

Extreme intrusive force affects the expression of c-Fos and matrix metalloproteinase 9 in human dental pulp tissues

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Abstract

This study aimed to investigate the expression of c-Fos and matrix metalloproteinase 9 (MMP-9) in dental pulp of patients receiving orthodontic treatment via wire appliance.

Fifteen patients (30 teeth in total) were randomly assigned to five groups: t=0, t=1, t=4, t=8 and t=12 (n=6). The first maxillary premolars of patients in the t=0 group were extracted without any orthodontic treatment. An intrusive force of 300 g was applied on first maxillary premolars in the other four groups via wire appliances. This force was maintained for 1 week for t=1 group, 4 weeks for t=4 group, 8 weeks for t=8 group, or 12 weeks for t=12 group, before the teeth were extracted.

The expression of c-Fos and MMP-9 in the pulps of each group was analyzed by immunohistochemical staining and real-time PCR. The relationship in the protein expression between c-Fos and MMP-9 in the dental pulp was analyzed by Pearson correlation analysis.

Intrusive force of 300 g increased the expression of both c-Fos and MMP-9 in the dental pulp. The protein expression of MMP-9 in the dental pulp was significantly correlated with the expression of c-Fos ($P < .001$).

Extreme intrusive force upregulates c-Fos and MMP-9 expression in the dental pulp. Moreover, protein expression of c-Fos and MMP-9 is significantly correlated under intrusive force.

Abbreviations: AP-1 = activator protein-1, AP-1MMP-9 = matrix metalloproteinase 9, PDL = periodontal ligament.

Keywords: c-Fos, dental pulp, MMP-9, orthodontic, protein expression

1. Introduction

Orthodontic treatment has been extensively used in dental clinics to straighten or move crowded or crooked teeth into better position.^[1,2] Orthodontic treatment is expected to improve oral functions including mastication and speech, and dental aesthetics, thus avoiding potential health issues with the teeth. During this treatment, a proper external force is applied on the

teeth through proper appliances. The exact mechanisms underlying orthodontic treatment is far from fully understood.^[3] It is believed that the application of continuous mechanical force to the teeth induces inflammation in the periodontal ligament (PDL) and thus remodels alveolar bone. Studies reported in the literature showed that the force applied during orthodontic treatment induce molecular changes not only in the cells of the periodontal ligament and alveolar bone, but also in the pulp-dentine complex.^[4–8] Transient pulpitis and periodontitis have been observed because of serious inflammatory response such as circulatory disturbances caused by mechanical force used to induce orthodontic tooth movement. It was reported that patients receiving intrusive treatment with a force of 300 g up to 12 weeks still kept vital dental pulp in those treated teeth and no necrosis was observed.^[9] However, the molecular changes in pulpal cells upon the severe force application remain unclear.

Therefore, this study aimed to investigate the expression of c-Fos and matrix metalloproteinase 9 (MMP-9) in dental pulp of patients receiving extreme orthodontic force. We applied an intrusive force of 300 g on the bilateral first maxillary premolars in patients for 12 weeks. These extracted teeth were processed for the analysis of c-Fos and MMP-9 expression in the dental pulps using immunohistochemistry and real-time PCR.

2. Materials and methods

2.1. Patient enrollment

Approval of this study was obtained from institutional review board of Jilin University (Approval No. 23871), and informed written consent was obtained from all enrolled human subjects and/or their parents.

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Fifteen orthodontic patients were enrolled whose bilateral first maxillary premolars (30 teeth in total) had to be extracted due to orthodontic issues. They were 14 to 24 years old (mean 17.9 years). They were randomly assigned to 5 groups (6 teeth per group) base on the treatment duration: $t=0$ (without treatment), $t=1$ week, $t=4$ weeks, $t=8$ weeks, and $t=12$ weeks. Patients in the group of $t=0$ received no treatment with straight-wire appliances; while patients in the other groups received a stress of 300 g impressed on their bilateral maxillary first premolar for one to 12 weeks.^[11] All patients were subjected to weekly observation post-operation before the treatments were ended according to the group assignment and the treated teeth were extracted.

The inclusion criteria of enrollment were: no major systemic diseases; no history of antibiotic treatment within three months, and no use of calcium channel blockers such as phenytoin; non-pregnancy; health periodontal tissue (patient might having slight gingival inflammation with periodontal exploration depth < 3 mm and without alveolar bone absorption shown by X-ray); no root canal treatment history for the bilateral maxillary first premolar; full development of first premolar apical foramen.

2.2. Teeth treatment

Orthodontic separators, orthodontic bands, lingual buttons were purchased from New Asia Dental Materials Co., Ltd. (Hangzhou, China). Lingual buttons were adhered to the buccal sides of the first maxillary premolars (two teeth per patient) in patients (only those in $t=1$, $t=4$, $t=8$ and $t=12$ groups). Intrusive force was applied using a transparent and close-fitting orthodontic elastic rubber band from ORMAER (USA) (Fig. 1A) and adjusted to the desired value of 300 g. A transpalatal arch was used to strengthen the support (Fig. 1B). Patients were instructed to revisit the hospital once a week to replace the orthodontic elastic rubber bands. The residual force of each orthodontic elastic rubber band was measured using the orthodontic force gauge dynamometer (Tianmei dental equipment, Changsha, China) and recorded before being replaced during the revisit.

2.3. Histological staining and immunohistochemistry

The first maxillary premolars were extracted with minimal traumas at the time pre-designed for each group by one same surgeon. The teeth were dissected along the coronal pulp horns to expose the pulp for maximum use. The dissected teeth were fixed in 4% paraformaldehyde solution for 48 hours and then

decalcified using alumina formate and 19% EDTA solution, followed by dehydration and wax-embedment in paraffin. The wax-embedded tissues were sliced ($5 \mu\text{m}$) along the long axial mesial-distal direction. Ten slices from each tooth were used for the following experiment, with 5 slices for c-Fos protein immunohistochemical staining and the other 5 slices for MMP-9 immunohistochemical staining.

The slices were de-waxed in toluene twice (20 minutes each), and then hydrated in anhydrous ethanol, 90% ethanol, and finally 80% ethanol. After being washed with PBS (0.01 M, pH 7.5) for 3 times, the slices were processed to retrieve antigen with compound digestion solution. Afterwards, each slice was incubated with $50 \mu\text{l}$ peroxidase blocking solution for 10 minutes, followed by washing with PBS for 3 times. Each slice was incubated with primary antibody for c-Fos or MMP-9 (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight, followed by washing with PBS for 3 times. Each slice was incubated with biotinylated secondary antibody (Zhongshan Golden Bridge, Beijing, China) at room temperature for 10 minutes, followed by washing with PBS. Afterwards, each slice was incubated with Streptomyces avidin-peroxidase solution (Zhongshan Golden Bridge, Beijing, China) at room temperature for 10 minutes, followed by washing with PBS. The sections were visualized by adding freshly prepared 3,3'-diaminobenzidine tetrahydrochloride solution (Zhongshan Golden Bridge, Beijing, China). The sections were then counterstained using hematoxylin, dehydrated, and sealed for observation under microscope. For negative controls PBS was used instead of the primary antibody. Three sections stained for c-Fos or MMP-9 were randomly selected from each tooth for observation under microscope at $20\times$. Image-Pro Plus software was used to analyze the average grayscale value.

2.4. Real-time PCR

Total RNA was extracted from the pulps of tooth tissues by using RNeasy Fibrous Mini kit (Qiagen, Germany) following the recommended protocols. cDNA was synthesized with total RNA as the template by using random primer and Superscript III Reverse Transcriptase (Invitrogen, USA). cDNA was used as the template for real-time PCR on a ABI7300 PCR system by using TaqMan Master Mix (Applied Biosystems, USA). The primers were as follows: c-fos 5'-CTGGCGTTGTGAAGACCAT-3' and 5'-TCCCTTCGGATTCTCCTTTT-3'; MMP-9 5'-CACTGTC-CACCCCTCAGAGC and 5'-GCCACTTGTCGGCGATAAGG; GAPDH 5'-CCATGGAGAAGGCTGGGG and 5'-CAAAGTT



Figure 1. A: an intrusive force of 300 g was applied to the first maxillary premolar via an orthodontic elastic rubber band (red arrow); B: a transpalatal arch (black arrow) was used to reinforce anchorage.

GTCATGGATGACC. The mRNA levels were expressed as relative changes after normalization to GAPDH.

2.5. Statistical analysis

Statistics software SPSS 11.5 (SPSS Inc., Chicago, IL) was used to analyze numerical data expressed as mean \pm standard deviation. The differences among multiple groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test if necessary. $P < .05$ was considered as significant difference. Pearson correlation Bonferroni correction to the P values based on the number of genes per cancer type shared between methylation and expression data sets. The correlation of c-Fos and MMP-9 expression was analyzed using Pearson's correlation with a Bonferroni-corrected $P < .05$.

3. Results

3.1. Intrusive force upregulated c-Fos expression in human dental pulp

By immunohistochemical staining, c-Fos protein in the specimens was stained to show brownish or brown colors in the cytoplasm

and nuclear membranes (Fig. 2A and B). The specimens from the group of $t=0$ showed only weak expression of c-Fos in the odontoblast layer, pre-dentin, dental pulp cells, and blood vessels in the pulp tissues (Fig. 2A). After 1 week's treatment, strong staining of c-Fos was found in the odontoblasts cell layer and their perivascular (Fig. 2C). Moreover, vacuolization was observed in some odontoblast cells. After 4 weeks' treatment, strong staining of c-Fos was found in the odontoblasts, dental pulp cells, and the blood vessels (Fig. 2D). The arrangement of the odontoblast cell layer and pulp fibers became disordered, and the blood vessels dilated and congested. After 8 weeks' treatment, strong staining of c-Fos was found in the odontoblasts and the blood vessels (Fig. 2E). The pulp tissues were disordered, with a decrease in cell numbers, and an increase of fibers. The blood vessels dilated and congested. After 12 weeks' treatment, strong staining of c-Fos was found in the odontoblasts and the pulp cells (Fig. 2F). The odontoblasts showed more vacuolization, and the dental pulp cells showed a net atrophy. Semi-quantitative analysis of staining intensity showed that protein expression of c-Fos increased with the treatment duration (Fig. 3A). Real-time PCR analysis confirmed that the expression of c-Fos mRNA increased with the treatment duration (Fig. 3B).

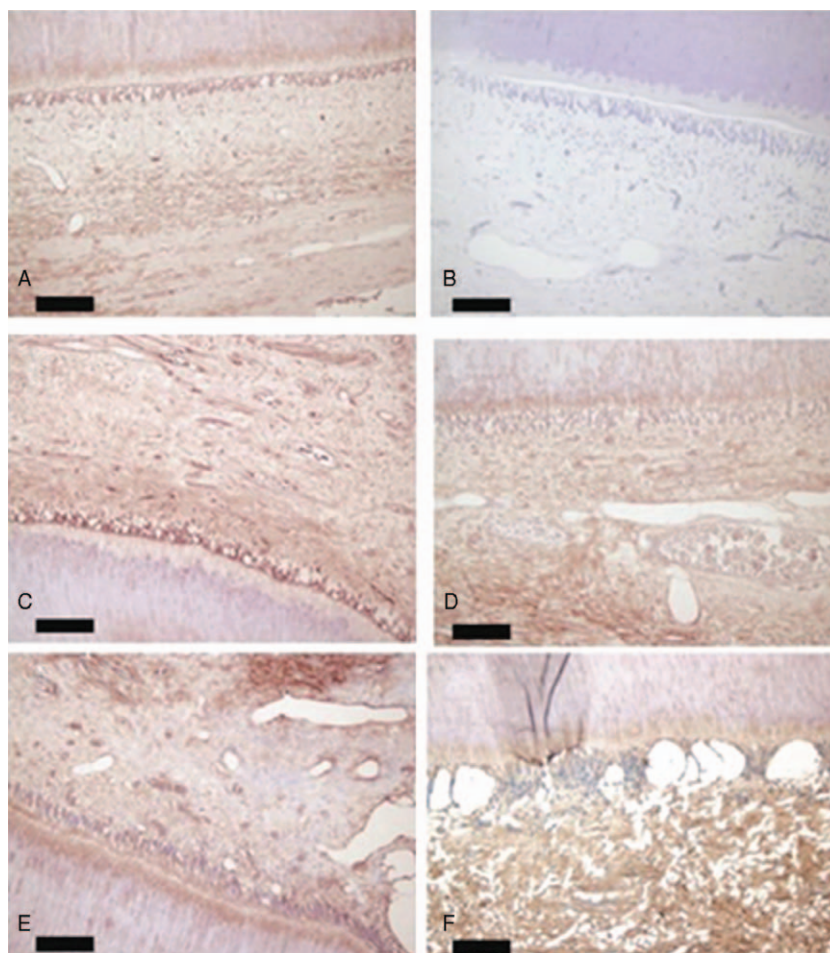


Figure 2. Typical images of immunohistochemical staining of c-Fos in coronal dental pulp tissues. A: c-Fos staining was weak in the odontoblast layer, pre-dentin, dental pulp cells, and blood vessels in the pulp tissues from patient group $t=0$; B: c-Fos staining was absent in negative control staining; C: c-Fos staining was strong in the odontoblasts cell layer and their perivascular from patient group $t=1$; D: c-Fos staining was strong in the odontoblasts, dental pulp cells, and the blood vessels from patient group $t=4$; E: c-Fos staining was strong in the odontoblasts and the blood vessels from patient group $t=8$; F: c-Fos staining was strong in the odontoblasts and the pulp cells from patient group $t=12$. Scale bar: 20 μm .

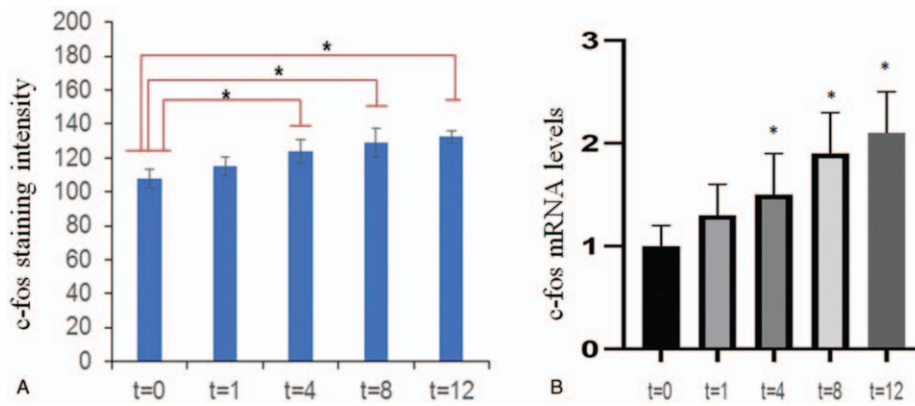


Figure 3. c-Fos expression in coronal dental pulp tissues of patients receiving intrusive force of 300g on their first maxillary premolars for different time periods. A. Semi-quantitative analysis of c-Fos staining intensity with the use of Image-Pro Plus software to calculate the average grayscale value. B. Real-time PCR analysis of relative c-fos mRNA levels with the t=0 group set as 1. *p < 0.05 compared to t=0 group.

3.2. Intrusive force upregulated MMP-9 expression in human dental pulp

By immunohistochemical staining, MMP-9 protein in the specimens was stained to show brownish or brown colors in

the cytoplasm (Fig. 4A and B). The specimens from the group of t=0 showed only weak expression of MMP-9 in the cytoplasm of odontoblasts and dental pulp cells (Fig. 4A). After one week’s treatment, strong staining of MMP-9 was found in the

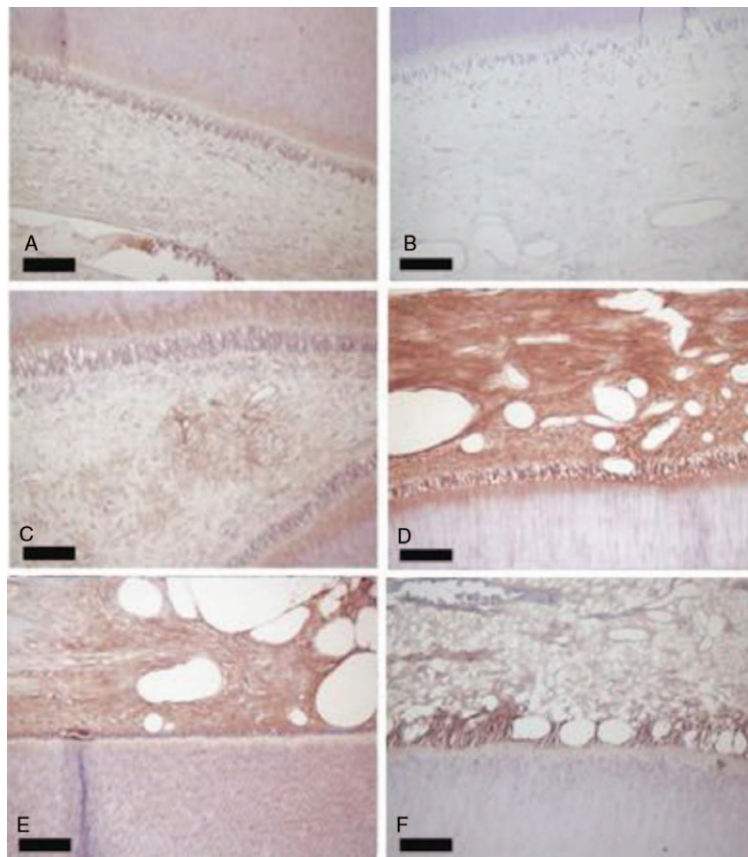


Figure 4. Typical images of immunohistochemical staining of MMP-9 in coronal dental pulp tissues. A: MMP-9 staining was weak in the cytoplasm of odontoblasts and dental pulp cells from patient group t=0; B: MMP-9 staining was absent in negative control staining; C: MMP-9 staining was strong in the odontoblasts layer and underneath and around the blood vessels from patient group t=1; D: MMP-9 staining was strong in the odontoblasts, dental pulp cells, and the blood vessels from patient group t=4; E: MMP-9 staining was strong in the odontoblasts and perivascular vessels from patient group t=8; F: MMP-9 staining was strong in the odontoblasts and the pulp cells from patient group t=12. Scale bar: 20µm.

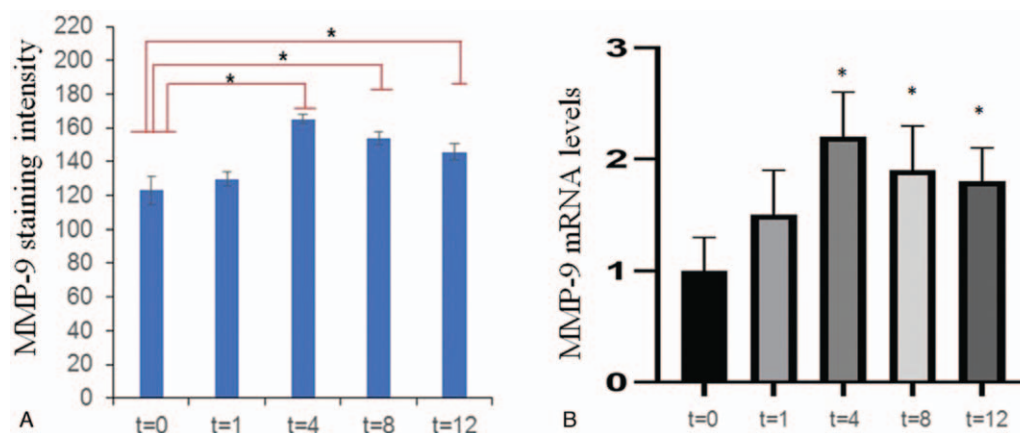


Figure 5. MMP-9 expression in coronal dental pulp tissues of patients receiving intrusive force of 300 g on their first maxillary premolars for different time periods. A. Semi-quantitative analysis of MMP-9 staining intensity with the use of Image-Pro Plus software to calculate the average grayscale value. B. Real-time PCR analysis of relative MMP-9 mRNA levels with the t=0 group set as 1. * $p < 0.05$ compared to t=0 group.

odontoblasts layer and underneath and around the blood vessels (Fig. 4C). After 4 weeks' treatment, strong staining of MMP-9 was found in the odontoblasts, dental pulp cells, and the blood vessels (Fig. 4D). After 8 weeks' treatment, strong staining of MMP-9 was found in the odontoblasts and perivascular vessels (Fig. 4E). After 12 weeks' treatment, strong staining of MMP-9 was found in the odontoblasts and dental pulp cells (Fig. 4F). Semi-quantitative analysis of staining intensity showed that protein expression of MMP-9 first increased with the treatment duration till 4 weeks, and then steady decreased thereafter till 12 weeks (Fig. 5A). Real-time PCR analysis confirmed that the expression of c-Fos mRNA showed similar trend of change with the treatment duration (Fig. 5B).

3.3. Correlation of c-Fos and MMP-9 expression in human dental pulp

The Pearson correlation analysis revealed that there was a positive correlation in protein expression between c-Fos protein and MMP-9 in human dental pulp, with a statistically significant difference ($P < .001$).

4. Discussion

During the orthodontic treatment to re-arrange the tooth positions, an external force was constantly applied to the treated teeth. This external force serves as the mechanical stimulus. It is well-known that such extracellular mechanical or physical stimuli induce a coordinated cellular response via various transcription factors. These transcription factors are involved in the proliferation and differentiation of various cells including those in the pulp tissues. C-Fos is one of such transcription factors belonging to the activator protein-1 (AP-1) family. In normal conditions, the expression of c-Fos in cells is low,^[10] as demonstrated in c-Fos immunochemical staining of the specimens from the t=0 group in this study. c-Fos has similar expression in both osteoblasts and odontoblast differentiation.^[11] When cells are mechanically stimulated, AP-1 gene is activated which promotes the synthesis of Fos. However, few studies have reported the expression of c-Fos during the orthodontic treatment. Therefore, in this present study we enrolled fifteen

patients to study the effects of intrusive force on the protein expression of c-Fos in the dental pulps using immunochemistry, and its changes with the time under intrusive force.

Our results showed that c-Fos protein was expressed in the odontoblast layer, pre-dentin layer, dental pulp cells, and blood vessels in the pulp tissues, although its expression was very weak under normal condition without intrusive force. Under the stimuli of 300g intrusive force, the expression of c-Fos increased with the increase of time duration, especially in the odontoblasts and around blood vessels, consistent with previous report that c-Fos mRNA in human periodontal fibroblasts increased rapidly and peaked at 30 minutes after mechanical stimulation. Moreover, we observed an increase of fibrosis in the pulp tissues under the stimuli of 300g depression force after 8 weeks. This is consistent with previous report that periodontal fibroblasts under mechanical stimulation had increased expression of c-Fos, resulting in enhanced proliferation of periodontal fibroblasts.^[12] This observation is also consistent with significant fibrous tissue formation in pulp tissues of teeth receiving extrusive force for 3 weeks.^[13] Therefore, we hypothesize that the increased expression of c-Fos might contribute to the observed vacuolization and net atrophy of dental pulp cells in dental pulp of patients receiving intrusive force on their first maxillary premolars.

MMP-9 is one of the major enzymes degrading extracellular matrix during physiological and pathological processes.^[14] The synthesis, secretion and activation of MMP-9 are tightly regulated. Therefore, MMP-9 is a biomarker indicating the degree of inflammation and damage. Orthodontic movements have been considered to cause inflammatory alterations in the dental pulp. Therefore, we determined the expression of MMP-9 protein in the dental pulp of teeth exposed to intrusive force of 300g, and examined the changes of MMP-9 expression with the time under intrusive force. We found that MMP-9 protein is commonly expressed in the cytoplasm of odontoblasts and dental pulp cells, although its expression is very weak in normal conditions without intrusive force. This observation was consistent with previous report that dental pulp cells and odontoblasts are the main cells for the synthesis of MMP-9 in the dental pulp tissue.^[15] Under the stimuli of 300g intrusive force, the expression of MMP-9 increased with time until 4 weeks after operation. Thereafter, the expression of MMP-9 slowly

decreased from the peak but was still stronger than that in $t=0$ group. These observations are consistent with previous *in vivo* studies that MMP-9 mRNA in the periodontal ligament increased after receiving an orthodontic force for 7 days and began to decline after 14 days, and that the expression of MMP-9 was enhanced in the odontoblasts and pulp fibroblasts of human dental pulp receiving an orthodontic force.^[16] However, these observations were inconsistent with the *in vitro* studies which reported reduced MMP-9 expression in dental pulp tissue after mechanical injury.^[17] The inconsistency might be attributed to the different conditions for the *in vitro* and *in vivo* studies.

It has been shown that c-fos promotes tumor cell invasion by upregulating MMP-9.^[18] Therefore, we analyzed the relationship between c-Fos and MMP-9 expression. We found a positive correlation between the expression of c-Fos protein and MMP-9 protein in the human dental pulp under an intrusive force of 300 g. Therefore, we speculated that c-Fos protein may participate in the repair of dental pulp tissue by upregulating the expression of MMP-9. On the other hand, force application may induce inflammation reaction in the teeth and MMP-9 is known to be involved in inflammation. However, it is a limitation of this study that we could not investigate the pathological role of c-Fos and MMP-9 in the inflammation of dental pulp tissue under force application. In addition, the sample size of this study is small and need more samples to confirm our conclusion.

In summary, intrusive force of 300 g significantly increased c-Fos and MMP-9 expression in the dental pulp. Moreover, protein expression of c-Fos and MMP-9 was significantly correlated in pulp tissues under intrusive force of 300 g. c-Fos and MMP-9 may participate in molecular changes in the cells of the dental pulp upon force application.

Author contributions

Conceptualization: Min Hu.

Funding acquisition: Min Hu.

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