Effects of piezosurgery in accelerating the movement of orthodontic alveolar bone tooth of rats and the expression mechanism of BMP-2

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Abstract. The aim of the study was to investigate the effects of piezosurgery in accelerating the movement of orthodontic alveolar bone tooth of rats and the expression mechanism of bone morphogenetic protein-2 (BMP-2). Adult male Wistar rats (n=30), with an age range of 14-15 weeks, and an average weight of 250±16 g were used. The animals were randomly divided into the control and observation groups. The rats in the control group were injected with 25-dihydroxyvitamin (1,25-dihydroxycholecalciferol) into their dental ligament. The rats in the observation group were placed with an orthodontic device between the first molar and central incisor in the maxillary. On the first day after animal treatment, piezosurgery stimulation was performed on the first molar in maxillary. The changes of the movement distance of the first molar and gum surface temperature on days 1, 3, 5, 7 and 14 were then compared. Immunohistochemical staining was performed to detect the expression of BMP-2 of periodontal tissue in the tension side of the first molar. Tooth movement distance in the observation group on days 5, 7 and 14 was significantly longer than that in the control group ($p<0.05$). The gum surface temperature of the observation group was elevated to some extent, peaking after 20 min. BMP-2 mRNA and protein levels in the observation group were significantly higher than those of the control group at days 3, 5, 7 and 14 ($p<0.05$). In conclusion, piezosurgery may significantly accelerate the movement of orthodontic alveolar bone tooth of rats and be associated with an increasing BMP-2 expression.

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Introduction

Orthodontic treatment on adults usually lasts 2-3 years (1-3). However, patients under exodontia require a longer period of time (4). The longer the treatment course is, the greater the risks of gingivitis, enamel demineralization, decayed tooth, and even root resorption (5-6). At present, the therapies that expedite the movement of orthodontic tooth mainly include drugs, physiotherapy and surgical operations.

As piezosurgery becomes widely applied in tumor resection, fracture healing promotion, bone non-union treatment, maturity and remodeling acceleration after distraction osteogenesis, it is gradually introduced into orthodontic alveolar bone remodeling to promote the movement effect of tooth (7). Bone morphogenetic protein-2 (BMP-2) is a multifunctional growth factor (8). As a type of signal protein, BMP-2 promotes the proliferation, differentiation and apoptosis of many cells, and is involved in the regeneration and repair of tissues (8). Specifically, this protein plays a significant role in the remodeling of bone tissues (8).

The aim of the present study was to establish an animal model to investigate the effects of piezosurgery in accelerating the movement of orthodontic alveolar bone tooth of rats and the expression mechanism of BMP-2.

Materials and methods

Animals. Thirty healthy adult male Wistar rats provided by the Experimental Animal Center of Wuhan University were included in the present study. The age range of the rats was 14-15 weeks, with an average weight of 250±16 g. The rats were kept in cages at a temperature of 22.5±0.5˚C, with a 12-h light/dark cycle, were fed a normal diet and had access to water. The present study was approved by the ethics committee of Wuhan University.

Reagents. The reagents used in the study included, isoflurane (SurgiVet, Inc., Waukesha, WI, USA), superfine diamond bar (Dentsply Maillefer, Tulsa, OK, USA), ligature wire (Changsha Tianmei Co., Ltd., Changsha, China), NiTi coil spring (3M Unitek, St. Paul, MN, USA), orthodontic stress and tension gauge (Hangzhou Tianmei Co., Ltd., Xiaoshan, China), ultrasonic

coupling agent (Aquasonic; Parker Laboratories, Inc., Fairfield, NJ, USA), silicone impression material (Heraeus, Hanau, German), goat serum (Invitrogen Life-Technologies, Carlsbad, CA, USA), BMP-2 antibody (Abcam, Cambridge, MA, USA), horseradish peroxidase-labeled sheep anti-rabbit IgG (Sigma, St. Louis, MO, USA), methanol (Merck, Darmstadt, Germany), streptavidin-biotin complex (SABC) immunohistochemistry kit (Dako, Glostrup, Denmark), 3,3'-diaminobenzidine (DAB) colour-producing reagent kit (Boster Inc., Wuhan, China), hematoxylin (Biosharp, Hefei, China), BCA protein quantitative detection kit (Boster Inc.), polyvinylidene fluoride membrane (Millipore Corp., Billerica, MA, USA), enhanced chemiluminescence (ECL) kit (Pierce Biotechnology, Inc., Rockford, IL, USA), β-actin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), primer (R&F; Sangon Biotech Co., Ltd., Shanghai, China), total RNA extraction kit (Omega Bio-Tek, Inc., Norcross, GA, USA), quantitative polymerase chain reacyion (qPCR) kit (Takara Bio, Inc., Otsu, Japan), reverse transcription kit (Takara Bio, Inc.), and TRIzol reagent (Invitrogen Life-Technologies, Carlsbad, CA, USA).

Instruments. The instruments used in the study were Piezosurgery (Exploiter TM UOSS-II; Beijing Boda Hi-Tech Co., Ltd., Beijing, China), thermometer (Shanghai Medical University Instrument Factory, Shanghai, China), turbine mixer (Shanghai Medical University Instrument Factory), water bath (Shanghai Pudong Physical Optical Instrument Factory, Shanghai, China), refrigerator (Haier Co., Ltd., Qingdao, China), inverted microscope (Olympus Corp., Tokyo, Japan), and qPCR instrument (Applied Biosystems Life Technologies, Foster City, CA, USA).

Establishment of animal model. After anesthesia by xylazine (5 mg/kg, i.m) and ketamine (100 mg/kg, i.m), the superfine diamond bar of the turbine motor was used to form a small groove of 0.15-0.25 mm in the cervical margin parallel to gingival margin on the flip side of the two central incisors in the rat's maxillary, and in the centrifugal axial surface corner of the neck of the first molar parallel to the gingival margin to fix the ligature wire. Then, 0.25 mm orthodontic stainless steel ligation wire was used to bind the NiTi coil spring with a diameter of 0.030" between the first molar and the central incisor on the left side and right side, respectively. The force value was set to 0.1 N, and the first molars were dragged on the two sides to move towards the mesial position, and thus the direction of coil spring stress segmentation was guaranteed to make the first molar move towards the mesial position horizontally. Any damages or detachment was closely monitored and repaired. If the retention was unfavorable or the stress value was changed due to the growth of the rats' central incisors, a new retention groove was made on the centrifugal cervical margin of the central incisors, and the retention equipment was relocated.

Experimental grouping. The animals were randomly divided them into 5 groups, with control and observation groups. Rats in the control group were injected with 25-dihydroxyvitamin [1,25-dihydroxycholecalciferol (DHCC)] into their dental ligament. Rats in the observation group were placed with an orthodontic device between the first molar and central incisor in the maxillary. On the first day after the model was successfully established, piezosurgery stimulation was performed on the first molar in maxillary. The animals were anesthetized with 3% isoflurane, and the first molar regions were defeathered in bilateral maxillary when the activity of rats was significantly decreased. Low-intensity pulse piezosurgery stimulation was carried out on the first molar region in the maxillary of the experimental side. The stimulation intensity was 30 mW/cm^2, frequency was 1.5 MHz, pulse width was 200 μsec, repeat frequency was 1 kHz, once a day, each time for 20 min.

Observation index and detection methods. Changes of the movement distance of the first molar and gum surface temperature were compared on days 1, 3, 5, 7 and 14. Immunohistochemical staining was used to detect the expression of BMP-2 of periodontal tissue in the tension side of the first molar. RT-PCR and western blot analysis was performed to detect the BMP-2 mRNA and protein expression. The distance between the central fossas of the first molar and the second molar in the maxillary was measured using a vernier caliper, and a temperature laser infrared thermometer was used to measure temperature changes on the gum surface.

Tissue preparation. The animals were sacrificed using 5% isoflurane followed by cervical dislocation. The thoracic cavity was opened to expose the heart and the aorta was separated. A thread was used to prepare for the ligation of the perfusion needle. A small mouth on the left ventricle was opened to insert the perfusion needle into the left ventricle until it was inside the ascending aorta. The perfusion needle was fixed using silk thread, then perfused with normal saline, at a flow rate of ~20 ml/min. The auricula dextra was then opened to allow the blood to flow out, followed by suspended perfusion until the liver gradually turned white, as the blood color of the excurrent liquid became thickened and then clear.

The procedure used was as follows: Internal fixation (perfusion of 4% paraformaldehyde for approximately 2 h), external fixation (the tissue blocks containing the first molar and the surrounding alveolar bone were extracted. These blocks were placed in 4% paraformaldehyde fixation liquid at 4˚C), decalcification (fixed for 48 h, decalcified with 5% EDTA at 4˚C for 10 days), dehydration (dehydration of tissues using 75% alcohol, 85% alcohol, 95% alcohol, 95% alcohol, 100% alcohol, and 100% alcohol, for 12 h each), transparentizing (immersion of the tissue blocks into dimethylbenzene until the tissue blocks became transparent), waxdip (at 60˚C, the transparent tissue blocks were dipped into the transparent agent and paraffin was melted for 20 min, after which the blocks were dipped into the melted pure paraffin wax for 1 h), embedding (the wax was melted to liquid, poured into the embedding frame, slightly heated tweezers were used to clip the tissue blocks into the embedding frame, the surface was placed in such a position as to be cut downward, the embedding frame was then moved until the paraffin wax was completely coagulated), and section (serial section was produced alongside the long axis of the molar from near, far to middle, with each section at 5 μm).

Immunohistochemical staining. Paraffin sections (3 μm) mounted on poly-L-lysine-coated slides were deparaffinized and rehydrated. Using 0.6% H_2O_2 in methanol, peroxidase activity was removed. Then, the sections were washed in tap

Table I. Primers for quantitative polymerase chain reaction.

Gene	Sequence
BMP-2	5'-CTACATGCTAGACCTGTATCGC-3' 5'-CCCACTCGTTTCTGGTAGTTC-3'
β-actin (human)	5'-CACCACACCTTCTACAATGAG-3' 5'-GCATACCCCTCGTAGATGGGC-3'

BMP-2, bone morphogenetic protein-2.

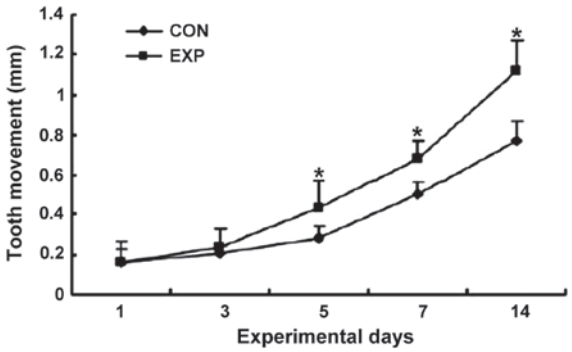

Figure 1. Comparison of tooth movement distance between the two groups.

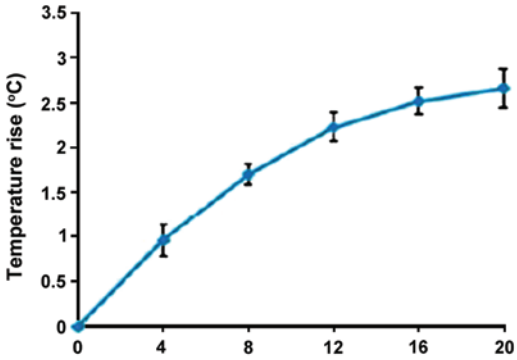

Figure 2. Gum surface temperature of the observation group.

water and in PBS (pH 7.4) for 10 min, treated with normal 1.5% goat serum for 20 min at room temperature and incubated with the first antibody: BMP-2 (diluted 1:250) overnight at 4˚C. Subsequently, the sections were washed in PBS and incubated with the secondary antibody (sheep anti rabbit IgG) for 1 h at room temperature. Peroxidatic activity was detected with a DAB colour-producing reagent kit. The sections were then counterstained with hematoxylin.

RNA extraction. For RNA extraction, 2 mm alveolar bone was collected and frozen in liquid nitrogen at -80˚C. Tissue (100 mg) was immersed in 1 ml TRIzol and placed at room temperature for 2-5 min. Subsequently, 200 µl chloroform was added to each tube and placed at room temperature for 5 min. The contents were centrifuged at 10,000 x g for 15 min at 4˚C, the supernatant was collected in the tube, and pre-cooled isopropanol of the same volume was added to the tube. The solution was placed at room temperature for 10 min and the contents were centrifuged at 10,000 x g for 10 min. Then, 1 ml of 75% alcohol was used to wash the precipitate. RNA extracted in the form of pellets was dried at room temperature for 5 min until it became transparent. This was followed by the addition of 20 µl RNase-free water to dissolve the pellet. The RNA concentration was quantified using UV spectrophotometry (Thermo Fisher Scientific Inc., Orlando, FL, USA).

Preparation of cDNA. In detail, 1 µl Oligo dT and 2.0 µg total RNA were mixed in a PCR tube, and DEPC-treated water was added until 9 µl was reached. The contents were centrifuged after blending, washed in 70˚C warm water for 5 min and then washed in ice. The reaction mixture was prepared on ice with 4 µl 5X RT buffer, 2 µl 10 mM dNTPs, 0.5 µl RNase inhibitor, 1 µl M-MLV-RTase, 3.5 µl DEPC H_2O, vortexed uniformly, and centrifuged. The reaction mixture was reacted at 42˚C for 1 h, followed by 70˚C for 10 min. The cDNA was then preserved at -80˚C for subsequent use.

qPCR. For qPCR, 1.0 µl cDNA, 10 µl SYBR Premix Ex Taq II (2X), 0.5 µl upstream primer (5 µM), 0.5 µl downstream primer (5 µM), and 8.0 µl ddH_2O were mixed on ice, vortexed and centrifuged. Table I shows the primers used. PCR conditions included pre-denaturation at 95˚C for 5 sec, annealing and extension at 60˚C for 30 sec for a total of 45 cycles, denaturation at 95˚C for 1 min, then cooled down to 55˚C. Starting from 55˚C, the temperature was raised by 0.5˚C in each stage until 95˚C for 30 sec.

Western blotting. Western blot analysis included protein extraction, electrophoresis, membrane transfer, and immune coloration. Total protein (30 µg) was loaded to SDS-PAGE gel and then transferred to the nitrocellulose membrane. The membrane was incubated at room temperature with TBST containing 50 g/l milk. The membrane was incubated with primary rabbit polyclonal BMP-2 antibody (Abcam, Cambridge, MA, USA; catalog no.: ab14933) overnight at 4˚C. The membrane was washed repeatedly with TBST and incubated with corresponding secondary antibody (1:5,000). The antibody was exposed with ECL solution and the gray value was calculated. The assay was performed in triplicate.

Statistical analysis. The SPSS 20.0 software (IBM SPSS Armonk, NY, USA), was used for statistical analysis. The measurement data were presented as means ± standard deviation. An independent sample t-test was used to compare between groups. Countable data were presented as case or percentage, and the χ^2 test was applied for intra-group comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Tooth movement distance. Tooth movement distance in the observation group on days 5, 7 and 14 was significantly longer than that in the control group ($p<0.05$) (Fig. 1).

Gum surface temperature of observation group. The gum surface temperature of the observation group was elevated to some extent, and peaked after 20 min (Fig. 2).

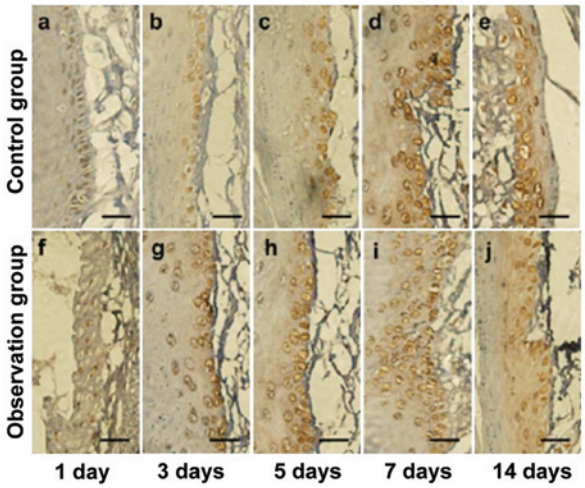

Figure 3. Immunohistochemical staining of bone morphogenetic protein-2 for the control and experimental groups at different time intervals from day 1 to 14.

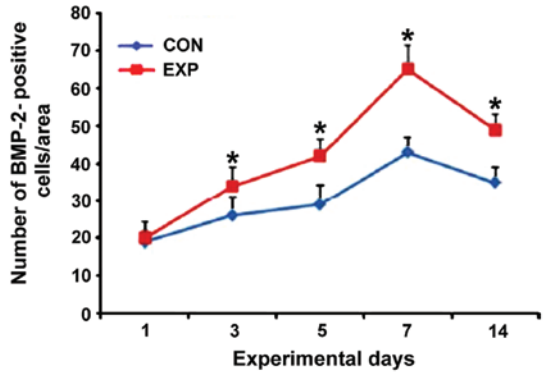

Figure 4. Comparison of the number of bone morphogenetic protein-2 (BMP-2)-positive cells for the control and experimental groups at different time intervals from day 1 to 14.

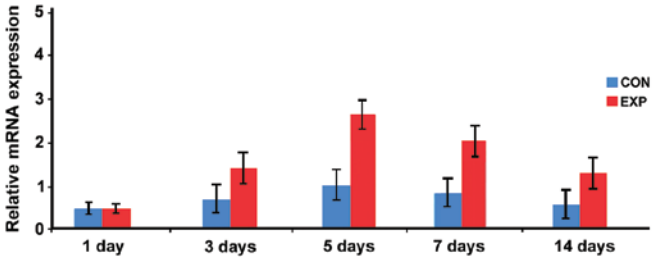

Figure 5. Expression of bone morphogenetic protein-2 mRNA for the control and experimental groups at different time intervals from day 1 to 14.

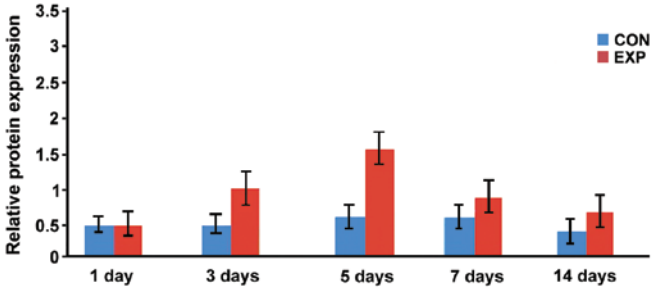

Figure 6. Expression level of bone morphogenetic protein-2 protein for the control and experimental groups at different time intervals from day 1 to 14.

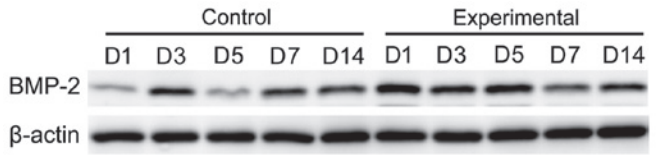

Figure 7. Expression level of bone morphogenetic protein-2 protein for control and experimental groups at different time intervals from day 1 (lane 1), day 3 (lane 2), day 5 (lane 3), day 7 (lane 4), day 14 (lane 5).

BMP-2 immunohistochemical staining. The number of positive cells in the observation group was significantly higher than that in the control group ats day 3, 5, 7 and 14, and the difference was statistically significant ($p<0.05$) (Figs. 3 and 4).

Expression of BMP-2 mRNA. The expression level of BMP-2 mRNA in the observation group was significantly higher than that in the control group at days 3, 5, 7 and 14, and the difference was statistically significant ($p<0.05$; Fig. 5).

Expression level of BMP-2 protein. The expression levels of BMP-2 protein in the observation group at days 3, 5, 7 and 14 were significantly higher than that in the control group, and the difference was statistically significant ($p<0.05$; Figs. 6 and 7).

Discussion

Tooth movement is closely associated with the application of orthodontic force. Orthodontic force is the key factor of alveolar bone remodeling (10). The periodontal ligament is a thin layer of dense connective tissue between the alveolar bone and cementum with the capacity for self-renewal and self-restoration. Orthodontic force is important in keeping the dynamic balance of periodontal tissues (11). In the process of orthodontic treatment on tooth movement, a tension zone and pressure zone may appear in the parodontium area of the tooth under pressure. The periodontal ligament in the tension area may be in tensional state, the expression of genes related

to bone formation, such as osteocalcin and bone sialoprotein, were upregulated, and thus promoted the formation of new bones in the tension area. The periodontal ligament in the tension area has a compression force, which may activate the osteoclast, promote the absorption of alveolar bone in the tension area, and eventually promote the balance between bone formation and bone absorption of alveolar bone surrounding the stressed tooth (12).

1,25-DHCC is the most active metabolite of vitamin D, which can promote bone deposition and inhibit the release of parathyroid hormone. A physiological dose of 1,25-DHCC would not stimulate bone resorption while a low dose of 1,25-DHCC promotes the differentiation of osteoclasts by upregulating the expression of receptor activator of nuclear factor-κB ligand (13). Collins *et al* injected 1,25-DHCC into the dental ligament of cats' cuspid teeth and found that 3 weeks later, the movement speed of the cuspid teeth of the observation group was 60% faster than that of the control group (14). The main effects of the ultrasonic cavitation effect on the human body mainly included cavitation, mechanical, thermal, thixotropic, dispersion, fragmentation and hemostatic effect. As a type of high frequency soundwave, ultrasonic was able to transmit the wave into the organs via the form of mechanical energy. Ultrasonic has been widely applied in the medical field. Low intensity-pulsed ultrasound treatment is the best known parameter for promoting fracture healing. A large number of animal experiments and clinical studies have demonstrated that low intensity-pulsed ultrasound can reduce the healing time of fracture, and effectively treat the delay fracture healing and bone non-union. Compared with other treatments, it is safer and more invasive (15).

In the process of proliferation and differentiation, osteoblasts may undergo four stages: cell proliferation, matrix secretion, matrix maturation and mineralization formation. This process is regulated by a series of cytokines, signaling molecules, and the surrounding environment, such as BMPs, transforming growth factor-β. BMPs play an important role in the metabolism of bone. BMP-2 is important in controlling the proliferation, differentiation and bone matrix secretion of osteoblasts by stimulating specific transcription procedures in the period of embryonic skeletal development and bone remodeling after birth (16,17).

In the present study, we found that tooth movement distance in the observation group at days 5, 7 and 14 was significantly longer than that in the control group. The number of positive cells in the observation group was significantly more than that in the control group at days 3, 5, 7 and 14. The levels of BMP-2 mRNA and protein expression in the observation group were significantly higher than those in the control group at days 3, 5, 7 and 14. The data show that piezosurgery may significantly accelerate the movement of rat orthodontic alveolar bone tooth. It may be associated with an increasing BMP-2 expression.

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