Experimental animal study on BMP-3 expression in periodontal tissues in the process of orthodontic tooth movement

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Abstract. Expression characteristics of bone morphogenetic protein-3 (BMP-3) in the process of orthodontic tooth movement was investigated. Forty-eight rats were randomly divided into the 3-day group (n=12), the 7-day group (n=12), the 14-day group (n=12) and the 21-day group (n=12). The maxillary left molar of each rat was used as the experimental tooth, the orthodontic tooth model was established, and the contralateral molar was used as the control tooth. The tooth movement distance was measured using a vernier caliper at day 1, 3, 7 and 14 after modeling, and rats in each group were sacrificed and sampled at the corresponding time-point. The tissue morphology was observed via hematoxylin and eosin (H&E) staining, and the expression of BMP-3 was detected via immunohistochemistry. The protein expression of BMP-3 was detected via western blotting, and the messenger ribonucleic acid (mRNA) expression of BMP-3 was detected via quantitative polymerase chain reaction (qPCR). At day 14 after modeling, the periodontal space was significantly uneven, the form of periodontal tissues was disordered, and a large number of multinucleated osteoclasts could be seen. The expression levels of BMP-3 in other groups were significantly increased compared with that in the control group (P<0.05). The expression level of BMP-3 reached the peak at day 14 after modeling, and differences were statistically significant compared with those in other time points after modeling (P<0.05). The orthodontic tooth movement distance after modeling was significantly longer with the extension of time (P<0.05). In the process of orthodontic tooth movement, the expression level of BMP-3 is gradually increased and reaches the peak at day 14, promoting the increase of osteoclasts and benefiting the orthodontic tooth movement.

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Introduction

Tooth orthodontics is to exert the external mechanical force to the deformed tooth in clinical treatment, so as to remodel the periodontal tissues around the tooth, thus moving and correcting the tooth. Periodontal tissues have rich structures, including alveolar bone, periodontal membrane and gingiva. During the tooth orthodontic treatment, periodontal membrane remodeling is the most important, which is a dynamic equilibrium state between bone formation and bone resorption (1). The periodontal membrane, as the mediator of orthodontic treatment, is a kind of variant periosteum. Bone morphogenetic protein-3 (BMP-3) is one of the important members of the transforming growth factor-β (TGF-β) family, and mainly involved in the bone activity (2). Moreover, BMP-3, as an important cytokine, plays an important regulatory role in bone remodeling (3). At first, scholars believed that BMP-3 is a kind of bone response factor, but they recognized that BMP-3 is a negative regulatory factor of bone formation with the deepening of research on BMP-3 (4,5). However, the role and expression characteristics of BMP-3 in the process of orthodontic tooth movement remain unclear. Therefore, this study investigated the role and expression characteristics of BMP-3 in the process of orthodontic tooth movement of rats.

Materials and methods

Experimental animals and grouping. A total of 48 Sprague-Dawley rats (half male and half female) weighing 220±20 g were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) [license no.: SCXK (Shanghai) 2014-0003]. The rats were kept in cages with controlled temperature and light cycles (24˚C and 12/12 light/dark cycle). The humidity was 60±10% and had free access to food and water. The above 48 rats were randomly divided into the the 3-day group (n=12), the 7-day group (n=12), the 14-day group (n=12) and the 21-day group (n=12) using the random number table method. The maxillary left molar of each rat was used to establish the orthodontic tooth movement model, while the contralateral tooth received no treatment as the control group. The study was approved by the Ethics Committee of Qingdao Women and Children's Hospital (Qingdao, China).

Experimental reagents. Anti-BMP-3 antibody (Abcam, Cambridge, MA, USA), ethylene diamine tetraacetic acid (EDTA) (Sinopharm Group), hematoxylin and eosin (H&E) staining kit (Solarbio, Beijing, China), immunohistochemistry kit (Maxim, Fuzhou, China), AceQ quantitative polymerase chain reaction (qPCR) SYBR Green Master Mix kit (Vazyme, Nanjing, China), HiScript II Q RT SuperMix for qPCR (+gDNA wiper) kit (Vazyme), optical microscope (Leica DMI 4000B/DFC425C; Leica Microsystems GmbH, Wetzlar, Germany), fluorescence qPCR instrument (ABI 7500, USA), Image-lab image analysis system and Image-Pro image analysis system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), orthodontic clinically-special nickel-titanium push spring (Dalian Tongdali Co., Ltd., Dalian, China) and orthodontic clinically-special micro implant (Dentaurum, Ispringen, Germany).

Establishment of orthodontic tooth movement model. After successful anesthesia via intraperitoneal injection of 7% chloral hydrate into rats (5 ml/kg), the maxilla of rats was fully exposed, and the micro-implant was implanted as the support into the left upper incisor at a speed of 1,020 x g at 20˚C for 5 min. A 0.02 mm orthodontic ligature wire was passed between the maxillary left first molar and the second molar, one end of which was connected to the nickel-titanium push spring, and the other end was connected to the implant. An orthodontic dynamometer was connected to the other end of the nickel-titanium push spring to measure its length at 40 g, and the length of both ends of the nickel-titanium push spring should be the same.

Treatment in each group. The maxillary left molar of rats in each group was used as the experimental tooth and the incisor as the anchorage tooth to prepare the orthodontic tooth movement model. The corresponding maxillary right molar received no treatment as the control group. The tooth movement distance of each rat was measured using a vernier caliper at day 1, 3, 7, 14 and 21 after modeling. The rats were sacrificed at day 3 after modeling in the 3-day group, at day 7 after modeling in the 7-day group, at day 14 after modeling in the 14-day group and at day 21 after modeling in the 21-day group.

Material acquisition. After successful anesthesia, the maxillary left and right molars of each rat were taken directly. The molars of 6 rats in each group were fixed in 4% paraformaldehyde at 4˚C for 48 h, decalcified in EDTA solution for 2 months, and prepared into paraffin-embedded tissue sections for H&E staining and immunohistochemical detection. The molars of the remaining 6 rats were placed into Eppendorf (EP) tubes for western blotting and qPCR.

H&E staining. After 5 μm-thick paraffin-embedded tissue sections were routinely deparaffinized with xylene, they were dehydrated with gradient alcohol, stained with hematoxylin for 5 min, rinsed with tap water, differentiated with hydrochloric acid alcohol for 30 sec, soaked in tap water for 15 min, stained with eosin for 2 min, routinely dehydrated again with gradient alcohol, made transparent with xylene and sealed with neutral gum.

Immunohistochemistry. Tissues were fixed with 10% formaldehyde at 20˚C for 16 h. Paraffin-embedded tissue sections (5 μm in thickness) were routinely deparaffinized, placed into water, added with the citrate buffer and heated in a microwave oven for antigen retrieval. After sections were rinsed with phosphate-buffered saline (PBS), the endogenous peroxidase blocker was added for incubation for 10 min, followed by rinsing with PBS. Then, sections were sealed with goat serum for 20 min, the serum sealing solution was removed, and rabbit anti-rat BMP-3 primary polyclonal antibody (1:200; cat no. ab134724; Abcam) was added for incubation at 4˚C overnight. After being rinsed with PBS, sections were incubated with goat anti-rabbit secondary antibody for 10 min, rinsed again with PBS and added with streptavidin-peroxidase solution for 10 min of incubation, followed by color development via diaminobenzidine (DAB), counterstaining via hematoxylin, sealing with neutral gum and observation and photography under the microscope (Olympus, Tokyo, Japan).

Table I. Primer sequences.

Name	Primer sequences
BMP-3	F: 5'-ACATCGCTAACCAAGTCTGA-3'
	R: 5'-GAGCAATAATAGGCATCAAAG-3'
GAPDH	F: 5'-ACGGCAAGTTCAACGGCACAG-3'
	R: 5'-GAAGACGCCAGTAGACTCCACGAC-3'

F, forward; R, reverse.

Western blotting. Tissues stored at -20˚C to be used were added with lysis solution, followed by ice bath for 60 min and centrifugation at 14,000 x g for 10 min at 4˚C. The protein was extracted with ProteoPrep® Total Extraction Sample Kit (Sigma-Aldrich, Darmstadt, Germany) and quantified using the bicinchoninic acid (BCA) method. The standard curve and absorbance were obtained in a microplate reader (Bio-Rad Laboratories, Inc.), based on which the protein concentration was calculated. After protein denaturation, 12 μl sample was added and separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the corresponding concentration. When the Marker protein ran to the bottom of the glass plate, and the sample protein sank to the bottom basically in a straight line, the gel running was terminated. The protein was transferred onto a polyvinylidene fluoride (PVDF) membrane, sealed with 5% milk at 20˚C for 1.5 h and washed with TPBS 3 times. Then, anti-BMP-3 primary antibody (1:1,000) and secondary antibody (1:1,000) were added successively, and the membrane was rinsed with Tris-buffered saline with Tween®-20 (TBST) once between every two steps. The color was developed after the secondary antibody was removed using TBST. The membrane was placed into the chemiluminesence reagent for reaction for 1 min, and the color was developed in the dark, followed by densitometry using the gel scanning imaging system Quantity One software (Bio-Rad, Laboratories, Inc., Hercules, CA, USA).

qPCR. The total ribonucleic acid (RNA) was extracted from the tissues stored at -20˚C using the RNA extraction kit and it

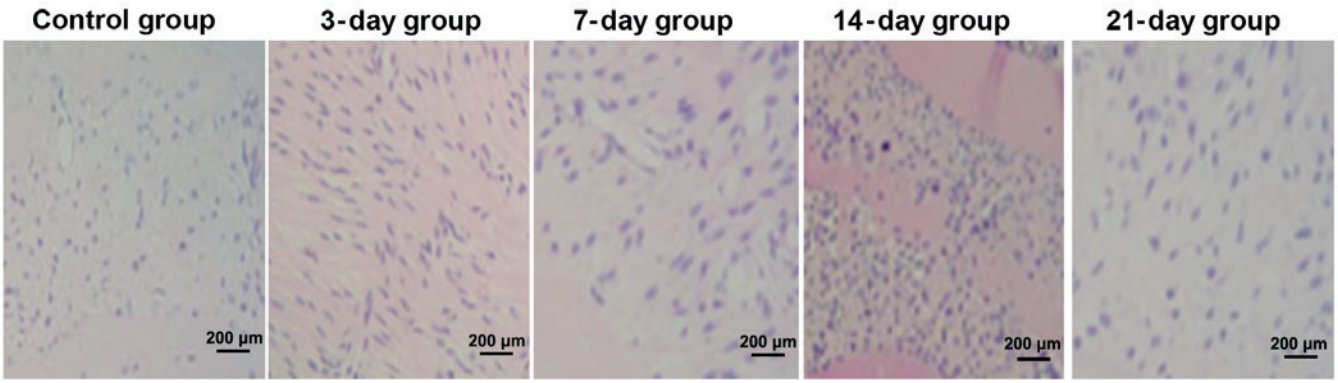

Figure 1. Tissue morphology in each group observed via H&E staining (magnification, x200).

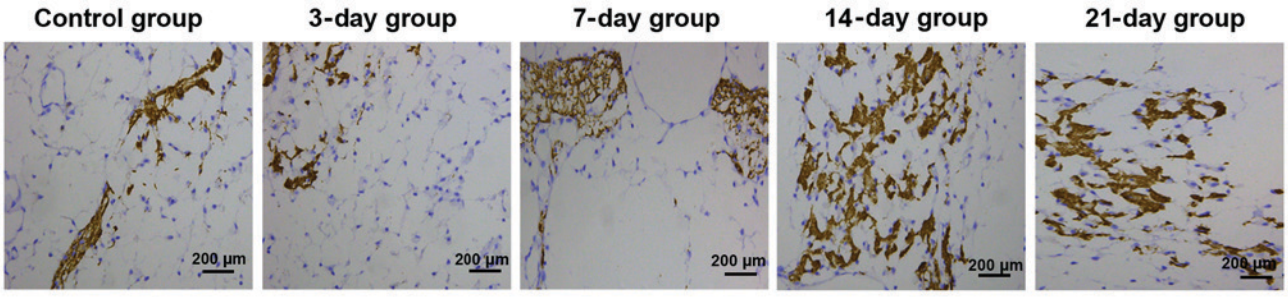

Figure 2. BMP-3 expression detected via immunohistochemistry (magnification, x200).

was reverse transcribed into complementary deoxyribonucleic acid (cDNA) using the reverse transcription kit. The reaction system was 20 μl, and reaction conditions were as follows: reaction at 51˚C for 2 min, predenaturation at 96˚C for 10 min, denaturation at 96˚C for 10 sec and annealing at 60˚C for 30 sec, a total of 40 cycles. The relative expression level of BMP-3 messenger RNA (mRNA) was analyzed using the $2^{-\Delta\Delta Cq}$ method (6). Primer sequences are shown in Table I.

Statistical analysis. Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Enumeration data are presented as mean ± standard deviation. The t-test was used for data in line with the normal distribution and homogeneity of variance, corrected t-test was used for data in line with the normal distribution and heterogeneity of variance, and non-parametric test was used for data not in line with the normal distribution and homogeneity of variance. Chi-square test was used for enumeration data. ANOVA was used for comparison between multiple groups and the post hoc test was LSD test.

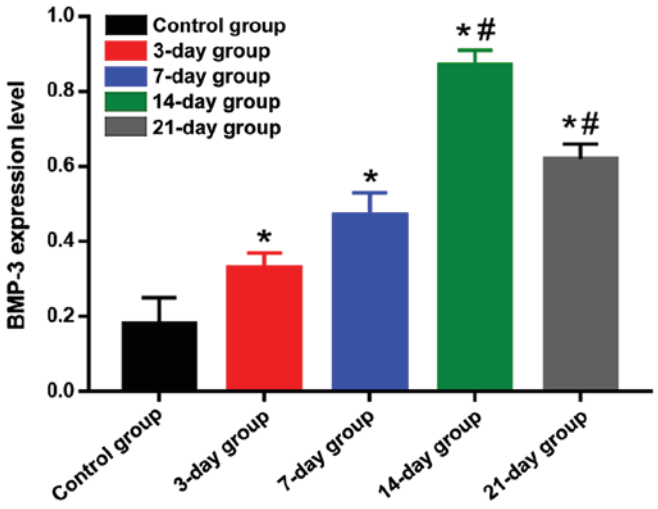

Figure 3. BMP-3 expression level in each group. *P<0.05 vs. the control group; #P<0.05 vs. the 3- and 7-day groups.

Results

H&E staining. In the control group, the periodontal space was uniform, the periodontal membrane was tidy and distributed in an orderly manner without compression and traction, and there were no obvious multinucleated osteoclasts. In the 3- and 7-day groups, the periodontal space was not uniform, the periodontal membrane was not tidy with slight compression and traction, and multinucleated osteoclasts were visible. In the 14- and 21-day groups, the periodontal space was uneven, the periodontal membrane was not uniform and distributed in a disorderly manner with obvious compression and traction, and there were many multinucleated osteoclasts (Fig. 1).

BMP-3 expression level detected through immunohistochemistry. In the control group, there was no significant positive expression of BMP-3, and the color of periodontal membrane was uniform. In the 3-day group, slightly positive expression of BMP-3 could be seen on the tension side. In the 7-day group, the positive expression of BMP-3 was enhanced on the tension

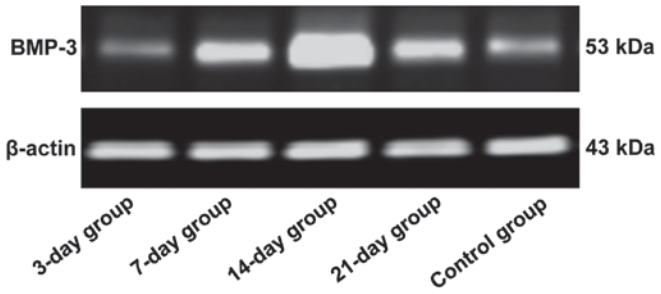

Figure 4. BMP-3 protein expression detected via western blotting.

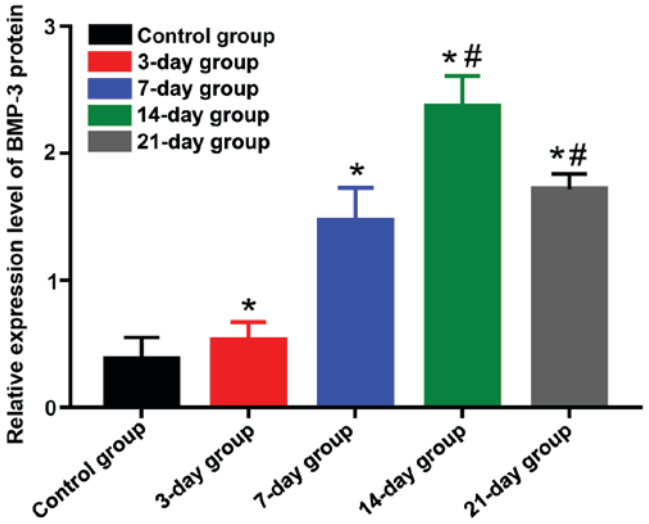

Figure 5. Relative expression level of BMP-3 protein. *P<0.05 vs. the control group; #P<0.05 vs. the 3- and 7-day groups.

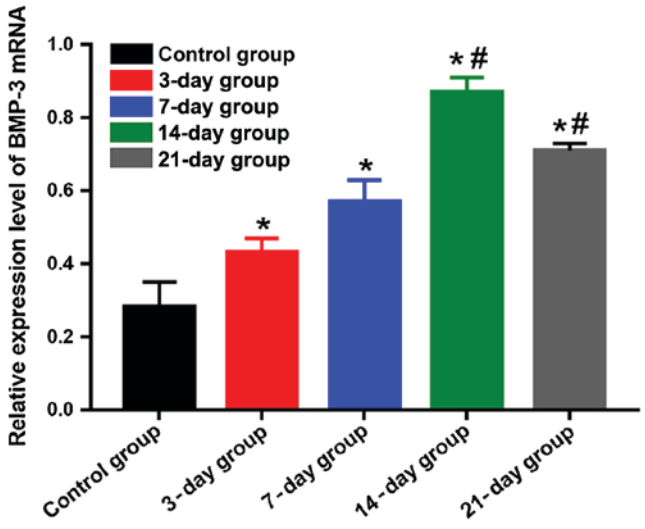

Figure 6. Relative expression level of BMP-3 mRNA. *P<0.05 vs. the control group; #P<0.05 vs. the 3- and 7-day groups.

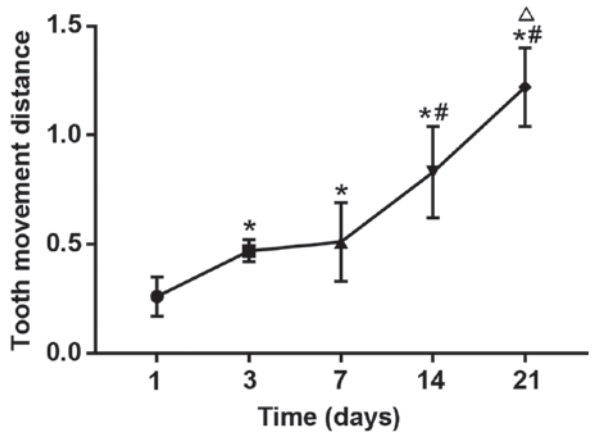

Figure 7. Tooth movement distance. *P<0.05 vs. the 1-day group; #P<0.05 vs. the 3- and 7-day groups; ΔP<0.05 vs. the 14-day group.

side, showing bead string-like distribution. In the 14-day group, there was significantly positive expression of BMP-3 on the tension side, with deep color and numerous quantity, showing plate-like distribution. In the 21-day group, the positive expression of BMP-3 on the tension side was weakened and light brown (Fig. 2). Statistical analysis results of the positive expression are shown in Fig. 3. Compared with that in the control group, the positive expression of BMP-3 on the tension side in the other groups was significantly increased, and differences were statistically significant (P<0.05). The positive expression of BMP-3 on the tension side in the 14-day group was the highest, which was significantly higher than those in the 3-, 7- and 21-day groups, suggesting that the expression level of BMP-3 on the tension side is gradually increased in the process of orthodontic tooth movement, and reaches the peak at day 14 and begins to decline gradually after that.

BMP-3 protein expression level detected via western blotting. The protein expression level of BMP-3 was lower in the control group, but significantly increased in the other groups, displaying statistically significant differences (P<0.05). The protein expression level of BMP-3 in the 14-day group was significantly higher than those in the 3-, 7- and 21-day groups, and differences were of statistical significance (P<0.05) (Figs. 4 and 5), indicating that the protein expression level of BMP-3 is gradually increased, and the translation ability is enhanced in the process of orthodontic tooth movement. The protein expression level reached the peak at day 14 and began to decline gradually after that.

BMP-3 mRNA expression level detected using qPCR. The mRNA expression level of BMP-3 was lower in the control group, but it had obvious increases in the other groups, showing statistically significant differences (P<0.05). The mRNA expression level of BMP-3 in the 14-day group was the highest, which was obviously higher than those in the 3-, 7- and 21-day groups, and differences were statistically significant (P<0.05) (Fig. 6), indicating that the mRNA expression level of BMP-3 is gradually elevated, and the transcription ability is enhanced in the process of orthodontic tooth movement. The mRNA expression level reached the peak at day 14 and began to decline gradually after that.

Average tooth movement distance. The tooth movement distance at day 3 after modeling was remarkably longer than that at day 1 after modeling, and there was a statistically significant difference (P<0.05). The tooth movement

distances at day 14 and 21 after modeling were increased significantly, which were obviously longer than those at day 3 and 7 after modeling, and differences were statistically significant ($P<0.05$). The tooth movement distance at day 21 after modeling was remarkably longer than that at day 14 after modeling, and there was a statistically significant difference ($P<0.05$) (Fig. 7), suggesting that the tooth movement distance is shorter in the early stage of orthodontic treatment, and it is gradually increased with the extension of time. The longer the time is, the greater the movement distance will be.

Discussion

Tooth orthodontics is a commonly-used oral orthodontic method in clinic, which is widely used with a good curative effect (7). When orthodontic external forces are exerted on the tooth to be corrected, osteoblasts will accumulate in the alveolar bone on the tension side, while osteoclasts will accumulate in the alveolar bone on the pressure side, thereby mediating bone formation and bone resorption (7-9). In this study, the movement distance of the rat's molars was measured using the vernier caliper. It was found that the rat molars moved rapidly in a near-medium distance in the early orthodontic stage, namely within 1-3 days, moved slowly within 3-7 days and moved significantly quickly from 7 to 14 days. The reason is that the orthodontic external forces lead to periodontal compression on the pressure side of the rat's molars in the early orthodontic stage, and the rapid tooth movement is closely related to the concession of periodontal membrane (10). The hyalinization of periodontal membrane at 3-7 days after orthodontic treatment is a possible cause of the slow movement of the rat's molars (11). The external force is positively correlated with the scope of hyalinization, so the tooth displacement is difficult, with underlying absorption (12). The rapid tooth movement at 7-14 days after orthodontic treatment is related to the maximum physiological remodeling function of periodontal membrane and alveolar bone (13).

BMP is one of the important subfamilies of TGF-β superfamily. BMP-3, as a member of the family, has only approximately 40% similarity to other BMP members (14). BMP-3 has an effect of chemotaxis of dorsal development (15), which is similar to the antagonists of other BMP members (16). Therefore, BMP-3 has a certain inhibitory effect on BMP-2 and BMP-4, two members with the bone induction effect in the BMP family, thereby inhibiting the BMP-2- and BMP-4-mediated downstream signaling pathways and suppressing osteoblasts (17,18). BMP-3, through regulating TGF-β/activin signaling pathways, can act on downstream Smad-2, Smad-3 and BMP-2- and BMP-4-activated Smad-1, Smad-5 and Smad-8 to compete for Smad-4, thus inhibiting BMP-2 and BMP-4 (17,19). In addition, ActRII-β, as a co-receptor of BMP-2, BMP-3 and BMP-4, binds to BMP-3 via the chemical bond and is invulnerable to hydrolysis, and there is a high affinity between them (20). Therefore, BMP-3 competitively combines with the ActRII-β receptor of BMP-2 and BMP-4 to inhibit BMP-2 and BMP-4. In this study, the implant anchorage was used to pull the rat's molars to study the process of tooth movement under the orthodontic external forces. BMP-3 antagonized the orthodontic tooth movement process and was expressed more on the tension side (21). Results of this study indicated that the staining of osteoclasts in the rat alveolar bone was deepened with time, and there were increasingly more osteoclasts, indicating that the orthodontic tooth becomes loose under the external force, thus facilitating the tooth movement. At the same time, the expression level of BMP-3 on the tension side of the alveolar bone was increased with time, and BMP-3 accumulated on the tension side, inhibiting osteoblasts, so that the microenvironment of the alveolar bone is conducive to tooth loosening, facilitating the tooth movement (22). Moreover, the increase of BMP-3 expression was displayed not only at the protein translation level, but also at the mRNA transcription level, suggesting that BMP-3 plays a positive role of inhibiting the osteogenic components in the alveolar bone and facilitating the tooth movement in the process of tooth orthodontic treatment. As a result, in the process of orthodontic tooth movement, the expression level of BMP-3 is gradually increased with time and reaches the peak at day 14, promoting the increase of osteoclasts and benefiting the tooth movement.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YG drafted the manuscript. YG and MZ were mainly devoted to the establishment of orthodontic tooth movement model. XT and MW helped with immunohistochemistry and H&E staining. YG and FZ were responsible for qPCR and western blotting. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Qingdao Women and Children's Hospital (Qingdao, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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