Research article

Compressive force regulates GSK-3β in osteoclasts contributing to alveolar bone resorption during orthodontic tooth movement in vivo

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ABSTRACT

Background: Orthodontic tooth movement mainly depends on biological and mechanical reactions in the periodontium, such as the indispensable reconstruction process of the periodontal ligament and alveolar bone. To explore whether orthodontic compressive force can induce bone resorption during orthodontic tooth movement by regulating the GSK-3β/β-catenin pathway.
Methods: We established orthodontic tooth movement models in Sprague-Dawley rats. In addition, compressive force-induced bone resorption that occurred during orthodontic tooth movement was analyzed by HE staining and micro-CT. The number and distribution of osteoclasts were observed by TRAP staining. Furthermore, pressure-induced bone resorption mediated by the GSK-3β/β-catenin signaling pathway was analyzed by immunohistochemistry.
Results: As shown by the micro-CT results, bone parameters, such as bone mineral density (BMD), the bone volume fraction (BV/TV), and trabecular thickness (Tb. Th), were significantly decreased under orthodontic compressive force stimulation, in contrast with the dramatically increased trabecular spacing (Tb. Sp). During the process of tooth movement, the compressive force can induce bone resorption on the side with the force, which increases the expression of phosphorylated Ser-GSK-3β and activation of the β-catenin signaling pathway. Additionally, downregulation of the GSK-3β activity further caused the downregulation of bone parameters, leading to bone loss. The TRAP staining and immunohistochemistry staining results indicated that orthodontic compressive force influenced osteoclast formation and the secretion of osteoclast-related cytokines, matrix metallopeptidase 9 (MMP-9) and receptor activator of nuclear factor-κB ligands (RANKLs), which is also related to the duration of orthodontic force.
Conclusions: These results indicated that the GSK-3β inhibitor can promote osteoclast formation on the side with orthodontic compressive force. In addition, the activation of the GSK-3β/β-catenin signaling pathway contributes to bone reconstruction caused by orthodontic compressive force. Therefore, the GSK-3β/β-catenin signaling pathway can be a potential target for further clinical applications.

1. Introduction

In orthodontic clinical treatment, the major purpose of intraoral or extraoral orthodontic devices is to move teeth to achieve ideal orthodontic effects [1]. Orthodontic tooth movement mainly depends on biological and mechanical reactions in the periodontium, such as the indispensable reconstruction process of the periodontal ligament and alveolar bone [2]. Similar to that of other bone tissues, the process of moving teeth mainly involves osteoclasts and osteoblasts [3]. These detailed steps, including the activation of osteoclasts on the pressure side, lead to bone resorption. When a tooth moves, the alveolar bone on the

tension side forms a vacancy, and the new bone can fill this vacancy [4]. During orthodontic teeth movement, on the compression side of the periodontal ligament, osteoblasts proliferate in large numbers to generate and deposit new bone tissue along the alveolar bone; on the tension side where the periodontal ligament is compressed, a large number of osteoclasts accumulate, causing the alveolar bone to resorb.

Bone resorption on the compression side provides a movable space for orthodontics. Hence, osteoclasts play an important role in the process of transforming mechanical force into biological signals. A study showed that osteoclasts and the RANKL of osteoclast precursor cells can sequentially bind receptors on the osteoclast membrane [5]. This

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combination promotes the activation of osteoclasts and forms a unique signal cascade [6]. Furthermore, RANKL upregulation can promote the secretion of matrix metalloproteinases-9 (MMP-9) and induce bone resorption [7]. In recent years, research has found that mechanical stimulation has a certain regulatory effect on periodontal ligament cells, thereby affecting the reconstruction of periodontal tissue on the side with tension [8]. The situation is opposite on the side with compressive force; the gaps among the periodontal ligament on the compressive force side are relatively small, the fibers are relatively concentrated, the blood vessels are compressed, and osteoclasts are easily differentiated [9]. Therefore, osteoclasts on the compressive force side of the orthodontic process are essential, but their underlying mechanism is still unclear.

In our previous study, we found that the tension force can induce bone formation via regulated GSK3β, and the binding between Wnt protein and its transmembrane receptor can lead to the inhibition of GSK-3β and the stabilization of β-catenin. In addition, β-catenin accumulation in the nucleus activates downstream Wnt target genes, leading to osteogenic differentiation [10]. However, the role of GSK3β on the side with compressive force is less known. A better understanding of the mechanisms by which GSK-3β regulates osteoclast formation on the compressive side is very important. In this study, we found that a GSK-3β inhibitor could accelerate osteoclast formation on the compressive side. Furthermore, the promoted nuclear translocation of β-catenin results in decreased RANKL expression by inhibiting GSK-3β. Finally, the activation of the GSK-3β/β-catenin signaling pathway is beneficial to orthodontic compressive force-induced bone reconstruction (Scheme 1).

2. Materials and methods

2.1. Experimental animals

Thirty Sprague-Dawley (SD) male rats that were 8 weeks old and had body weights of 200–250 g were purchased from the Experimental Animal Center of Soochow University (Suzhou, China). The animal handling and surgical procedures were conducted in accordance with protocols approved by the Ethics Committee at the First Affiliated Hospital of Soochow University (201709A472).
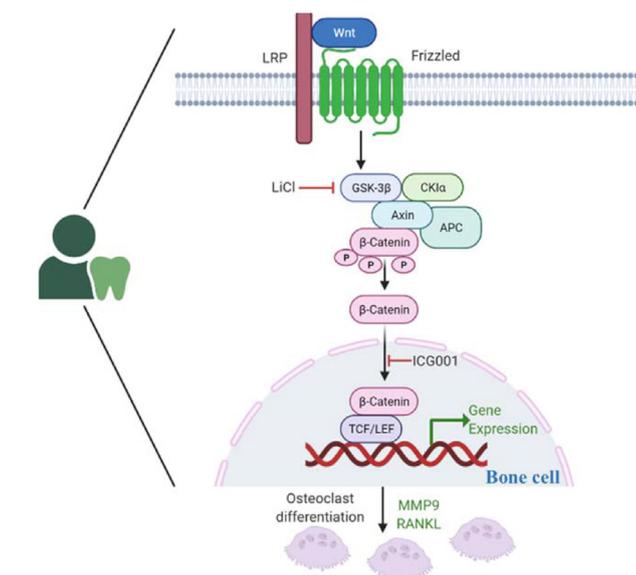
Scheme 1. The GSK-3β/β-catenin signaling pathway participates in alveolar bone resorption and reconstruction on the pressure side. Concretely, the GSK-3β inhibitor can accelerate osteoclast formation on the pressure side, and activation of the GSK-3β/β-catenin signaling pathway is beneficial for orthodontic compressive force-induced bone reconstruction.

2.2. Establishing orthodontic tooth movement models and drug administration procedures

The SD rats were divided into the following groups: SD rats treated with orthodontic tooth movement (OTM) (denoted as OTM, n = 15), LiCl (denoted as OTM + LiCl, n = 5), ICG001 (denoted as OTM + ICG001, n = 5), and LiCl and ICG001 (denoted as OTM + LiCl + ICG001, n = 5). The rats were anesthetized by intraperitoneal administration of 1.5% pentobarbital sodium. Then, the OTM model was established by applying a closed nitinol spring (50-g force) between the maxillary incisor and left first molar to cause the first molar to move to the middle. LiCl (200 mg/kg/day) was intragastrically administered to the rats. Additionally, ICG001 (10 μg/day) was locally injected into the mucoperiosteal of the left first molars. Rats in the OTM group were sacrificed at days 0, 7 and 14 (n = 5 at each time point, denoted as the control, 7 d and 14 d). At each point, rats were euthanized, and the whole maxilla, including the left first, secondly, and third molars, was collected. Micro CT was used to analyze the histology. The distance between the nearest points of dental crown of the first and second molars was measured on the digital radiographs.

2.3. Micro CT scanning and quantified analysis

High-resolution micro-CT (SkyScan 1176, Belgium) scanned the maxilla with fixed parameters (resolution: 18 μm, voltage: 80 kV, current: 100 μA). After scanning, three-dimensional image reconstructions were performed. For the quantified analysis, the region of interest (ROI) was the alveolar bone area located in the middle of the mesial cheek root. The top of the cube (500 μm × 500 μm × 500 μm) was located at the interface between $1/3^{rd}$ of the tooth root and $1/3^{rd}$ of the root tip. After scanning, three-dimensional (3D) digital image reconstruction was carried out using the supporting analysis software (Mimics). Then the resident quantitative analysis software (CTAn, Skyscan) was used to obtain the following parameters: bone mineral density (BMD), bone volume fraction (BV/TV), trabecular thickness (Tb. Th) and trabecular spacing (Tb. Sp).

2.4. Hematoxylin-eosin staining and tartrate-resistant acid phosphatase staining

After decalcification, the tissues were embedded in paraffin and sliced. Then, these specimens were dewaxed with xylene and stained with hematoxylin for 5 min. The residual hematoxylin was cleaned with deionized (DI) water, and then the specimen was immersed in a 75% hydrochloric acid ethanol solution for 30 s. After being cleaned with DI water for 2 min, these specimens were immersed in ammonia for 30 s. Then, the specimens were washed with DI water and stained with eosin dye for 2 min. Finally, these specimens were successively immersed in 95% ethanol and xylene for two cycles (1 min for each solution) and sealed with neutral resin.

The specimens were fixed with fixative for 30 s and then washed with DI water for 1 min. Then, the specimens were immersed in the prepared TRAP staining solution and incubated for 1 h at 37 °C under light protection. After that, the residual TRAP staining solution was cleaned with DI water. Finally, the specimens were stained with hematoxylin and sealed with neutral resin.

2.5. Immunohistochemistry

After Micro-CT scanning, the maxillae were dissected and trimmed into smaller blocks containing the first molars. All blocks were decalcified in 10% (w/v) ethylene diamine tetraacetic acid for 3 weeks, and then embedded in paraffin. The blocks were cross-sectioned to 5 μm, mounted on protein-coated glass slides, dewaxed. For immunohistological staining, the specimens were incubated with GSK-3β antibody (1:200, ab32391), phosphorylation-Ser9-GSK-3β antibody (1:250, ab75814), β-catenin (1:500, ab32572), RANKL antibody (1:500,

sc71955), MMP-9 antibody (1:500, ab10375), and sclerostin antibody (1:100, ab63097) overnight at 4 °C.

Then, the specimens were washed and incubated with the secondary antibody (coupled with biotin) for 30 min. After that, the specimens were further incubated with avidin-biotinase for another 30 min and processed with a 3,3N-diaminobenzidine tetrahydrochloride (DAB) color development kit. Cells showing brown particles in the cytoplasm or intracell were marked as positive cells, and 10 random views were selected to count the number of positive cells. The results were calculated by two different observers separately. Briefly, the histomorphometric analysis was performed on the most central section. A round-shaped region of interest was defined as previously recommended. Using 20× magnification, the bone tissue was circled by the operator, and the software automatically recorded and calculated the determined area with positive cells.

2.6. Statistical analysis

Non-parametric T test was used to measure significant differences among the treatment groups (*p values <0.05, and **p values <0.01). Data were taken as the mean ± standard error.

3. Results

3.1. Compressive force-induced bone resorption during the process of orthodontic tooth movement

As the H&E staining and micro-CT 3D reconstruction results indicated when orthodontic compressive force was applied, there was an increased gap between the first molar that experienced mesial movement and the second molar, as shown in Figure 1A. Under orthodontic compressive force, the periodontal ligament fibers of rats became arranged irregularly with an incomplete morphology. After an orthodontic compressive force of 7 d, the periodontal space was very

narrow; the alveolar bone was destroyed, and the bony edge was obviously absorbed with resorption lacuna. In contrast, rats in the control group had regular arrangements of periodontal ligament fibers and uniform periodontal spaces. The fibroblasts were evenly distributed in the periodontal ligament fibers with a clear cytoplasmic nucleus structure. After an orthodontic compressive force for 14 d, the rats had an obviously larger ~7.32 fold ($P < 0.01$) periodontal space between the first molar and the second molar than that of the rats without an orthodontic compressive force (control group), as shown in Figure 1A. Additionally, the alveolar bone treated with orthodontic compressive force was analyzed by micro-CT. Specifically, BMD (~0.68 fold, $P < 0.01$), BV/TV (~0.65 fold, $P < 0.01$) and Tb. Th (~0.73 fold, $P < 0.01$) significantly decreased with the stimulation of orthodontic compressive force, but the Tb. Sp (~1.55 fold, $P < 0.01$) and orthodontic tooth movement (~7.32 fold, $P < 0.01$) was dramatically increased in 14 d (Figure 1B to 1F), which indicates that orthodontic compressive forces can induce bone resorption on the pressured side, leading to bone loss with time pass.

The TRAP and HE staining results further indicated that the orthodontic compressive force affected the formation of osteoclasts on the pressured side. Specifically, as shown in Figures 2A and 2B, compared with that of the control group, more TRAP-positive cells (~3.34 fold, $P < 0.01$) on the pressured side were observed in the rats treated with orthodontic compressive force in 7 d with obvious alveolar bone resorption. Importantly, most of the osteoclasts are distributed at the pressured side of the alveolar bone edge and few osteoclasts were also observed at the pressured side of the remote end of the alveolar bone, which indicates that subtle bone resorption occurred in the remote end.

However, after treatment for 14 d, the markers of TRAP, MMP-9 and RANKL decreased significantly ($P < 0.01$) (Figure 2C to 2F). These results revealed that the orthodontic compressive force at the pressured side affected the formation of osteoclasts and osteoclast-related cytokines.
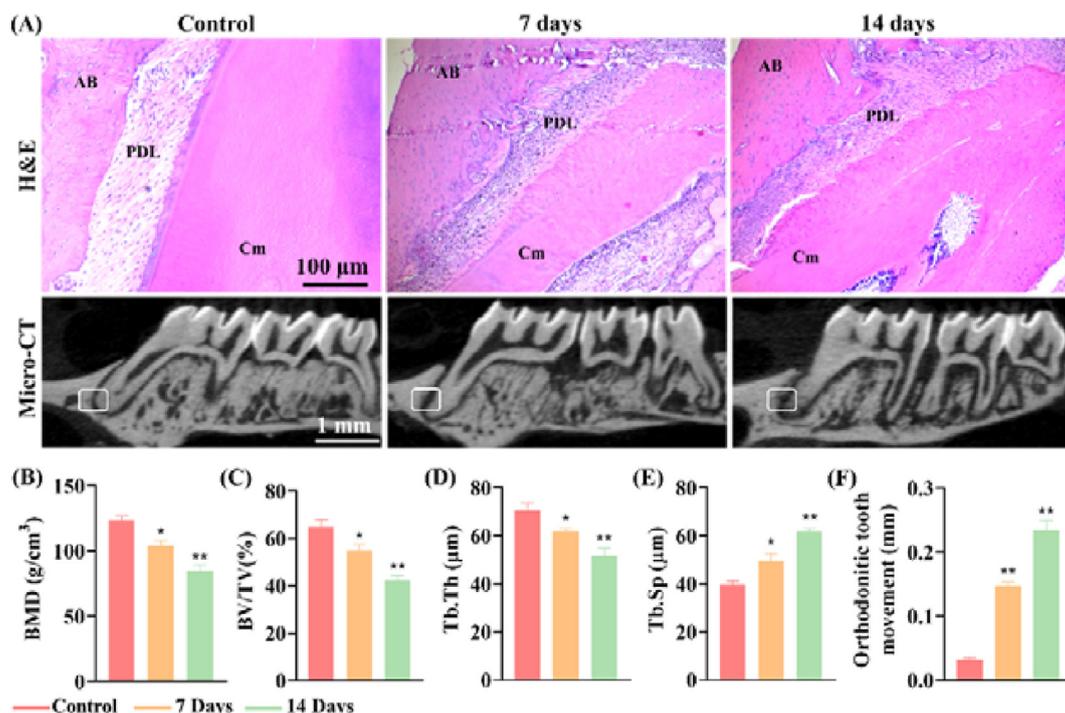

Figure 1. Orthodontic force promotes orthodontic tooth movement and bone resorption on the compressive pressure side (A) HE staining (scale bar = 100 μm) and micro-CT (scale bar = 1 mm) results of orthodontic tooth movement after different durations of orthodontic compressive force (AB: Alveolar Bone, PDL: Periodontal Ligament, Cm: Cementum). The white box is an interest area, that is, the micro-CT analysis area. Quantitative analysis results of micro-CT in (B) BMD, (C) BV/TV, (D) Tb. Th (E) Tb. Sp (F) Orthodonitic tooth movement. (Control *vs.* 7 Days and Control *vs.* 14 Days, n = 5; *$P < 0.05$, **$P < 0.01$).
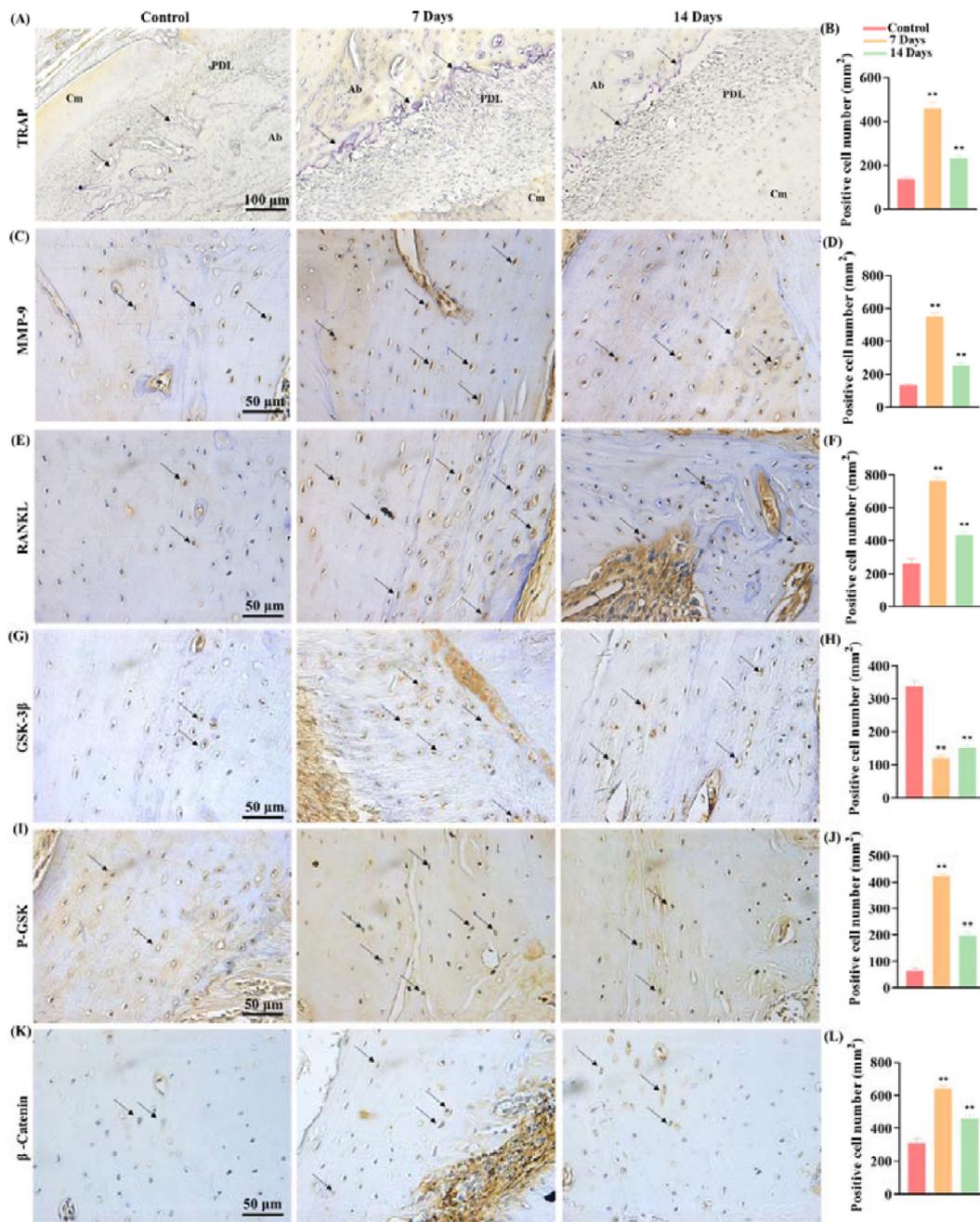
Figure 2. Immunohistochemistry staining and quantitative analysis. Immunohistochemical staining for (A) TRAP, (C) MMP-9, (E) RANKL, (G) GSK-3β, (I) phospho-Ser9-GSK-3β, and (K) β-catenin at the pressure site of the tooth that moved (AB: Alveolar Bone, PDL: Periodontal Ligament, Cm: Cementum) (scale bar = 50 μm). The counting results of the positive cells in (B) TRAP, (D) MMP-9, (F) RANKL, (H) GSK-3β, (J) phospho-ser9-GSK-3β and (L) β-catenin. Brown dots and arrows indicate the positive cells. (Control *vs.* 7 Days and Control *vs.* 14 Days, n = 5; *$P < 0.05$, **$P < 0.01$).

3.2. The relationship between the GSK-3β/β-catenin signaling pathway and compressive force side-bone resorption during orthodontic tooth movement

As shown in Figure 2G, GSK-3β staining results were more significant for the pressure side than for the control group at different time points. The semiquantitative results indicated that the expression level of GSK-3β decreased after treatment for 7 d (~0.35 fold) but increased after treatment for 14 d (~0.45 fold) compared with control group ($P < 0.01$) (Figure 2H). However, the expression of phosphorylated Ser9-GSK-3β (p-GSK) was obviously increased in the pressure-exposed alveolar bone after treatment for 7 d (~6.71 fold), and then the expression level decreased after treatment for 14 d (~3.13 fold) with control group ($P < 0.01$)

(Figures 2I and 2J). Since GSK-3β plays an important role in β-catenin expression, we further explored the expression of β-catenin. As shown in Figures 2K and 2L, the expression of β-catenin increased on the pressure side after 7 d (~2.07 fold, $P < 0.01$) compared with control group. These results revealed that the relationship between the GSK-3β/β-catenin signaling pathway and pressure side bone resorption was time-dependent during the process of orthodontic tooth movement.

3.3. Pressure-induced bone resorption mediated by the GSK-3β/β-catenin signaling pathway

To explore whether compressive force can induce bone resorption by mediating the GSK-3β/β-catenin signaling pathway during the process of

orthodontic tooth movement, the orthodontic compressive force-treated rats were be treated by the GSK-3β inhibitor (LiCl) for 7 d. The distance of tooth movement is affected by many factors, due to differences in the crown structure, the measurement results sometimes may not reflect the accurate movement of the teeth (Figure 3B). Therefore, the Micro-CT results at the alveolar bone and the roots could show the changes more accurately. As shown in Figure 3C to 3F, LiCl-treated rats exhibited significantly decreased, BV/TV and Tb. Th (~0.78 fold and ~0.79 fold, $P < 0.01$) indicating bone loss, which is consistent with the decrease in new bone deposition seen in the HE staining results (Figure 3A). Additionally, for the TRAP staining results, osteoclasts were observed in the pressure side periodontal tissue in all the experimental rats (Figure 3G). However, the percentage of positive cells was different among these groups, and the rats treated with OTM + LiCl showed the highest percentage of positive cells.

The expression of MMP-9 and RANKL was evaluated by immuno-chemistry staining to explore whether the enhanced osteoclasts could contribute to the decreased bone density. As shown in Figure 4A to 4D, daily administration of LiCl obviously enhanced the percentage of MMP-9- and RANKL-positive cells (~1.37 fold and ~1.40 fold, $P < 0.01$) in the pressure side alveolar bone. Moreover, after treatment of LiCl for 7 d, the population of β-catenin- and P-GSK positive cells increased (~1.37 fold and ~1.87 fold, $P < 0.01$) with the decreased number of GSK-3β-positive cells (~0.45 fold, $P < 0.01$) compared with OTM group, and the GSK-3β/β-catenin signaling pathway was activated. After local administration of ICG001 for 7 d, the GSK-3β/β-catenin signaling pathway was inhibited, leading to decreased numbers of β-catenin- and P-GSK positive cells (~0.68 fold and ~0.80 fold, $P < 0.01$) and increased numbers of GSK-3β-positive cells (~3.85 fold, $P < 0.01$) compared with OTM + LiCl group (Figures 4E-4J). Moreover, the MMP-9- and RANKL-positive populations were reduced and had improved bone mass. All these results indicated that the GSK-3β/β-catenin signaling pathway mediated bone resorption on the pressure side during the process of orthodontic tooth movement.

4. Discussion

Orthodontic tooth movement is a type of biological response that allows the reconstruction of alveolar bone and periodontal tissue under mechanical stimulation [11]. The orthodontic compressive force causes bone formation and bone resorption during the process of orthodontic tooth movement. When the process reaches a certain balance, the normal morphology and function of alveolar bone can be maintained. Additionally, one of the most important factors in orthodontic tooth movement is bone resorption caused by osteoclasts [12]. Recently, many studies have focused on how osteoclast differentiation influences orthodontic tooth movement and the mechanism behind this process [13]. According to our results, the orthodontic compressive force interacts with osteoclast-induced bone resorption. Specifically, mechanical stimulation contributes to osteoclast activation and bone resorption, but long-term mechanical stimulation weakens bone resorption.

In addition, with an orthodontic compressive force, the periodontal ligament can process mechanical stimulation to biological signals, which further leads to a series of biological responses, such as changes in periodontal tissue metabolism and the reconstruction of alveolar bone [14]. This is a very complicated process involving various signal transduction molecules and pathways, diverse matrix proteins and cytokines [15, 16]. With the knowledge that RANKL/RANK signaling is essential for osteoclast formation, major efforts have been made to identify the signaling pathways that are activated downstream and to determine the full extent of the involvement of RANKL in osteoclast biology and common bone diseases. During the differentiation and maturation of osteoclasts, RANKLs produced by bone cells also participate in orthodontic tooth movement [17]. In a previous study, as an osteoclast differentiation factor, the production of RANKL was positively correlated with the duration of mechanical stimulation [18]. On the one hand, MMPs can degrade the ECM, and on the other hand, MMPs can induce bone resorption and regulate the activity of osteoclasts [19]. In particular, MMPs can specifically degrade nonmineralized cartilage and release vascular endothelial growth factor, which leads to osteoclast activation and delayed osteoclast recruitment [20]. Our immunohistochemistry results indicate a positive correlation between the positive cell population of MMP-9 and RANKL and the duration of mechanical stimulation. After an orthodontic compressive force of 7 d, alveolar bone reconstruction reached the most active status, which is also consistent with previous research.
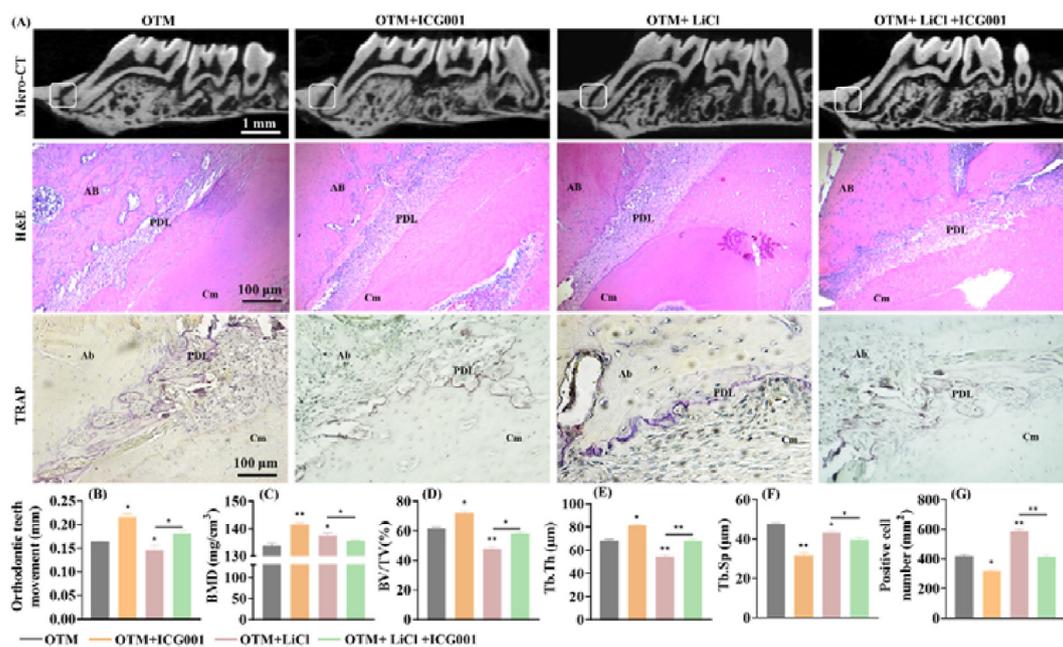

Figure 3. Pharmaceutical inhibition of GSK-3β promoted compressive force-induced bone resorption (A) Micro CT (scale bar = 1 mm) and HE (scar bar = 100 μm) images of the maxillae of rats (AB: Alveolar Bone, PDL: Periodontal Ligament, Cm: Cementum) (B) The distance of the orthodontic tooth movement. The white box is an interest area, that is, the micro-CT analysis area. Quantitative analysis results of micro-CT in (C) BMD, (D) BV/TV, (E) Tb. Th, and (F) Tb. Sp (G) The counting results of TRAP-positive cells. (OTM *vs.* OTM + ICG001, OTM *vs.* OTM + LiCl and OTM + LiCl *vs.* OTM + LiCl + ICG001, n = 5; *$P < 0.05$, **$P < 0.01$).
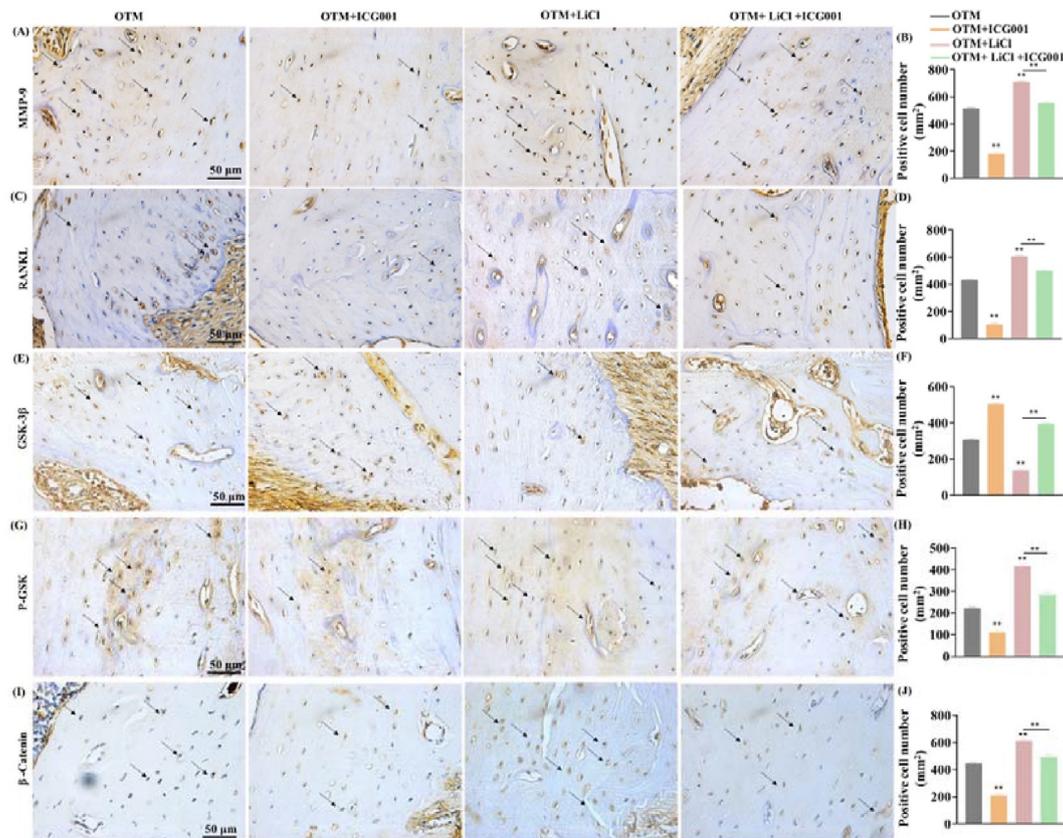
Figure 4. Immunohistochemistry staining and quantitative analysis. Immunohistochemical staining for (A) MMP-9, (C) RANKL, (E) GSK-3β, (G) phospho-Ser9-GSK-3β, and (I) β-catenin at the pressure site of the tooth that moved (scale bar = 50 μm). The counting results of the positive cells in (B) MMP-9, (D) RANKL, (F) GSK-3β, (H) phospho-ser9-GSK-3β and (J) β-catenin. Brown dots and arrows indicate the positive cells. (OTM *vs.* OTM + ICG001, OTM *vs.* OTM + LiCl and OTM + LiCl *vs.* OTM + LiCl + ICG001, n = 5; *$P < 0.05$, **$P < 0.01$).

The periodontal ligament can process mechanical stimulation to biological signals. The Wnt signaling pathway plays an important role in this process; specifically, mechanical stimulation can activate this pathway, leading to stimulation duration and stimulation force-dependent bone reconstruction [21]. In mouse bone cells, only short-term mechanical stimulation can induce the activation of the Wnt/β-catenin signaling pathway, and long-term stimulation significantly downregulates the expression level of Wnt/β-catenin [22]. Moreover, on the pressure side, a time-dependent relationship was observed between mechanical stimulation and the GSK-3β/β-catenin signaling pathway. GSK-3β is a multifunctional protein kinase that plays an important role in signal transmission and cell differentiation, mediating multiple signaling pathways. GSK-3β can activate osteoclasts in the process of orthodontic tooth movement, and it can also accelerate the root resorption. Some research has also indicated that LiCl can prevent bone loss. ICG001 competes with β-catenin in combination with CBP and inhibits TCF/β-catenin-mediated transcription, which can be used to specifically evaluate the interaction between CBP/β-catenin and the Wnt signaling pathway [23]. In addition, we further investigated GSK-3β-mediated osteoclast bone resorption on the pressure side during orthodontic tooth movement. As the TRAP staining results revealed, after 7 d of mechanical stimulation, the OTM + LiCl treatment groups had the most osteoclast populations; in contrast, the OTM + ICG001 treatment groups had the least number of osteoclast populations. After antagonism of LiCl, bone resorption was enhanced; however, ICG001 inhibited bone resorption. Since ICG001 is an inhibitor of β-catenin, it can further inhibit the expression of β-catenin. Therefore, we considered the positive correlation between the activation of the GSK-3β/β-catenin signaling pathway and bone resorption at the tooth pressure side. β-catenin is a key factor in mediating the Wnt signaling pathway; in addition, the Wnt signaling pathway has a close relationship to osteoclasts [24]. The activity of β-catenin is mediated by the phosphorylation of GSK-3β. When phosphorylation occurs at the Ser9 position of GSK-3β, GSK-3β loses its ability to activate β-catenin. The resulting GSK-3β/β-catenin can directly or indirectly influences osteoclast formation. However, the mechanism is still unclear, and some researchers have found that increasing osteoclasts with β-catenin gene silencing will induce accelerated bone resorption and decelerated bone formation [25]. Compared to our previous study, the activity of GSK-3β/β-catenin signaling pathway was positively correlated with bone formation on tension sites and bone resorption on the compressive force side during orthodontic tooth movement. GSK-3β inhibitors can accelerate alveolar bone remodeling and can be used as targets for clinical orthodontic treatment. In addition, we can use cell stretching model to explore the effect of GSK-3β/β-catenin on osteoblasts in *vitro*. However, the specific role and molecular mechanism still need to be further explored.

In conclusion, our results indicate that the GSK-3β inhibitor can promote osteoclast formation on the side with orthodontic compressive force. In addition, the activation of the GSK-3β/β-catenin signaling pathway contributes to bone reconstruction caused by orthodontic compressive force. Therefore, the GSK-3β/β-catenin signaling pathway can be a potential target for further clinical applications.

Declarations

Author contribution statement

Yan Liu; Ke Wu: Analyzed and interpreted the data; Performed the experiments; Wrote the paper.

Xing Cui: Analyzed and interpreted the data; Wrote the paper.

Yelin Mao: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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