Original Article
Levels of matrix metalloproteinases in saliva during orthodontic tooth movement

Xuejun Xu1,2, Qiong Zhang3, Yan Lv1,2, Tianming Yu4, Jianing Chen3, Ping Zeng3, Lin Wang3, Tianxing Liu5, Hongyan Diao3

1The Affiliated Stomatology Hospital, Zhejiang University School of Medicine, Hangzhou 310006, Zhejiang, China; 2Key Laboratory of Oral Biomedical Research of Zhejiang Province, Zhejiang University School of Stomatology, Hangzhou 310006, Zhejiang, China; 3State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Center for Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310006, Zhejiang, China; 4Ningbo No. 6 Hospital, Ningbo 315040, Zhejiang, China; 5Hangzhou Foreign Languages School, Hangzhou 310023, Zhejiang, China

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Abstract: Purpose: To determine the levels of matrix metalloproteinases (MMPs) in response to orthodontic tooth movement, in order to clarify the relationship between the change of MMPs and distance of tooth retraction. Materials and methods: In total, 11 patients who needed tooth extraction and 11 patients who didn’t before being subjected to fixed orthodontic appliance therapy were assigned into two groups: The extraction group and the non-extraction group. The saliva samples were collected from each patient at four indicated time points, including the first visit time (T1), time before fitting the orthodontic appliance (T2), one hour after (T3) and eight weeks after fitting the appliance (T4). Salivary MMPs concentration was determined using multiplexed bead immunoassay. Alginate impressions were taken at T1 and T4. Results: In the non-extraction group, the concentration of MMP-8 and MMP-9 were significantly increased at T3. In the extraction group, the concentration of MMP-8, MMP-9, and MMP-12 increased during the orthodontic tooth movement process, reaching a peak at T3. In particular, orthodontic tooth movement was positively associated with MMP-8, MMP-9, and MMP-12 levels in saliva at T3 in the extraction group. Conclusions: The result of our present study suggested that orthodontic force could modulate MMPs levels in saliva. MMP-8, MMP-9 and MMP-12 in saliva may represent novel indicators of the degree of orthodontic tooth movement.

Keywords: Matrix metalloproteinases, orthodontic tooth movement, saliva, multiplexed bead immunoassay

Introduction
Orthodontic tooth movement by therapeutic mechanical stress results from remodeling of the periodontal ligament and alveolar bone [1-3]. It initiates with an inflammatory-like response involving the induction of various biological factors and degradation/synthesis of the extracellular matrix (ECM) in the periodontal ligament (PDL) [4, 5]. The continued force application in remodeling surrounding tissues of orthodontic teeth might affect cellular responses, including the recruitment of osteoblast and osteoclast precursors, as well as the extravasation and chemotaxis of inflammatory cells [6-8]. On the other hand, previous studies also found certain biologically active substances could affect orthodontic tooth movement [9]. Therefore, study on the relationship between remodeling of periodontal tissues and change of various cytokines in this process has been a hot topic of orthodontic treatment research.

MMPs are potentially involved in remodeling of periodontal tissues. They are a family of proteases that play a critical role in remodeling of the ECM [10]. MMPs are generally classified into several subgroups: Collagenases (MMP-1, MMP-8, and MMP-13) that disintegrate native fibrillar collagens; gelatinases (MMP-2 and MMP-9) that cleave denatured collagen, stromelysins (MMP-3 and MMP-10); matrilysins (MMP-7 and MMP-11); membrane-type MMPs (MMP-14, MMP-15, MMP-16, and MMP-17) and miscellaneous MMPs [11, 12]. They are mainly distributed in saliva, gingival crevicular fluid, dentin and dental pulp in the human oral cavity [13]. The expression of MMPs, including MMP-
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| Table 1. Patient demographics and parameters |
|-----------------|-----------------|-----------------|
| Parameter       | Non-extraction  | Extraction      |
|                 | group           | group           |
| Sex             | Male            | Female          |
|                 | 2               | 3               |
|                 | 9               | 8               |
| Age (y)         | 21.0±3.3        | 22.7±2.8        |
| Angel Classification | Class I relationship | Class II relationship |
|                 | 3               | 4               |
|                 | 7               | 6               |
|                 | 1               | 1               |
| Dental Arch Crowding | mild            | moderate        | severe          |
|                 | 8               | 3               | 0               |
|                 | 0               | 9               | 2               |

1, -8, -9, -12 and -13 in the gingival crevicular fluid (GCF) have been deemed to increase in patients with periodontitis [14, 15]. MMPs also play a role in periodontal ligament (PDL) remodeling during orthodontic tooth movement. The level and activity of MMP-1 was increased in the compression side of the gingiva during orthodontic treatment in dogs [16]. The expression of MMP-8 and MMP-13 mRNA were also increased in the PDL of rats during active tooth movement [17]. In addition, previous study reported levels of MMP1/2 in human GCF changes during tooth movement in a time-dependent manner [18]. Although the alteration of MMPs levels in the periodontium has been reported before, the correlation between distance of orthodontic tooth movement and the levels of MMPs has not been detailed yet. In this study, we aimed to determine the levels of various MMPs in response to orthodontic tooth movement using multiplexed bead immunoassay technique and try to clarify the potential correlation among levels of MMPs, specific time points during fixed orthodontic appliance therapy and distance of tooth retraction.

Material and methods

Patients

All 22 patients in extraction or non-extraction groups were collected from the Orthodontics Department of Oral Cavity Hospital affiliated to Zhejiang University between September 2014 and December 2015. All potential referred patients were screened by experienced orthodontists to confirm their suitability for study. The subjects were required to be in good general health and had no periodontal disease or a history of treatment. Individuals who had autoimmune diseases, pregnancy, lactation, or use of any medication that could interfere with orthodontic tooth movement (e.g. antibiotics, antihistamines, cortisone, and hormones) within a month preceding the beginning of the study were excluded [19]. All patients who were recruited completed the study with no loss of follow-up. Their ages ranged from 18 to 29 years old, with mean ages of 21.0 years old in the non-extraction group and 22.7 years old in the extraction group. The extraction group included 3 males and 8 females, and the non-extraction group consisted of 2 males and 9 females (Table 1). The subjects in the extraction group needed extraction of two first upper premolars and two second lower premolars. The criteria of tooth extraction before treating with fixed orthodontic appliance contained composite factors including severity of crowding, mesial drift of anchorage molar, jaw growth, curve of space, incisor retraction and so on. Malocclusion type and severity of dental crowding were well classified in each patient by experienced orthodontists. The former classification is based on Angle Classification, which is originally devised as a prescription for orthodontic treatment planning, including neutroclusion, distroclusion and mesioclusion. Dental crowding was also measured based on dental arch crowding. The protocol of the present study was approved by the Medical Ethic Committee of Affiliated Stomatology Hospital, School of Medicine, Zhejiang University, and complied with the requirements of the Declaration of Helsinki. All subjects gave their informed consent.

Saliva samples collection

Saliva samples were collected from the subjects of the two groups (the extraction group and the non-extraction group) at the first visit (T1), a week later after teeth extraction in the extraction group (T2) or before fitting the orthodontic appliances in the non-extraction group (T2). The third and fourth time points (T3 and T4) were one hour and eight weeks after orthodontic appliances were activated, respectively. About 1.2 mL of whole saliva was obtained using disposable sterile attractor. The uses of antiseptic mouth rinse and food intake were
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forbidden within 2 hours before collection. The collected saliva was clarified by centrifugation for 5 minutes at 8000×g immediately. The supernatants were retained and frozen at -80°C.

All patients were treated with a BioQuick prescription 0.018×0.025-in bracket slot appliance, which is bonded to the maxillary or mandibular teeth. A 0.014-in nickel-titanium conventional arch-wire was then placed for the initial leveling and alignment stage. Thereafter, saliva samples were taken at one hour (T3) and eight weeks (T4) after force application. No further appliance reactivations were performed during these sampling times.

**Measurement of orthodontic tooth movement**

Alginate impressions were taken at the first visit and eight weeks after fitting the orthodontic appliances in order to monitor the extent of tooth movement. The impressions were poured into with plaster (calcium sulfate). Vertical lines were drawn on the cast over the palatal surface of the canine and the lateral incisor from the middle of the incisal edge to the middle of the cervical line. The distance between the canine and the lateral incisor was assessed at T3 at three points: incisal, middle, and cervical thirds of the crowns (Figure 1C). All cast measurements were made using an electric digital caliper (Exploit, Japan) with an accuracy of 0.01 mm.

**MMPs measurement**

Salivary levels of MMPs were measured through the multiplexed bead immunoassay using the commercial kits bio-plex pro human MMP Panel (Bio-Plex Suspension Array System, Bio-Rad Laboratories, Inc, USA). The assay was performed in a 96-well filter plate which was pre-wetted with assay buffer. Microsphere beads which were coated with monoclonal antibodies

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**Figure 1.** Comparison of orthodontic tooth movement between the non-extraction group and extraction group. A. Yellow impressions from a patient of non-extraction group are taken at the first visit, representing as the upper permanent teeth, the lower permanent teeth and the type of malocclusion (Class II relationship in Angle Classification) respectively (upper). White impressions are taken at eight weeks after orthodontic appliances fitting from the same patient (lower). B. Impressions are from a patient of extraction group. The type of malocclusion is Class II in Angle Classification. “×” denotes two first upper premolars and two second lower premolars that need to be pulled. C. Tooth movement is calculated by measuring the distance between the two lines linked by three points: incisal edge, middle, and cervical thirds of the crowns. D. Orthodontic tooth movements are compared between the non-extraction group and extraction group. Each bar represents the mean ± SEM. A ANOVA was used for testing the significance between the groups (*P<0.05).
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against different target analysts were diluted with assay buffer and added to the wells. The plate was washed and then samples and standards were added, respectively. After incubation for 30 minutes, the sealing tape was slowly removed; the wells were washed again and biotinylated detection antibodies specific to the analyte of interest were added. After incubation for 1 hour, Phycoerythrin (PE)-conjugated Streptavidin was added for an additional 30 minutes. After washing to remove unbound reagents, the samples were measured with Bio-Plex™ 200 (Luminex®, MiraiBio, Alameda, Calif). The concentration of MMPs in the unknown samples was determined from the standard curve.

**Statistical analysis**

The data were analyzed by SPSS 22.0 (SPSS Inc., IL, USA) with average values and standard error of means (mean ± SEM). Chi-squared test was used for comparison of malocclusion type between 2 groups. A one-way analysis of variance (ANOVA) test was used for testing the significance of intra-group MMPs from T1 to T4. Pearson correlation test was used for analysis of correlation between MMPs concentration and the distance of tooth movement. P<0.05 was considered significant.

**Results**

The comparison of malocclusion types between extraction and non-extraction group

The malocclusion type of each patient was classified based on Angle Classification. In the non-extraction group, there were three patients of class I, seven patients of class II, and one patient of class III. In the extraction group, there were four patients of class I, six patients of class II, and one patient of class III. There was no significant difference in the types of malocclusion between the non-extraction and extraction groups (P>0.05) (Table 1). Dental arch crowding was also measured. The non-extraction group was comprised of eight patients with mild crowding and three with moderate crowding, while the extraction group included nine patients with moderate crowding and two with

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**Figure 2.** Salivary concentration of MMPs at different phases of orthodontic tooth movement in non-tooth extraction group. A-H present the concentration of MMP-1, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-12, and MMP-13. T1-T4 present the sampling time at the first visit, before fitting the orthodontic appliances, one hour and eight weeks after fitting the appliances, respectively. Each bar represents the mean + SEM. A ANOVA was used for testing the significance between the groups (*P<0.05, **P<0.01).
severe crowding. Thus, in terms of dental arch crowding, there was a significant difference between the two groups (P<0.05) (Table 1).

The measurement of tooth movement

We determined the orthodontic tooth movement at eight weeks after fitting the orthodontic appliances. We found an clear change in the distance between canine and the lateral incisor at eight weeks after fitting the orthodontic appliances compared with the first visit both in the non-extraction and the extraction groups (Figure 1A and 1B). In addition, dentition and occlusion were significantly corrected and the interdentium diminished at eight weeks after fitting the orthodontic appliances.

Moreover, we found that orthodontic tooth movement was significantly higher in the extraction group compared with that in the non-extraction group (Figure 1C and 1D, P<0.05).

The changes of MMPs concentration in the non-extraction group

To investigate the alterations of the expression of MMPs family proteins at different time points, the concentration of salivary MMPs including MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-12, and MMP-13 in both groups were tested by multiplexed bead immunoassay. All MMPs except MMP-2 were maintained at detectable levels. We found that there was no apparent difference in the protein concentration of MMP-1, MMP-3, MMP-7, MMP-10, MMP-12, and MMP-13 among the different time points before and after treatment in the non-extraction group (Figure 2A-C, 2F-H). Notably, the concentration of MMP-8 and MMP-9 were significantly increased at T3 after treatment. However, eight weeks after force application (T4), the MMPs concentration fell back to levels comparable with T1 and T2 (Figure 2D, 2E).

The changes in MMPs concentration in the extraction group

We also investigated the alterations of MMPs family proteins in the tooth extraction group. The protein concentration of MMP-8, MMP-9,
and MMP-12 gradually increased from T1 to T3 after the treatment, and then significantly decreased at T4 (Figure 3D, 3E, 3G). The results also indicated that the protein concentration of MMP-7 was remarkably reduced at T4 versus T2 after treatment and MMP-10 was significantly decreased at T4 compared with T3 (Figure 3C, 3F). There were no clear differences in the protein concentration of MMP-1, MMP-3, and MMP-13 among the different time points induced by extraction or force application (Figure 3A, 3B, 3H).

Correlation between MMPs concentration and the distance of tooth movement

Since the boost of the expression of MMP-8/9/12 by orthodontic forces was detected, we also wondered about the relationship between MMPs concentration and orthodontic tooth movement. The results demonstrated that the changes in MMP-8, MMP-9, and MMP-12 levels did not correlate with the degree of tooth movement in the non-extraction group (Figure 4A-C). However, in the extraction group, the changes in the MMP-8, MMP-9, and MMP-12 levels correlated positively with the degree of tooth movement (Figure 4D-F).

Discussion

Several studies have demonstrated that orthodontic forces change the levels of MMPs in GCF during orthodontic tooth movement. However, MMPs measurements in saliva have rarely been reported. It is believed that different factors in GCF involved in alveolar bone and periodontal ligament remodeling are continuously drained into the saliva, which makes the saliva an easy alternative for GCF, offering the basis for a phase-specific screening of analytes linked to bone turnover during orthodontic tooth movement.

Previous study has shown MMP-8 was elevated shortly after application of orthodontic forces [20], which was consistent with our current study that salivary MMP-8 was increased significantly in one hour after force application, than before. Our study also demonstrated MMP-8 and MMP-9 accumulated after tooth extraction, which may reflect MMP-8/9 as being good biomarkers of inflammation in PDL during extraction. In addition, in both the extraction and non-extraction group, the concentration of MMPs almost regressed to the baseline value eight weeks after appliance fitting, which indicated application of orthodontic forces didn’t cause further damage to periodontal conditions despite having a tooth extraction or not.

Almeida et al has reported that the orthodontically moved and control teeth have a difference in the level of MMP-1 at the 1-hour time point after force application, the levels of MMPs dur-
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ing the 21 days of application of orthodontic forces showed no statistically significant changes [21]. This may be due to the subjects employed in the different studies. Our study employed subjects with healthy periodontium while their study treated individuals with a history of periodontitis.

In the present study, MMP-1, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-12, and MMP-13 levels were detected in saliva at various times during orthodontic tooth movement. In the non-extraction group, the levels of MMP-8 and MMP-9 increased one hour after force application. In the extraction group, the constantly fluctuating levels of MMP-8, MMP-9, and MMP-12 suggested that they are the major collagenolytic MMPs in saliva samples associated with orthodontic tooth movement. MMP-8 and MMP-9 secretion can be stimulated by mechanical stress within 1 hour, which was observed in both groups. The MMP-12 level only changed in the extraction group. Furthermore, MMP-7 and MMP-10 were also detected, but at much lower levels. MMP-7 is a matrilysin and MMP-10 is a macrophage elastase. No significant changes were found in non-extraction group during different sampling times. In the extraction group, MMP-7 peaked at T2 and MMP-10 peaked at T3. In terms of correlation analysis, there was no correlation between MMP levels and the tooth movement in the non-extraction group. This might be caused by insufficient space for the teeth to move. Of note, we first demonstrated that the expression levels of MMP-8/9/12 were positively correlated with the distance of orthodontic tooth movement in extraction group.

Conclusion

The result of our present study suggested that orthodontic force could modulate MMPs levels in saliva. MMP-8, MMP-9 and perhaps, MMP-12 in saliva may represent novel indicators of the degree of orthodontic tooth movement.

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Disclosure of conflict of interest

None.

Address correspondence to: Qiong Zhang and Hongyan Diao, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Center for Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou 310006, Zhejiang, China. E-mail: zhangqiong530@zju.edu.cn (QZ); diaohy@zju.edu.cn (HYD)

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