

Original Article

Effects of psoralen, a traditional Chinese medicine, on relapse and bone remodeling after orthodontic tooth movement in rats

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Abstract: This study was to investigate effects of psoralen on relapse after orthodontic tooth movement (OTM) and to seek the possible molecular mechanism. Thirty-six male Wistar rats (8-week old) were divided into two groups: the psoralen group and the control group. Nickel-titanium closed-coil springs were installed between the central incisor and the left maxillary first molars in all rats. After 21 days, the force appliances were removed, and rats in the psoralen group received psoralen 8 mg/kg per day, and control group got 1 ml/d 0.9% sodium chloride for 28 days. Maxillary casts were made every week and scanned by 3D Scanner to measure relapse distance. Relapse distances significantly decreased in psoralen group, 89.46±4.34% in control group and 62.22±1.60% in psoralen group in day 28 ($P<0.05$). Molars relapsed fastest in the first week, 0.07±0.01 mmd⁻¹ in psoralen group, 0.12±0.01 mmd⁻¹ in control group. Hematoxylin-eosin staining results implied that periodontal ligament distributed more regularly and the surface of alveolar bone are much smoother in psoralen group. Immunohistochemistry results showed that G protein coupled receptor 30 (GPR30) locates in periodontal tissue. GPR30, Bone Morphogenetic Protein 2 (BMP2) and Bone Morphogenetic Protein 4 (BMP4) expressed significantly high in psoralen group ($P<0.05$). These results suggest that psoralen is a bone-modify agent to prevent relapse after OTM, probably through activating GPR30 and up-regulating BMP2 and BMP4 expressions.

Keywords: Psoralen, bone remodeling, orthodontic relapse, BMP2, BMP4, GPR30

Introduction

Retention is the last phase of orthodontic treatment and it serves to maintain teeth in their new position after OTM. Acknowledged, teeth have a tendency to relapse back to their original position following OTM, which is a major concern in orthodontics. In rats, the mean relapse one day after removal of orthodontic appliances ranged from 62.5 percent to 73.3 percent, with the rate of relapse decreasing gradually over time [1-3]. In humans, Edman *et al.* [4] stated that the major part of relapse took place during the initial year of retention, whilst Kuijpers *et al.* [5] reported that almost 50 percent of the relapse occurred within the primary two years of retention. Above all, post-orthodontic relapse is a significant problem to deal with.

Recently, many researchers have used pharmacologic agents to prevent relapse after active orthodontic treatment. Kim *et al.* [6] clarified that systemic administration of bisphosphonate in rats decreased the extent of relapse of moved molars via a mechanism involving impairment of the structure and degradation functions of osteoclasts. Kanzaki *et al.* [7] reported that the osteoprotegerin (OPG) gene transfer to periodontal tissue inhibited the receptor activator of nuclear factor κ B ligand (RANKL)-mediated osteoclastogenesis and significantly inhibited experimental tooth movement. Kawakami *et al.* [8] demonstrated that 1, 25-dihydroxyvitamin D₃ enhanced bone formation for tooth stabilization after OTM. Collectively, these results suggest that any biologic tool, that regulates the catabolic or anabolic activity of the alveolar bone, possibly

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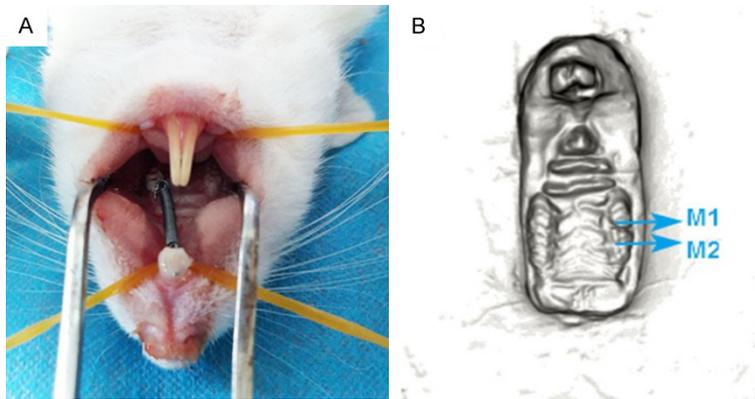


Figure 1. Orthodontic relapse model. A: Orthodontic tooth movement by inserting nickel-titanium coil embedded with enamel cement. B: Maxillary casts were made per week after appliance release, scanned and measured by 3D Scanner. (M1: distal groove of first molar, M2: distal groove of second molar).

yields a new procedure for decreasing time of retention after orthodontic treatment.

Psoralea corylifolia fruit (Buguzhi) is a well-known Chinese traditional medicine for kidney-tonifying and bone-nourishing applications. This herbal drug commonly used in formulas prescribed for the treatment of fractures, joint disorders, lower-back pain and knee weakness. Psoralen (molecular formula: $C_3H_6O_3$, molecular weight: 186.16) is the main active ingredient isolated from the seeds of *P. corylifolia* L. Psoralen, which has been documented to process a number of therapeutic properties, like anti-psoriasis [9], antioxidant [10], anticancer [11], antidepressant [12], antibacterial [13], anti-osteoporosis [14, 15] effects. Previous researches have revealed that psoralen facilitates bone formation and diminishes bone resorption [14-17]. So far, no study concerning the effect of psoralen on OTM and relapse has been reported. The purposes of this study were to investigate the role of psoralen in bone remodeling for tooth stabilization after OTM.

Material and method

Animals and groups

Thirty-six male Wistar rats (8 weeks old, mean weight 170 ± 10 g) obtained from the experimental animal center at Shandong University, Jinan, People's Republic of China were randomly divided into two groups (2×18 each): the control group (force appliance plus normal saline),

and the psoralen group (force appliance plus psoralen). All rats were divided into separate plastic cages under the temperature of 25°C , the interior noise below 60 dB and the artificial lighting by fluorescence lamps with a 12:12-hour light: dark cycle. Normal diets, including a minimum of 0.1% calcium content, 0.4% phosphorus content, and 2,000 IU/kg vitamin D content and fresh drinking water, were provided every day, and soft food was supplied for substitution when rats were installed with nickel-titanium coil. The study was

approved by the Institutional Animal Care and Use Committee of Shandong University, and was practiced following the National Institutes of Health Guidelines for the Use of Laboratory Animals.

Orthodontic relapse model

The appliance design and placement were as our team previously described [18]. A nickel-titanium coil (0.012 inch, Grikin Advanced Materials Co. Ltd, Peking, China) was ligated to the left maxillary first molars and the ipsilateral incisor and fixed with 0.2 mm staining wire around both teeth. A shallow groove was milled around the neck of incisor, 0.5 mm in depth. The stainless wire was embedded into the groove, and the two incisors were bonded together with enamel cement (Tianjin Synthetic Material Research Institute, Tianjin, China) to enhance anchorage (Figure 1A). The force was kept in 50 g measured with a gauge. Rats were under anesthesia throughout surgery. Experimental tooth movement was conducted for 21 days. At the end of the strength phase, the spring appliances were removed, and precise impressions of all rat maxillae were taken by using silicone material with individual resin trays with the rats under anesthesia; stone models of the maxillae were also made.

Administration of solution

Psoralen (purity >98%, Sigma, St Louis, Mo, USA) powder was dissolved in physiological

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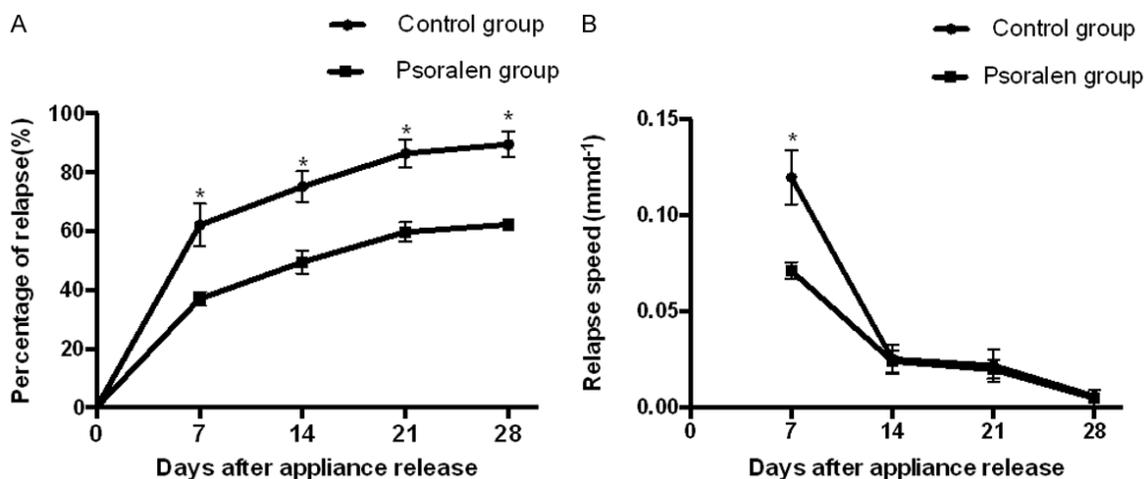


Figure 2. A: The percentage of relapse. The percentage of relapse is the proportion of the distance of relapse to the distance of tooth movement. *: Significant decrease versus control group at $P < 0.05$. B: The relapse speed. Molars relapsed fastest in the first week in both group, and decreased in the following days. *: Significant slow versus control group at $P < 0.05$.

saline to prepare a concentration of 2 mg/ml. After appliance release, rats in the psoralen group were given a gavage of psoralen 8 mg/kg per day for 4 weeks. Meanwhile, rats in the control group received daily gavage of 1 ml 0.9% sodium chloride every day. The rat maxillae in both groups were duplicated in stone models every week.

Measure the distance of relapse

The models were trimmed to be parallel to the occlusion plane of the molars; the height from the bottom to the occlusal plane was about 1 cm. Prepared models were scanned by 3D Scanner (ACTIVITY888, Smart Optics, Germany), and the distance between the distal grooves of the first and the second molar was also measured by 3D Scanner (**Figure 1B**). All measurements were repeated 3 times by an examiner, and the mean of these measurements was used as the representative value of each distance.

Specimen preparation

After drug treatment, the rats in both groups were euthanized by injecting an overdose of chloral hydrate after orthodontic treatment. The maxillae were dissected and fixed in 4% paraformaldehyde buffer at 4°C for 24 hours, and then decalcified in 10% EDTA (PH=7.4) at 4°C for 5 months. During decalcification, the solution was replaced every 3 days. The decal-

cified maxillae underwent a graded series of ethanol and cleared with xylene, then embedded in paraffin.

HE staining

Consecutive sagittal slices from the crowns to the apex of the mesial roots in the first left molars were cut at 4 μ m and mounted on slides coated with 3-aminopropyl-triethoxysilane. After deparaffinized and rehydrated, the sections were stained by hematoxylin and eosin (H&E) and observed under a light microscope (BX51, Olympus, Tokyo, Japan) to accept the variation of periodontal tissue during orthodontic relapse.

Immunohistochemical staining of BMPs and GPR30

BMPs and GPR30 were visualized in prepared sections that experienced deparaffinized in xylene, hydrated through a graded alcohol series, washed with phosphate-buffered saline, treated with 0.1% (w/v) trypsin at 37°C for 10 minutes to retrieve antigen, blocked with 3% H₂O₂ for 30 minutes to inhibit endogenous peroxidase activity, preincubated with goat serum for 30 minutes to block nonspecific binding, and subsequently incubated with BMP2 antibody (1:100 dilution, Abcam, Cambridge, UK)/BMP4 antibody (1:100 dilution, Abcam, Cambridge, UK)/GPR30 (1:100 dilution, Abcam, Cambridge, UK) at 4°C overnight. After rinsed,

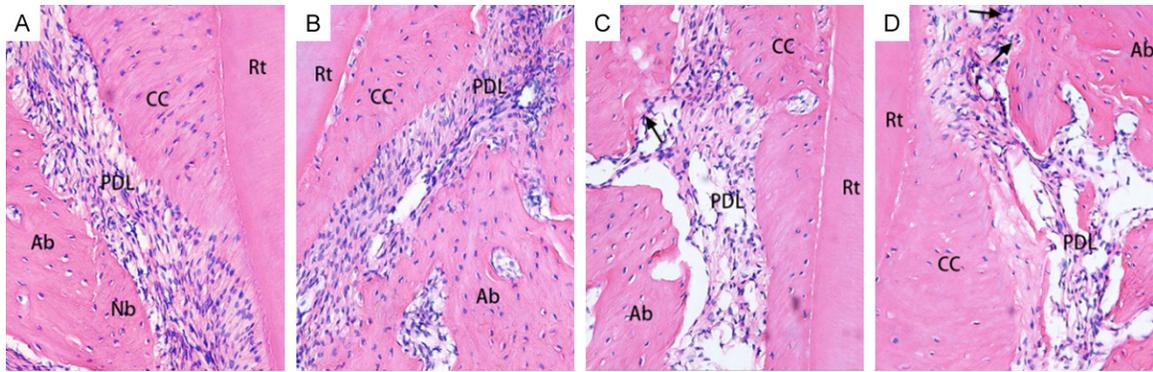


Figure 3. Histological examination of PDL. A. The tension side of psoralen group; B. The compression side of psoralen group; C. The tension side of control group; D. The compression side of control group. In the psoralen group, the red-stained PDL shows more organized arranged. Bone formed on the continuous alveolar bone on the tension side, but deposited on loose trabecular structure on the compression side. In the control group, PDL was irregular, especially on the compression side. Newly formed bone lay on the porous alveolar bone of the both sides. Much Howship's resorption lacunae on alveolar bone and root resorption in cellular cementum was showed on the compression side. (Ab: alveolar bone, Rt: root, CC: cellular cementum, PDL: periodontal ligament, Nb: new bone, Black arrow: Howship's resorption lacunae, magnification $\times 200$).

the sections were incubated with biotinylated goat anti-rabbit immunoglobulin G and streptavidin-biotin complex (Boster, Wuhan, China) at 37°C for 25 minutes. Color was developed with Diaminobenzidine solution (Boster) for 2 minutes and hematoxylin for counterstain.

Stained by BMP2/BMP4, cells with brownish-yellow granules within cytoplasm were deemed to be positive cells, unlikely, GPR30-positive cells with granules on cell membrane. The slides were analyzed by Image-Pro Plus 6.0 software to determine the mean optical density of the immunohistochemical images, under 400 fields.

Statistic analysis

The evaluated data were presented as the mean \pm standard deviation and analyzed by SPSS Statistics 21.0. Differences between each group were examined in one-way analysis of variance (ANOVA) and Fisher's protected least significant difference (PLSD). $P < 0.05$ was considered to be statistically significant.

Results

Distance of tooth relapse

After 21 days of active tooth movement, all treated first molars exhibited mesial movement and the mean distance was 1.35 ± 0.16 mm. In day 28 after appliance release, significant decrease of relapse distance was observed in

the psoralen group compared with the control group ($69.79 \pm 1.39\%$ in psoralen group, $89.46 \pm 4.34\%$ in control group) ($P > 0.05$) (**Figure 2A**). **Figure 2B** shows that, the molars relapse fastest in the first week in both groups (psoralen group: 0.07 ± 0.01 mmd^{-1} , control group: 0.12 ± 0.01 mmd^{-1}), with a subsequent decrease in the following days. Psoralen group showed lower relapse rate in every detected time, but significant difference was witnessed only in day 7 compared to control group ($P < 0.05$).

HE staining

Removal of force appliance reversed the loading direction, leading the distal side of the mesial root to the compressed side, and the mesial side to the stretched side. **Figure 3** shows the histological change on day 28. In the psoralen group, periodontal ligament (PDL) distributed regularly, with plenty of cementum generated around the apex of mesial root. New bone deposited on the surface of alveolar bone, which was smooth on the tension side, but rough on the compression side. Besides, osteoblasts, cementoblasts and odontoblasts lay in a line on the surface of alveolar bone, cementum and pulp cavity. In the control group, the periodontal fibers arranged in a mess on the compression side. Little new bone precipitated on the porous alveolar bone on both sides. In addition, much resorption of cellular cementum and Howship's resorption lacuna were observed on the compression side.

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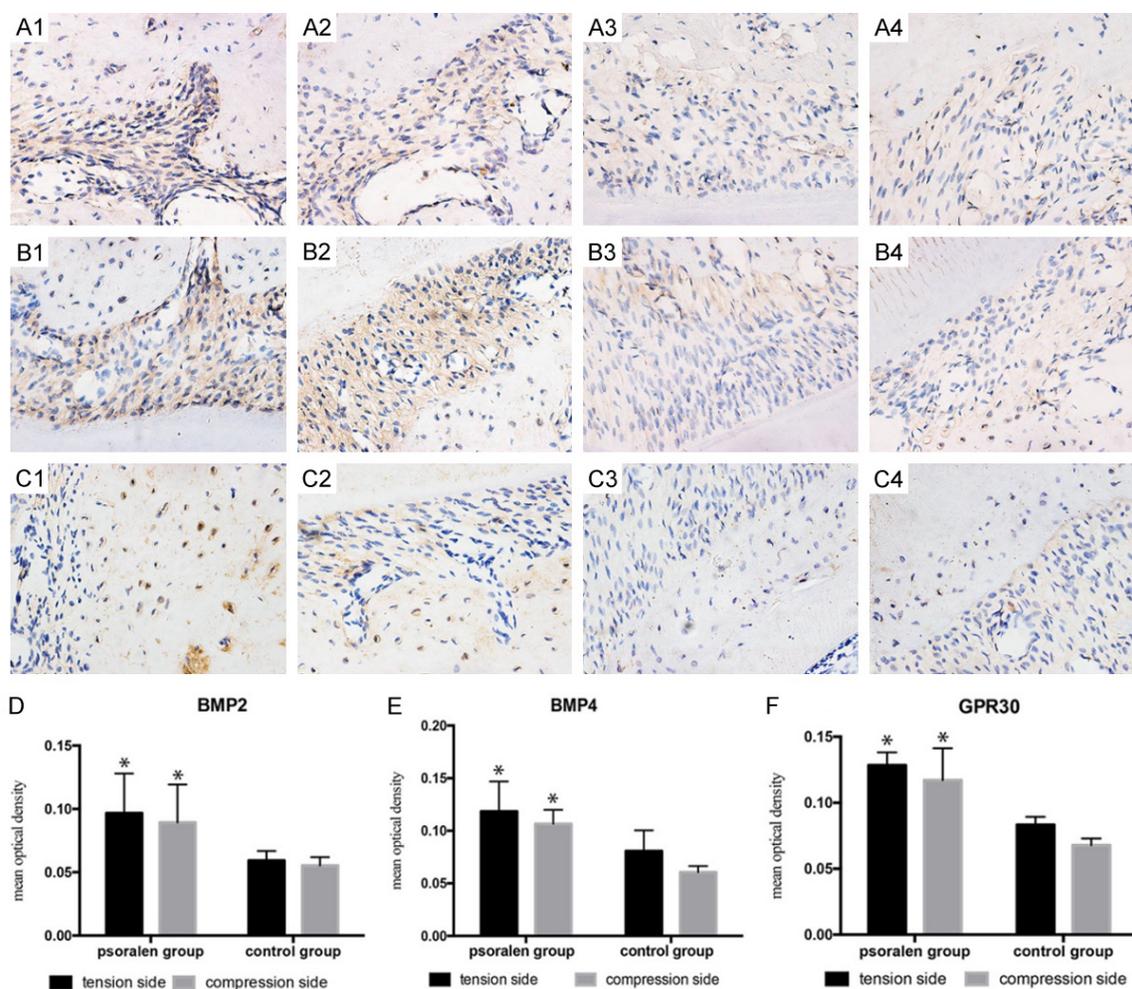


Figure 4. Immunohistochemical examination of BMP2 (A1-A4), BMP4 (B1-B4) and GPR30 (C1-C4). BMP2, BMP4 and GPR30 expressed in PDL. GPR30 was also examined in the alveolar bone and cellular cementum. (A1, B1, C1) the tension side of psoralen group. (A2, B2, C2) the compression side of psoralen group. (A3, B3, C3) the tension side of control group. (A4, B4, C4) the compression side of control group. Mean optical density of BMP2 (D), BMP4 (E) and GPR30 (F) expressions. * $P < 0.05$, significant high versus control group. (magnification $\times 400$).

Immunohistochemistry

BMP2 and BMP4, important to bone formation, were detected to determine the mechanism of psoralen on bone remodeling. In this study, positive staining for BMP2 and BMP4 appeared in PDL and inside the blood vessels on both sides in two groups. Compared with the control group, the mean optical density value of BMP2 and BMP4 immunoreactivity in the psoralen group was significantly higher ($P < 0.05$). BMP2 and BMP4 expression on the tension side was slightly intense, but no statistical difference existed (Figure 4A1-A4, 4B1-B4, 4D, 4E).

An intracellular transmembrane G protein-coupled estrogen receptor (GPR30), newly report-

ed, was examined to explore the relevance to psoralen. From Figure 4C1-C4, we got that the expression of GPR30 was observed in PDL. Significantly higher expression was observed within the psoralen group compared to the control group ($P < 0.05$) (Figure 4F).

Discussion

This study demonstrated that psoralen has an active effect on bone remodeling after orthodontic movement. Relapse post-tooth movement has been thought to be multifactorial, varying from muscle disorders, harmful oral habits, changes in dental arch form, unfavorable growth pattern, to the stretch of the transseptal fiber. Though the mechanisms of action

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are varied, relapse is ultimately decreased by modification during the remodeling process of the dental supporting tissues.

Psoralen is a classic Traditional Chinese medicine for kidney-tonifying and bone-nourishing applications. The traditional Chinese theories, “kidney controlling bones”, and “kidney producing bone marrow” mean that, kidney has a great role in the growth and development of bone marrow. Psoralen has a well-defined bone protective effect to rescue osteoporosis [19], probably by elevating the serum level of transforming growth factor-beta (TGF- β) and the expression of TGF- β gene in bone [20], and also possibly related to the stimulation of differentiation of bone marrow mesenchymal stem cells (bMSCs) to osteoblasts [15]. Psoralen is a useful bioactive component for activating the cartilaginous cellular functions of chondrocytes [21]. It is considered that tooth and bone are highly correlated to kidney in traditional Chinese medicine theory. So our study sought to apply psoralen to mitigate the clinically significant problem of post-orthodontic relapse and explore the mechanism.

Previous studies revealed that a 40 to 60 g force stimulated substantial molar tooth movement in rats [22], thereby, 50 g force was loaded in rats by compromise. Additionally, psoralen was administrated in 8 mg/kg per day, according to Ying et al. [20] taking on ovariectomized osteoporosis models of rats.

By examining relapse distances, we found that, the psoralen group showed a significant decrease in relapse distance than that of the control group. This indicates that psoralen has a stimulatory effect on inhibiting relapse after OTM. Molars relapsed fastest in the first week in both groups. Once orthodontic force has been removed, molars tend to relapse distally, resulting from the resumption of physiological distal drift [22]. After removal of spring, the relapse energy stored in the collagenous periodontal and transseptal fiber systems. Then the energy gradually releases, leading to faster and greater relapse within 1 week [23]. As the energy has dissipated, the speed and extent of relapse step down until the remodeling of the PDL finishes.

In contrast to active tooth movement [18], more bone formation was in the mesial side, and

more bone resorption was detected in the distal side. The change of loading direction possibly accounted for it. Like simvastatin [23] and relaxin [24], psoralen takes effect on bone remodeling as well, enhancing bone formation and inhibiting bone resorption after OTM. However, a phenomenon was observed that more cellular cementum generated around root and fewer cementum degraded in the psoralen group. Recently, reports have demonstrated that BMPs trigger follicle cells to differentiate toward cementoblast phenotype and stimulate cementoblast-mediated biomineralization [25-27]. Therefore, psoralen possibly simulates cementoblast differentiation and cementum biomineralization by BMPs pathway. Psoralen may inhibit root resorption by reducing cementoclast differentiation, as we revealed in the pamidronate [28]. It needs further study.

BMP family is a member of transforming growth factor beta (TGF- β) superfamily. About 30 members have been explored in BMP family. Besides, BMP2 and BMP4 were confirmed to induce differentiation of mesenchymal stem cells into osteoblasts and promote proliferation and differentiation of osteoblasts [29-31]. Immunohistochemical results showed that BMP2 and BMP4 expressions were significantly higher among the psoralen group. *In vitro*, psoralen has been verified to enhance phosphorylation of Smad1/5/8, the central molecules in BMP signaling, and increase the expression of osterix (Osx). Moreover, Osx is the downstream target gene of BMP signaling to promote osteoblast differentiation [14]. Therefore, psoralen may have a stimulatory effect on bone formation in post-orthodontic movement, by stimulating BMPs expression.

Psoralen, a phytoestrogen, has potent estrogenic activity. Previous studies reported that psoralen could bind to nuclear estrogen receptor alpha and beta (ER α , ER β) [32], and mainly activated ER β to rescue bone [20]. Recently, a new ER, an intracellular transmembrane G protein-coupled estrogen receptor (GPR30), was revealed in the endoplasmic reticulum, mediating rapid signaling events. Some studies demonstrated GPR30 located in numerous tissues, such as female reproductive system, nervous system, immune system and cardiovascular system and bone tissue [33, 34]. Nevertheless, researches about GPR30 in periodontal tissue were very few. In addition, no studies demon-

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strated the interaction between psoralen and GPR30. In this study, we observed GPR30 expressed in PDL, and expression increased in the psoralen group. It was consistent with Luo *et al.*, who revealed GPR30 expressed in periodontal ligament cells in a *vitro* study [35]. Furthermore, deficiency of the G protein-coupled estrogen receptor GPR30 leads to reduced bone growth and bone mineral density in mice [33, 36]. Collectively, GPR30 was observed in PDL *in vivo*, and psoralen may bind to and activate GPR30 to play osteoprotective effect in PDL.

GPR30 has been revealed in PDL, but which types of cells it exists and how psoralen enhances GPR30 expression in these cells are unknown. So, we will examine cell markers to show which cells express GPR30, and take more detailed studies to explore the mechanism in psoralen enhancing GPR30 expression next time.

In addition, this study was designed to evaluate the effects of psoralen on PDL remodeling in the moved rat molars, but specimens were got only in day 28, leading to modification of periodontal tissue in different time unknown. Next, we would increase experimental specimen and obtain periodontal tissue variation in different time.

In conclusion, the present study indicates that psoralen could inhibit orthodontic relapse and plays a great role in bone remodeling, probably through binding to GPR30 in PDL and stimulating the expressions of BMPs. GPR30 also locates in periodontal tissue to take estrogenic effects. Psoralen may have a therapeutic effect on inhibiting relapse after OTM, especially for menopausal and post-menopausal women.

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Disclosure of conflict of interest

None.

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