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Research Article

Bone Marrow Mesenchymal Stem Cells Facilitate Alveolar Bone Remodeling in Periodontitis Rats During Orthodontic Tooth Movement Through STAT3/β-Catenin

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Abstract

We aimed to explore the performance of bone marrow mesenchymal stem cells (BMSCs) in modulating signal transducer and activator of transcription 3 (STAT3)/β-catenin to facilitate alveolar bone remodeling in periodontitis rats in the process of orthodontic tooth movement. Flow cytometry was employed to identify BMSCs isolated, and then their osteogenic capacity was examined by alizarin red staining assay. BMSCs group (n=10), periodontitis + orthodontic tooth movement (PO+OTM) group (n=10), BMSCs+Static group (n=10) and negative control (NC) group (n=10) were set up for random allocation of 40 rats. The PO+OTM group had significantly decreased bone mineral density (BMD), trabecular number (Tb.N), bone volume/total volume (BV/TV), trabecular thickness (Tb.Th), phosphorylated STAT3 (p-STAT3) ratio in alveolar bone tissues, but increased receptor activator of nuclear factor-κB ligand protein expression, trabecular space (Tb.Sp), cement-enamel junction to alveolar bone crest, and osteoclast count in comparison to those of the NC group ($P<0.05$). In contrast with the PO+OTM group, the BMSCs group had significantly increased BMD, Tb.N, BV/TV, Tb.Th, p-STAT3/STAT3 ratio, and protein expressions of β-catenin and osteoprotegerin, but decreased Tb.Sp ($P<0.05$). For periodontitis rats, BMSCs can promote osteogenic differentiation to facilitate alveolar bone remodeling in the process of orthodontic tooth movement.

Keywords: Alveolar bone loss, Bone marrow, Mesenchymal stem cells, Periodontitis

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Introduction

As a most frequent-occurring oral disease in clinical practice, periodontitis exhibits a prevalence rate of about 11% according to global data [1]. Periodontitis leads to injuries of periodontal tissues, usually accompanied by pathological tooth migration, which injures occlusion to speed up periodontal deterioration plus the loss of alveolar bone. Finally, secondary malocclusion is developed, severely affecting the oral health and facial aesthetics of people, which often requires orthodontic treatment [2,3]. Orthodontic treatment can reduce the mobility degree of teeth with periodontal disease, during which, however, there is a risk of alveolar bone loss. Hence, discovering methods able to effectively avoid the loss of alveolar bone in the period of orthodontic therapy for periodontitis is a hot research topic. Belonging to mesenchymal cells extracted from bone marrow, bone marrow mesenchymal stem cells (BMSCs) are potentially capable of not only differentiating into osteoblasts but also promoting bone tissue generation [4,5]. BMSCs are characterized by rich sources, convenient acquisition, multiple differentiation abilities, and anti-inflammatory and immunomodulatory properties, so they have been widely applied in clinical practice. BMSCs are of great significance for the repair of bone defects [6]. Signal transducer and activator of transcription 3 (STAT3) is a vital player in bone metabolism. After knockout of STAT3 in osteoblasts, the bone morphology of mice becomes obviously abnormal, and STAT3 can also mediate the osteogenic effect of osteoblasts on mechanical stress loading [7]. It is therefore speculated that STAT3 may be involved in regulating orthodontic tooth movement and bone remodeling. For periodontitis patients, β-catenin, a core regulatory factor

in the downstream of the typical Wnt pathway, has been proven to have a high expression in gingival tissues and a relation to periodontal destruction [8]. However, whether the STAT3/β-catenin pathway in periodontitis rats can act as a mechanism promoting alveolar bone remodeling throughout orthodontic tooth movement has not been reported yet.

In this study, therefore, whether BMSCs can promote alveolar bone remodeling in the course of orthodontic tooth movement by modulating the STAT3/β-catenin pathway in periodontitis rats was explored.

Material and Methods

Ethical Approval

This study has been approved by the ethics committee of Tai'an 88 Hospital on November 15th, 2022 (approval No. 2022-273).

Laboratory Animals

Liaoning Changsheng Biotechnology Co., Ltd. provided 40 SPF-grade male Sprague-Dawley (SD) rats [license No.: SCXK (Liaoning) 2020-0001], with an age of 6-8 weeks old plus a weight of 220-240 g. Besides, 31-week-old female SD mice weighing 18-20 g were bought. The rats and mice were kept in a unified animal house under 45-55% indoor relative humidity and 22-26°C room temperature, with a 12 h/12 h light/dark cycle, which were permitted to eat food and drink water freely during 1 week of adaptive feeding.

Reagents and Apparatus

Static, a STAT3 small molecule inhibitor, was bought from Selleck (USA). Wuhan Boster Biological Technology Co., Ltd. (China) supplied the antibodies against receptor activator of nuclear factor-κB ligand (RANKL), STAT3, phosphorylated STAT3 (p-STAT3), osteoprotegerin (OPG), β-catenin, and alkaline phosphatase (ALP), and rabbit anti-rat cluster of differentiation 29 (CD29), CD34, CD44 and CD45 antibodies were provided by Shanghai Qiming Biotechnology Co., Ltd. (China). A kit for bicinchoninic acid (BCA) was offered by Beijing Solarbio Science & Technology Co., Ltd. (China). The instruments used in this study included a dynamometer (Hangzhou Aosu Dental, China), a desktop micro-computed tomography (CT) scanner (Bruker, Siemens, Germany), and an optical microscope (Guangzhou Micro Domain Optical Instrument Co. Ltd., China).

Isolation and Identification of BMSCs

Bilateral femurs of the 3 1-week-old mice were taken out, from which the metaphyseal red bone marrow was aspirated, followed by culture in IMEM (containing 20 μg/mL heparin). Next, bone marrow cells were resuspended and subjected to 10 min of centrifugation at 1500 rpm. Afterwards, following the absorption and discarding of the supernatant, Dulbecco's modified Eagle medium was applied to dilute bone marrow cells to 10 mL, which were sieved using a 90-mesh filter screen. Thereafter, bone marrow cells were cultured in a 37°C incubator containing 5% CO_2 for 24 h. With the 80-90% confluence achieved, trypsin (0.25%) was added for cell digestion, and then the cells were passaged (1:2), followed by observation of morphology of the third- to fifth-generation cells under the microscope. The third-generation BMSCs were harvested for 5 min of 1000 rpm centrifugation. Next, monoclonal antibodies against CD29, CD90, CD45 and CD34 were added to each tube in turn and incubated in a dark ice box for 45 min. After that, 1% BSA-containing PBS was used for cell resuspending, and CD45, CD29, CD34, and CD90 as the cell surface markers were detected by a flow cytometer.

Alizarin Red Staining

BMSCs passaged to the third generation were harvested, inoculated in each well of a 6-well plate after adjusting the density to 2×10^5/cells and subjected to 24 h of incubation. Next, osteogenesis induction medium (10 mm/L β-glycerophosphate, 10 mL of fetal bovine serum, 50 μm/L antithrombotic, 89 mL of Dulbecco's modified Eagle medium/F12, 1 mL of double antibody, and 100 nm/L dexamethasone) was utilized to substitute the conventional culture medium, followed by 21 d of continuous culture, during which the medium was replaced once every 3 d. Afterwards, the original culture medium was discarded, and then paraformaldehyde solution (4%) was added for 30-min fixation of washed cells. Thereafter, alizarin red staining was performed on the cells for 3 min, followed by cell washing. Finally, cells were observed under the microscope for their osteogenic capacity.

Grouping and Modeling

A random number table was employed to set up groups for totally 40 rats, namely model group (n=30) plus negative control (NC) group (n=10). Then models of periodontitis were established using all rats from model group according to the following steps. Firstly, 10% chloral hydrate was injected intraperitoneally into rats for anesthesia. Then the first molar on the left maxilla of rats was peeled off with a probe, a 0.2 mm deep retention groove was ground in the gingival sulcus near the mesial tooth neck of the molar with a rapid hand grinding machine, and the first molar was ligated with a 0.2 mm ligature. Next, rats were fed with high sugar water (100 g/L) and softened feed for 1 month. Red and swollen gums, bleeding on probing and periodontal pocket formation suggested successful modeling of periodontitis [9].

All the 30 rats were successfully prepared into models

of periodontitis, and the model of orthodontic tooth movement was prepared. In brief, after anesthetizing rats, a retention groove with a depth of 0.2 mm was ground on the distal surfaces of the necks of the two maxillary central incisors. Then a tension spring was utilized, with one end connected to the molar of rats and the other end connected to the incisors of rats. The force of tension spring was adjusted to 50 g by the dynamometer, and the force applying device was removed after force application for 21 d. Next, the first molar and two incisors of rats were ligated with ligature wires to keep the first molar at the position after movement. One day before the removal of the force applying device, the 30 rats were then evenly and randomly divided into periodontitis + orthodontic tooth movement (PO+OTM) group, BMSCs group and BMSCs+Static group. No treatment was conducted on rats in NC group.

Drug Intervention

One day before the removal of the force applying device, 15 μL of BMSC suspension with a cell density of 1×10^7 cells/mL was injected into the distal local gingival tissues and buccal and palatine mucosae of the left maxillary first molar in BMSCs group. For BMSCs+Static group, based on the treatment in BMSCs group, 10 μL of Static solution (prepared as follows: Dimethyl sulfoxide was added to dissolve the STAT3 small molecule inhibitor Static, whose final concentration of 50 μmol/L was reached through normal saline dilution) was injected into the buccal and palatine mucosae of the maxillary first molar once every two days for 14 consecutive days. In NC group and PO+OTM group, an equal volume of normal saline was injected at the same site. Following 14 d of drug intervention, all rats were anesthetized by the intraperitoneal injection of 3% pentobarbital sodium and sacrificed through cervical dislocation. Then alveolar bone tissues of the left maxillary first molar were separated, some of which were quickly frozen for 30 min by liquid nitrogen for -80°C preservation using a refrigerator. Finally, 4% paraformaldehyde solution was utilized for fixation of the remaining tissues, which were decalcified for 30 d.

Micro-CT

The maxillary alveolar bone tissues were collected, followed by removal of skin and muscle tissues. Next, the alveolar bone was scanned with the micro-CT scanner under the following conditions: tube voltage: 70 kV, current: 353 μA, and scanning thickness: 8 μm, followed by three-dimensional image reconstruction. Afterwards, the degrees of alveolar bone loss and tissue damage were evaluated. The three-dimensional images were used to measure the distance from cement-enamel junction to alveolar bone crest (CEJ-ABC), bone mineral density (BMD) of rats was calculated for all groups, and trabecular thickness (Tb.Th), trabecular space (Tb.Sp), bone volume/total volume (BV/TV), and trabecular number (Tb.N) were measured.

Hematoxylin-Eosin (HE) Staining

Decalcified alveolar bone tissues were collected for paraffin embedding and tissue section (thickness: 5 μm) preparation using a slicer. Then tissue sections were deparaffinized in xylene, dehydrated with ethanol gradient, and treated with HE staining. Afterwards, they underwent dehydration together with transparentization, followed by observation under the microscope. The degree of bone defect repair was scored according to the Lane-Sandhu histological scoring criteria [10], and a higher score indicated a higher repair degree.

Tartrate-Resistant acid Phosphatase (TRAP) Staining

After deparaffinization plus rehydration, the tissue sections were put in glycollate solution and incubated in the dark at 37°C for 1 h. Thereafter, tissue sections were stained in accordance with the instructions of TRAP/ALP staining kit. Afterwards, 5 sections were randomly selected, and 5 clear positive visual fields were selected at the root of each section to count the number of osteoclasts. The cytoplasm of positive osteoclasts was pink to carmine.

Measurement of Protein Expressions of STAT3, p-STAT3, β-catenin, RANKL and OPG in Alveolar Bone Tissues by Western Blotting

The alveolar bone tissues were taken, and the BCA assay was carried out to determine the concentration of total protein therein. The protein specimens with a final concentration of 2 μg/mL were boiled in hot water for 10 min, which were subsequently preserved by the -20°C refrigerator. Next, protein samples (50 μg) were collected, mixed with loading buffer, and denatured in boiling water. Thereafter, SDS-PAGE separation and PVDF membrane transfer were adopted for the protein specimens. Afterwards, 5% skim milk was supplemented to seal the membrane for 1 h, followed by membrane washing with Tris-buffered saline solution with Tween (1 mL/L). Afterwards, primary antibodies were added for overnight incubation (4°C) of the membrane, and then cleaning agent was used for rinsing. Thereafter, secondary antibodies (1:5000) were employed for room-temperature incubation of the membrane for 2 h. Then electrochemiluminescence liquid was added for development and exposure. Finally, Image Lab software was adopted to analyze the relative expression of each protein, with GAPDH as the internal reference.

Statistical Analysis

Experimental data were subjected to statistical analysis

through GraphPad Prism 8.0 software. Mean ± standard deviation (X±s) was used as the expressing format of all measurement data with normal distribution. The measurement data underwent statistical comparison among groups by one-way ANOVA and between groups through the LSD-t test. The difference of statistical significance was denoted with P<0.05.

Results

Microscopically, BMSCs passed to the third generation were mostly spindle-shaped and arranged radially *(Fig. 1-A)*. The results of flow cytometer exhibited a positive expression rate of 98.3%, 99.1%, 2.3%, and 3.6% for CD29, CD90, CD45, and CD34 antibodies, respectively, which are in line with the surface markers of mesenchymal stem cells, implying that the isolated cells were BMSCs *(Fig. 1-B-E)*.

According to alizarin red staining assay, BMSCs had more mineralized nodules and a significantly increased alizarin red stained area than Control cells (P<0.05), indicating that BMSCs possess osteogenic capacity *(Fig. 2)*.

Compared with those in NC group, Tb.Th, BMD, Tb.N, and BV/TV significantly declined while CEJ-ABC plus Tb.Sp significantly rose in PO+OTM group (P<0.05). In contrast with PO+OTM group, BMSCs group had significantly elevated BMD, Tb.Th, Tb.N, and BV/TV but significantly dropped CEJ-ABC and Tb.Sp (P<0.05). BMSCs+Static group presented significantly lower BMD, Tb.N, BV/TV, and Tb.Th, as well as significantly higher Tb.Sp and CEJ-ABC than BMSCs group (P<0.05) *(Fig. 3)*.

In NC group displayed neatly arranged periodontal membrane fibers together with smooth alveolar bone and root surfaces and no distinct osteoclasts. In PO+OTM group, periodontal membrane fibers were disorganized, and the alveolar bone was observed with scattered bone absorption lacunae on the pressure surface in addition to active osteoclasts on the noncontinuous surface, with a marked elevation in the number of osteoclasts compared with NC group (P<0.05). As for BMSCs group, periodontal ligament fibers exhibited gradually regularized arrangement, and by contrast to PO+OTM group, it had a significantly smaller number of osteoclasts (P<0.05). According to *Fig. 4*, BMSCs+Static group displayed similar changes to PO+OTM group, with obviously disordered or even broken periodontal ligament fibers and an obvious increase in the number of osteoclasts in comparison to BMSCs group (P<0.05).

PO+OTM group had lower p-STAT3/STAT3 ratio and protein expressions of β-catenin and OPG and a higher protein expression of RANKL in alveolar bone tissues

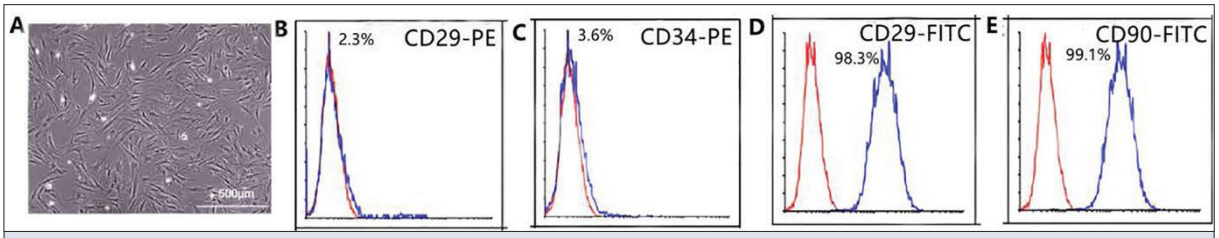

Fig 1. Morphology and identification of BMSCs. **A:** BMSCs typically spindle-shaped and arranged radially, **B-E:** Identification of cell surface markers by the flow cytometer

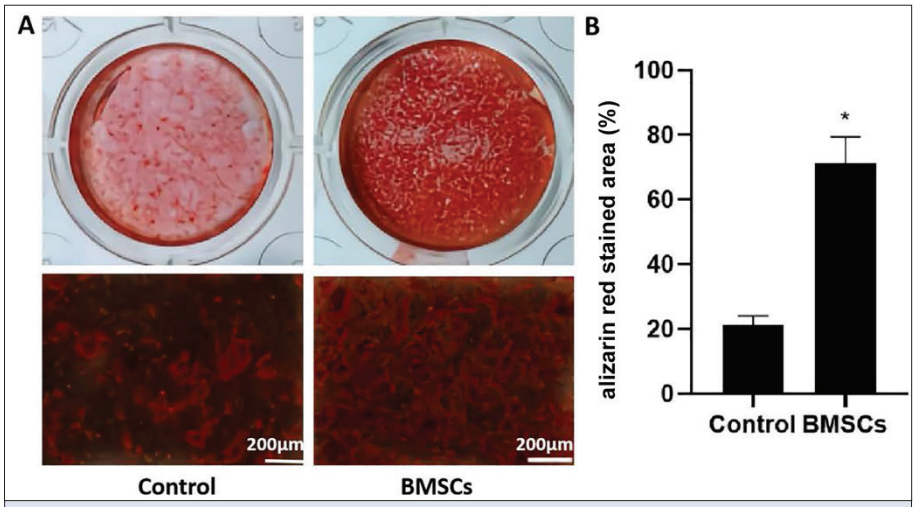

Fig 2. Identification of osteogenic capacity of BMSCs. **A:** Alizarin red staining assay, **B:** Comparison of alizarin red stained area. *P<0.05 *vs.* NC Group

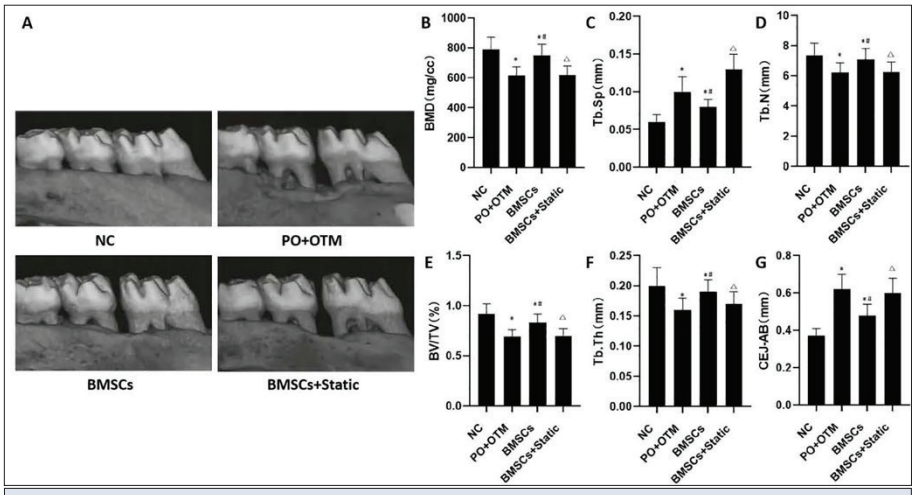

Fig 3. Alveolar bone remodeling of rats. **A:** f Alveolar bone under micro-CT scan, **B-G:** Comparisons of Tb.Th, BMD, BV/TV, Tb.Sp, Tb.N, and CEJ-ABC in alveolar bone. *P<0.05 *vs.* NC Group, #P<0.05 *vs.* PO+OTM Group, #P<0.05 *vs.* BMSCs Group

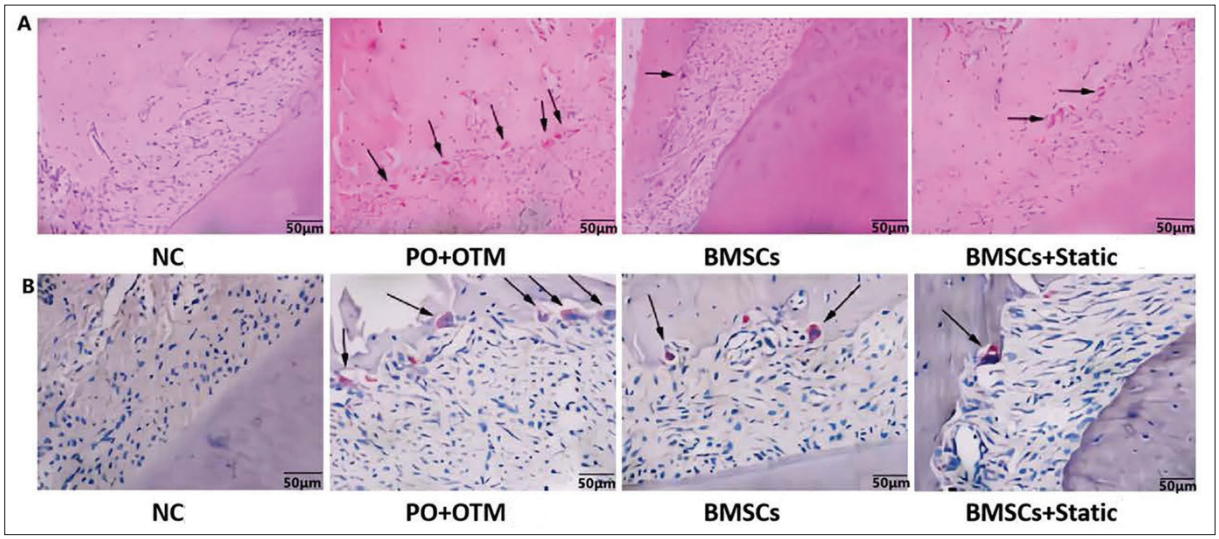

Fig 4. HE + TRAP staining assays on alveolar bone tissues. **A:** HE staining assay on alveolar bone tissues (×200), **B:** TRAP staining assay on alveolar bone tissues (×200). The black arrow represents osteoclasts

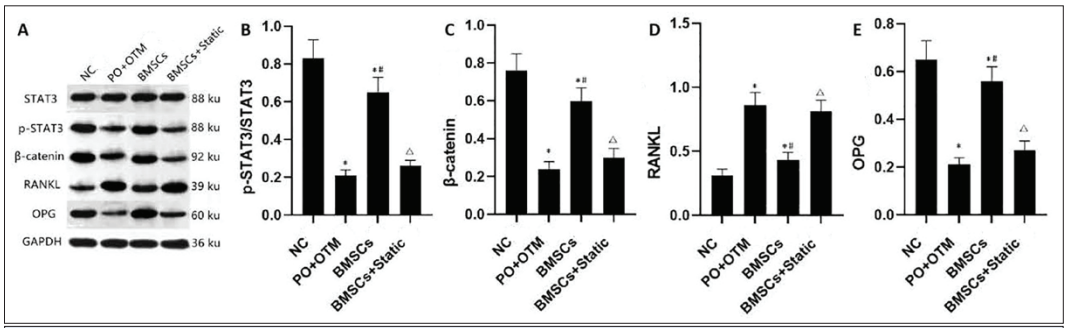

Fig 5. Expressions of STAT3, p-STAT3, β-catenin, RANKL and OPG proteins in alveolar bone tissues of rats. **A:** Protein bands of STAT3, p-STAT3, β-catenin, RANKL and OPG, **B:** Comparison of p-STAT3/STAT3 ratio in alveolar bone tissues, **C:** Comparison of protein expression of β-catenin from alveolar bone tissues, **D:** Comparison of expression of RANKL protein from alveolar bone tissues, **E:** Comparison of expression of OPG protein from alveolar bone tissues. *P<0.05 *vs.* NC Group, #P<0.05 *vs.* PO+OTM Group, △P<0.05 *vs.* BMSCs Group

than NC group (P<0.05). Compared to PO+OTM group, BMSCs group displayed elevated p-STAT3/STAT3 ratio and protein expressions of β-catenin and OPG and a decreased protein expression of RANKL in alveolar bone tissues (P<0.05). BMSCs+Static group possessed significantly lower p-STAT3/STAT3 ratio and protein expressions of β-catenin and OPG in alveolar bone tissues besides significantly higher protein expression of RANKL than BMSCs group (P<0.05) *(Fig. 5)*.

Discussion

As a chronic inflammation of periodontal tissues, periodontitis refers to gingivitis-induced inflammation spreading to the periodontal membrane, alveolar bone, cementum and so on, which can result in tooth loss in severe cases [11]. Orthodontic treatment can align dentition while bringing periodontitis under control, enabling the recovery of adjacent relationship and chewing function of teeth to normal levels [12]. However, alveolar bone loss or resorption occurs due to periodontitis-induced destruction of periodontal tissue, weakening tooth support and reducing the stability of tooth movement, which leads to the instability of teeth during orthodontic treatment to easily result in bad movement such as inclination and rotation.

Stem cell transplantation has become a new strategy for the treatment of bone defects due to the rapid development of tissue engineering [13,14]. BMSCs are considered ideal bone repair cells by tissue engineering due to such characteristics as easy isolation, convenient acquisition, abundant sources, stable biocompatibility, and osteogenic differentiation potential. BMSCs have become a hot spot in the repair of bone defects in tissue engineering in recent years since they can differentiate into many types of cells and have the potential of multi-directional differentiation. BMSCs are capable of differentiating into osteoblasts by proper induction [15], and can exert potential curative effects in the treatment of bone defects and soft tissue injuries by releasing various osteogenically active factors. Sun et al.[16] proved that BMSCs were beneficial for alveolar bone defects in rats in terms of regeneration and repair. Likewise, we herein found that BMSCs in periodontitis rats promoted alveolar bone remodeling throughout tooth movement in orthodontic treatment.

The regeneration and repair of alveolar bone defects are realized through a series of repeated tissue resorption and formation processes, during which osteoclasts are responsible for absorbing bone tissue, whereas osteoblasts are responsible for forming new bone tissue. The two kinds of cells are in a dynamic equilibrium state due to the mutual suppression between them [17]. Osteoclasts are a kind of highly differentiated cells, which are derived from hematopoietic stem cells and can be fused by peripheral blood mononuclear cells and osteoclast precursor cells. However, there are too many osteoclasts in the alveolar bone due to the inflammation caused by alveolar bone defects, and their function is abnormal, jointly giving rise to excessive bone resorption and thus weakening osteogenic function. As a result, the normal osteogenesis-osteoclast balance is destroyed, and thus the repair process of alveolar bone defects is hindered [18]. In this study, the micro-CT results shows that BMSCs significantly elevated BMD, Tb.Th, Tb.N, and BV/TV but significantly dropped CEJ-ABC and Tb.Sp. Moreover, BMSCs promoted OPG to express by impeding the production of RANKL, thus decreasing the osteoclast count in alveolar bone tissues while facilitating alveolar bone remodeling.

The Wnt/β-catenin pathway is able to mediate bone formation, i.e. bone formation can be triggered by activating the Wnt/β-catenin pathway, while it is hindered by repressing this pathway. The Wnt/β-catenin pathway can modulate osteogenesis plus osteoclast differentiation by keeping OPG/RANKL balance [19]. Lan et al.[20] reported that activating the Wnt/β-catenin pathway facilitated periodontal ligament stem cells to differentiate into osteoblasts. Besides, STAT3 may influence the Wnt/β-catenin pathway to modulate bone formation [21]. In this research, STAT3 attenuated the Wnt/β-catenin pathway from activation, inhibition of osteoclasts and promotion of alveolar bone remodeling by BMSCs in PO+OTM rats.

In conclusion, for periodontitis rats, BMSCs can promote osteogenic differentiation to facilitate alveolar bone remodeling in the process of orthodontic tooth movement, of which the action mechanism may be related to activating the STAT3/β-catenin signal to suppress osteoclast differentiation.

Declarations

Availability of Data and Materials: The datasets used and/or analyzed during the current study are available from the corresponding author (X. Tian) on reasonable request.

Ethical Approval: This study has been approved by the ethic committee of our hospital (approval No. 2022-273), and great efforts have been made to minimize the animals' suffering.

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Conflict of Interest: There is no conflict of interest.

Authors' Contributions: H. Wang and F. Gong designed the study and drafted the paper; Z. Wu, D. Lai, and K. Wu performed and analyzed the experiments; X. Tian designed the study and significantly revised the paper. All authors have approved the submission and publication of this paper.

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