment remain largely uncharted. Some studies suggest that both chemical and mechanical irritation, arising from dental

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cements and orthodontic equipment, may contribute to GE.[2] It has also been postulated that GE can create pseudo-pockets, providing an environment conducive to the colonization of anaerobic microbiota.[3] Moreover, the host's response to microbial challenges mediated by inflammatory cytokines such as interleukin-1β (IL-1β) and transforming growth factor-β1 can lead to increased production of amorphous ground substance, culminating in GE.[1,4] Furthermore, there is evidence to suggest that even the gradual release of nickel near orthodontic appliances at low levels can initiate epithelial proliferation, ultimately resulting in gingival overgrowth.[5] Orthodontic patients are often assumed to exhibit compromised periodontal health.[6] However, in contrast, some studies indicate that periodontal conditions do not necessarily worsen during orthodontic treatment, even in the presence of plaque-retentive devices.[7] There is a debate as to whether orthodontic therapy is detrimental or beneficial to the long-term status of periodontal structures.[8]

Orthodontic appliances create conditions favorable for dental plaque accumulation and pose challenges for maintaining adequate oral hygiene, particularly in interdental areas that require adjunct cleansing aids. Consequently, ensuring an optimal level of plaque control may require additional time, skills, and effort, and it is not always achieved successfully. In addition, orthodontic patients experience alterations in the prevalence of periodontal pathogens, specific to both local and site-specific contexts.[9]

Notably, GE can occur in orthodontic patients even when oral hygiene is acceptable and there are no clinical signs of inflammation.[10] This phenomenon can be attributed to the gingiva's response to the mechanical forces exerted by orthodontic appliances, resulting in the remodeling of the periodontium and tooth movement. Consequently, the inflammation observed in hypertrophied gingiva may be considered an additional event, especially when access to the tooth surface is already limited in these situations.

During orthodontic treatment, the remodeling process involves several cytokines and enzymes, with matrix metalloproteinases (MMPs) playing a prominent role. Among these MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are members of a zinc- and calcium-dependent endopeptidase family, also known as human gingival gelatinases or Type IV collagenases. Fibroblasts, endothelial cells, and osteoblasts express MMP-2, while keratinocytes, polymorphonuclear leukocytes, and macrophages synthesize MMP-9.[11,12] Previous research has delved into their roles in collagen turnover, inflammation initiation, wound healing, the development of periodontal disease, and periodontal remodeling during orthodontic therapy.[12,13] MMPs play a crucial role in the physiological remodeling of the periodontal ligament and the tissue's response to mechanical stressors induced by orthodontic treatment.[14] Dysregulation of MMP activity has been implicated in pathological processes, particularly chronic inflammation in periodontal diseases.[15,16] MMPs are typically expressed in low quantities in their latent form and are subsequently activated through proteolytic or oxidative/nitrosative mechanisms. Unlike enzyme-linked immunosorbent assay (ELISA), which can quantitatively measure protein levels, they cannot assess enzyme activity.[17]

Although Şurlin *et al*. conducted studies exploring the potential association between MMP concentration and GE, none of these studies considered the enzymes' degree of activity and functional capacity as potential influencing factors in GE.[10,15] To our knowledge, no studies have assessed the activity of MMPs in orthodontic treatment-induced GE. Therefore, this study aims to investigate the potential relationship between the activity of MMP-2 and MMP-9 in orthodontic patients with GE.

MATERIALS AND METHODS

Study participants

In this cross-sectional case–control study (IR.kums.REC.1397494), 50 individuals undergoing fixed orthodontic treatment provided informed consent and participated. The participants were divided into case and control groups, representing those with and without GE, respectively.

Eligibility criteria

Participants were eligible if they were aged between 10 and 35 years, had a minimum of 16 teeth, and exhibited GE at least at one site. In addition, only individuals who had completed 16–20 weeks of orthodontic treatment were included. Exclusion criteria encompassed systemic diseases or conditions that might affect gingival status, such as diabetes, immunodeficiency, vitamin deficiency, tobacco smoking, pregnancy, and breastfeeding. Subjects

displaying any signs of periodontitis, bone loss, or individuals using medications such as calcium channel blockers (e.g., nifedipine, verapamil, and diltiazem), cyclosporine, anticonvulsants (e.g., phenytoin), antibiotics, or corticosteroids within the last 3 months were also excluded.

Clinical examinations

All clinical examinations were conducted by a previously calibrated examiner and documented with the assistance of a dental assistant. Parameters recorded included the plaque index,[18] gingival index,[19] demographic information, probing depth, and attachment level. The rating of GE followed the index initially established by Bökenkamp *et al*.[20]

Saliva collection

Nonstimulated salivary samples were collected from patients in the 16th–20th weeks of orthodontic treatment. The samples were stored in tubes containing a nonspecific protease inhibitor (N-ethyl-maleimide) and phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO, USA) and were preserved at −20°C until testing.

Gelatin zymography

The activity of MMP-2 and MMP-9 enzymes was assessed using gelatin zymography as follows: an 8% sodium dodecyl sulfate-polyacrylamide gel containing 1 mg/mL of gelatin was prepared. Each acrylamide well received 30 μg of saliva samples, followed by vertical electrophoresis at 50 V for 30 min and then at 150 V until the dye in the buffer gels was depleted. After electrophoresis, the polyacrylamide gel was rinsed with renaturation buffer (a solution containing 2.5% Triton X-100 and 50 mM Tris-HCl, pH = 7.5) at room temperature. The buffer was refreshed every 20 min. Subsequently, the gels were washed with incubation buffer (comprising 0.15 M NaCl, 10 mM CaCl2, 0.02% NaN3, and 50 mM Tris-HCl) and incubated at 37°C for 18 h. Staining of the gels was accomplished using 0.05% Coomassie Brilliant Blue G-250 (Bio-Rad, Richmond, CA) for 30 min, followed by destaining with a solution of 7% methanol and 5% acetic acid for 1 h at room temperature. Densitometry of gelatinolytic activity, revealed clear bands against a dark background. The bands corresponding to MMP-2 and MMP-9, with molecular weights of 72 kDa and 92 kDa, respectively, were quantified using ImageJ software and compared to standard MMP values. Each assay was conducted in triplicate and repeated at least twice.

Statistical analysis

Data distribution normality was assessed using the Kolmogorov–Smirnov test. The age distribution of research participants was evaluated with the Chi-square test, while the independent samples *t*-test was utilized to compare the ages between the two study groups. The comparison of MMP activity between the two groups was performed with the Mann–Whitney *U* test, and data from the 4th to 5th months of treatment were compared using the Wilcoxon signed-rank test. All statistical analyses were carried out using SPSS version 23.0 (Inc., Chicago, IL, USA), with a significance level defined at 0.05.

RESULTS

A total of 50 subjects participated in our study, divided into two groups: those with GE (case group) and those without GE (control group). The sociodemographic characteristics of the study sample are summarized in Table 1, showing no significant differences between the case and control groups regarding gender and age.

Table 2 presents the results of the clinical examinations, where T1 represents the 4th month after the initiation of orthodontic treatment and T2 represents the 5th month. This notation is consistent throughout all tables and figures.

Table 1: Study sample demographic details (units: Count and percentage for gender, years±standard deviation for age)

Participant Characteristics	Case	Control	Total	*P*
Gender				
Male	10 (40)	8 (32)	18 (36)	0.556
Female	15 (60)	17 (68)	32 (64)	
Age (years)	17.28±5.77	19.44±4.6	18.36±5.8	0.15

Table 2: Mean plaque index and gingival index scores in two study groups (units: Score±standard deviation)

Index	Case	Control	*P*
PI			
T_1	1.4±0.15	1.3±0.1	0.092
T_2	1.5±0.13	1.3±0.14	0.064
GI			
T_1	0.65±0.24	0.34±0.22	0.085
T_2	0.71±0.45	0.41±0.27	0.063

PI: Plaque index; GI: Gingival index

In Figure 1, gelatin zymograms depicting MMP2 and MMP9 gelatinolytic activity in subjects with and without GE at T1 and T2 are presented. As shown in Figure 2, the activity of MMP-9 was more pronounced in the case group compared to the control group. However, this difference reached statistical significance only at T1 (the 4th month of orthodontic treatment), with $P = 0.004$. In T2 (the 5th month), the difference did not reach a statistically significant level, with $P = 0.132$.

Table 3 presents the distribution of the case group according to gingival enlargement score categories. Table 4 illustrates that MMP-9 activity increased in both the case and control groups from the 4th to 5th months of treatment. However, this change was not statistically significant in either of the groups, with $P > 0.05$.

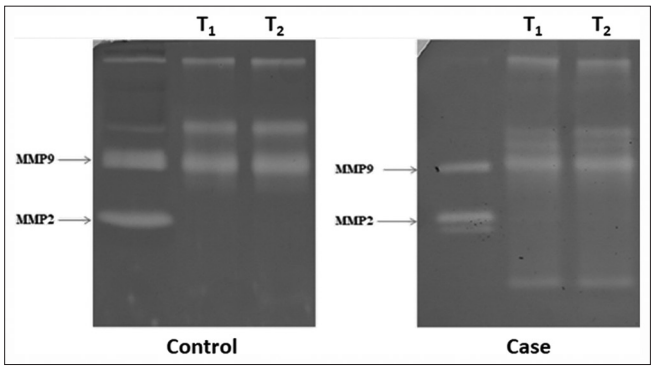

Figure 1: Gelatin zymograms show matrix metalloproteinase-2 (MMP-2) and MMP-9 gelatinolytic activity in subjects with and without gingival enlargement (case and control groups, respectively) at T_1 and T_2. The first lane in each picture is a serum control sample that confirms the reliability of test setup in detecting both gelatinases. MMP: Matrix metalloproteinase.

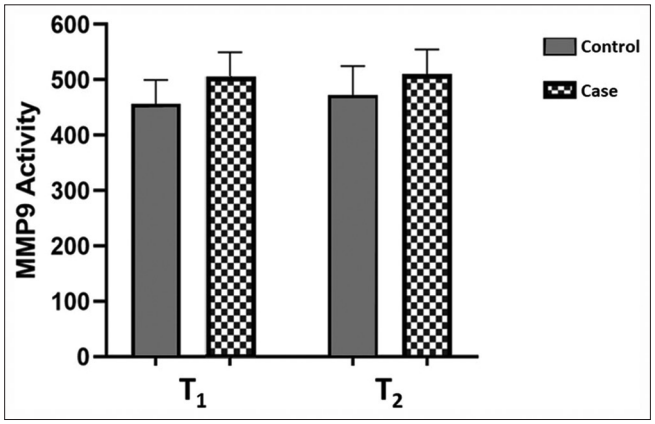

Figure 2: Matrix metalloproteinase-9 activity shown by an arbitrary unit (average amounts and standard deviation of three of tests) for case and control groups in T_1 and T_2.

Furthermore, no significant correlations were found between MMP-9 activity and the GE score at T1 and T2, with $P = 0.328$ and $P = 0.224$, respectively.

DISCUSSION

The conventional understanding of GE in orthodontic patients attributes it to inflammation resulting from poor dental hygiene and plaque accumulation. However, there is a growing body of research suggesting that mechanical stress may also play a role in gingival erosion and periodontal remodeling during orthodontic treatment.[10] MMPs, which are enzymes involved in tissue remodeling during tooth movement,[14,21] are potential candidates for influencing GE in individuals undergoing fixed orthodontic therapy.[10,15] The primary objective of our study was to investigate whether the activity of MMP-2 or MMP-9 undergoes changes in orthodontic patients with GE.

Previous studies have compared the levels of different MMPs in various periodontal health conditions using techniques such as ELISA, zymography, and Western immunoblotting.[22,23] Maeso *et al.* proposed that imbalanced levels of MMPs and tissue inhibitors of metalloproteinases (TIMPs) in gingival crevicular fluid (GCF) might be responsible for initiating tissue breakdown in periodontitis. They employed ELISA to measure concentrations of MMP-2, MMP-9, and TIMP1, finding a significant decrease in TIMP1, a modest increase in MMP-9, and a nonsignificant decrease in MMP-2 in periodontal

Table 3: Case group distribution according to gingival enlargement score category (units: Count and percentage)

GE	T_1	T_2
Grade 0	0	0
Grade 1	11 (44)	7 (28)
Grade 2	14 (56)	15 (60)
Grade 3	0	3 (12)

GE: Gingival enlargement

Table 4: Matrix metalloproteinase-9 activity of case and control groups in T_1 and T_2 (average amounts and standard deviation of three tests, measured in arbitrary units)

MMP-9 activity	T_1	T_2	*P*
Case	505/57±44.85	510/95±44/21	0.083
Control	456/91±43/21	472/55±52/26	0.76

MMP-9: Matrix metalloproteinase-9

disorders.[22] Similarly, Soell *et al*. argued that MMP-induced tissue destruction in periodontal disease is related to the imbalance between increased levels of enzymes and their inhibitors rather than the activation of proenzymes. They reported a substantial increase in MMP-2 levels when comparing GCF samples from periodontal patients to those from healthy individuals.[24] The roles of MMP-2 and MMP-9 in periodontal tissue degradation have been emphasized, and their activity levels have been shown to significantly decrease after effective periodontal treatment.[12] Séguier *et al*. indicated active forms of MMP-9 as a good indicator of tissue breakdown and clinical severity of periodontal disease. ProMMP-9 and active MMP-2, however, did not substantially rise. Their findings showed a slight but marginal reduction in pro MMP-2.[23] Some research shows that latent MMP-2 and MMP-9 are found in both chronic periodontitis and healthy periodontium, while active MMP-2 is exclusively present in cases of periodontitis.[25]

However, there are limited studies investigating the possible role of MMPs and their activity in GE. Şurlin *et al*. employed a double immunofluorescence technique on enlarged gingival tissues and found a higher expression of MMP-9 and type IV collagen in individuals with GE.[15] Despite the high expression of MMP-9 in the expanded gingival tissues, they concluded that the increased quantity of MMP-9 is insufficient to damage the basal membrane. Their hypothesis was that mechanical stress, rather than the inflammatory process, is the primary reason for the elevated MMP-9 levels in the GCF of orthodontic patients and the onset of GE.[15,26] However, the enzyme used in their immunohistochemical examination targeted a sequence of amino acids present in both active and latent forms of MMP-9 and could not differentiate between the proenzyme and active form of MMP-9.[15] The same authors observed elevated levels of MMP8 in GCF and hypothesized that MMP8 levels in GCF could serve as an indicator of GE onset. They found that MMP8 peaks 4–8 h after the initiation of orthodontic treatment, decreases in patients with normal gingiva, and continues to rise in certain individuals until their gingiva becomes enlarged. Immunohistological examination of excised tissue from gingivectomy procedures revealed higher expression of MMP8 in the overgrown gingiva of orthodontic patients. Thus, they postulated that the amount of MMP8 in GCF could be an indicator of GE onset.[10]

In our study, we opted to monitor the activity of gelatinases as an indicator of GE rather than their quantity, as the total amount of MMPs often includes latent or inactive enzyme forms. We employed zymography, which involves the electrophoretic separation of proteins using a polyacrylamide gel containing a proteolytic substrate. Following denaturing (but nonreducing) electrophoresis, the proteins were renatured and subjected to an appropriate solution for proteolytic activity. Clear zones of lysis on the stained gel indicated active proteinases, allowing us to distinguish between active and latent forms of MMPs based on their molecular weight and their ability to lyse the gelatin substrate, commonly used for MMP-2 and MMP-9.[27] Substrate zymography is a practical method for assessing the activity of MMP isoenzymes.[17]

Despite the structural similarities of MMP-2 and MMP-9 and their substrate selectivity, they are regulated by distinct mechanisms. For instance, the basal level of MMP-9 is typically low, and its secretion from inflammatory cells is induced by cytokines such as tumor necrosis factor-alpha (TNF-α), while MMP-2 is produced by various cell types and its secretion does not usually require cytokine induction. Furthermore, MMP-9 plays a pro-inflammatory role through the cleavage of IL-8, significantly amplifying neutrophil chemoattraction[28] and converting proTNF-α and proIL-1β into their active pro-inflammatory forms.[29,30] In contrast, MMP-2 acts to inhibit inflammation[31] and has been shown to degrade inflammatory mediators such as monocyte chemoattractant protein-3.[32,33]

Our study revealed a noticeable increase in salivary MMP-9 activity in subjects with GE compared to those without GE at T1. However, the intergroup difference diminished to a statistically insignificant level after 1 month (T2). In addition, our results did not demonstrate a correlation between MMP-9 enzyme activity and the GE score. Our findings align with those of Kim *et al*., who analyzed the expression levels of MMP-2 and MMP-9 in gingival tissues of periodontitis patients using Western blot analysis and assessed their enzymatic activity via gelatin zymography. However, after 1 month, the intergroup difference dropped to a statistically insignificant level. In addition, our results did not indicate a link between MMP-9 enzyme activity and GE score. In our investigation, the activity of MMP-9 differed between the case and control groups, corroborating

the results of Kim *et al*. They analyzed the expression level of MMP-2 and MMP-9 in gingival tissues of periodontitis patients using Western blot analysis and their enzymatic activity by gelatin zymography.[16] They concluded that the expression of MMP-9 was positively correlated with its activity; suggesting that MMP-9 amount and activity can be a predictive biomarker for the progression of periodontitis. However, this study did not observe any correlation between the expression level and activity of MMP-2. Similarly, our test on GE did not detect any activity of MMP-2 in saliva of patients with GE. In our study, the MMP-9 activity increased from the 4th to 5th months after the beginning of orthodontic treatment in subjects with GE, while there was not any correlation between enzyme activity and GE score. Kim *et al*. reported a positive correlation between the expression level and activity of MMP-9 and suggested that MMP-9 quantity and activity could serve as predictive biomarkers for the progression of periodontitis. However, they did not observe any correlation between the expression level and activity of MMP-2.

In our study, we did not detect any activity of MMP-2 in the saliva of patients with GE. Moreover, MMP-9 activity increased from the 4th to 5th months after the initiation of orthodontic treatment in subjects with GE. Nevertheless, no correlation was found between enzyme activity and the GE score.

One limitation of our study was our inability to quantify the enzyme quantity alongside assessing its activity. While measurements of MMP levels and activity might have been used to precisely determine the involvement of MMPs in GE, investigating the activity of enzyme inhibitors such as TIMP1 could provide further insight into the underlying mechanisms.

Furthermore, our data revealed a significant inter-individual heterogeneity. Factors such as variations in dental biofilm composition, its accumulation pattern, and differences in bacterial flora may have interfered with our measurements. In addition, we measured the target mediators in saliva, but site-specific monitoring of MMPs in the GCF of teeth exhibiting GE could have yielded different results.

In summary, this study contributes to our understanding of the role of MMPs, specifically MMP-9, in the pathogenesis of GE in orthodontic patients. While we observed significant differences in MMP-9 activity between individuals with and without GE at the 4th month of treatment, the exact mechanisms and factors involved require further investigation. The complex interplay between mechanical stress, inflammation, and MMP activity in the context of GE warrants continued exploration and a more in-depth understanding of this phenomenon.

CONCLUSION

Our study provides insights into the potential role of salivary MMP-2 and MMP-9 activity in orthodontic-induced GE. The study revealed that while MMP-2 activity was undetectable, MMP-9 activity increased during the 4th month of orthodontic treatment. However, this increase in MMP-9 activity did not exhibit a significant correlation with the degree of GE. These findings suggest that MMP-9 activity may play a role in the development of GE among orthodontic patients. Further research is needed to unravel the precise mechanisms underlying this phenomenon and its clinical implications.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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