

Original Article

Effects of icariin on orthodontic tooth movement in rats

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Abstract: Purpose: Orthodontic tooth movement (OTM) is achieved through bone remodeling of the alveolar bone. Icariin, the active ingredient isolated from Herba Epimedii which is a traditional Chinese medicine (TCM) commonly used for osteoporosis treatment in China. The purpose of the study is to explore the effect of icariin on OTM in rats, and analyze the possible mechanism involved. Methods: 48 rats were selected and divided into 2 groups: the control group and the experimental group. Rats in the experimental group were given 20 mg/kg/day icariin by intra-gastric administration, while the control group received the same volume solvent. All rats were placed a closed coil spring between their upper first molar and incisor, exerting a force of about 40 g to establish animal models of OTM. As the first molar moved mesially, a space between the first and second molar was created. The rats were sacrificed in batch on the 7th, 14th, 21st and 28th days after orthodontic treatment. The amount of tooth movement was measured, and histomorphometric analysis based on slices from periodontium adjacent to the maxillary first molars were used to observe new bone formation, bone resorption and quantify osteoclasts. Key results: Icariin increased OTM ($P < 0.05$) by 65.2%, 35.3%, 11.7% and 16.7% on day 7, 14, 21, 28 respectively compared with the control group. The number of osteoclasts in the icariin group showed a transient but sudden increase and then a persistent decrease. Conclusions: Icariin could accelerate OTM in rats through promoting bone remodeling of alveolar bone.

Keywords: Icariin, traditional Chinese medicine (TCM), orthodontic tooth movement (OTM), bone remodeling

Introduction

Orthodontic tooth movement (OTM) is achieved through the process of alveolar bone resorption on the pressure side and new bone deposition on the tension side [1]. That is to say, bone remodeling of the alveolar bone influences the efficiency of OTM. In the course of OTM, osteoclasts are activated to absorb old bone, and osteoblasts are recruited to the resorption area [2]. The appearance of osteoclasts was the requisite first step for tooth movement [3]. Therefore, any measures which could influence the functions of osteoclasts should make effects on OTM theoretically. To seek more efficient methods of OTM, a series of chemical agents have been attempted such as Non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroid hormone, cytokines and so on. Some agents accelerate such as thyroid hormone, Vitamin D, prostaglandin E₂ (PGE₂), and other inhibit such as alendronate, just as reviewed by Theodosia Bartzela [4]. However,

more scholars have turned their eyes to traditional Chinese medicine in China [5-7]. Herba Epimedii (commonly known as Yinyanghuo) has been one of the most frequently used herbs in formulas prescribed for 'nourishing kidney and strengthening bone' in China [8], with proven efficacy in treating several age-related diseases including osteoporosis, cardiovascular diseases, neurodegenerative diseases and sexual dysfunction [9, 10]. The therapeutic effects of HEP are attributed to icariin [9]. Icariin (C₃₃H₄₀O₁₅; molecular weight: 676.67; molecular structure **Figure 1**), the major efficient ingredient isolated from Epimedium pubescens (77%), which has been reported to enhance bone healing and reduce osteoporosis occurrence [11-13]. Over the years, many scientific studies about pharmacological activities of icariin have been carried out in China. Major of in vivo studies were about osteoporosis, which showed that bone formation was stimulated and bone mass was increased after the administration of icariin [14-16]. In vitro studies

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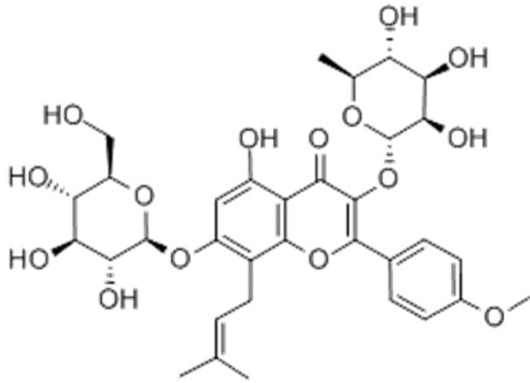


Figure 1. The molecular structure of icariin.

revealed that icariin enhanced osteogenesis and inhibit osteoclastogenesis [17-20]. However, the effect of this herb on OTM has not been reported at home or board so far. In consideration of its effect on the two basic processes of bone remodeling-bone resorption and new bone formation, we carried out this present study. The results suggested that OTM was induced by icariin. The possible cause was that icariin increasing the bone remodeling of the alveolar bone because of its well-balanced effects on bone formation and bone resorption.

Materials and methods

Animals and chemical agents

48 male Sprague-Dawley (SD) rats weighing 180-200 g (Shandong University experimental animal center) were selected. They were fed on normal diet with a minimum of 0.1% calcium content, 0.4% phosphorus content and 2000 IU/kg vitamin D content and continuous fresh water was provided. All rats were kept in cages in a room maintained at 25°C with 12-hour alternating light and dark schedule. The interior noise was controlled below 60 dB. All procedures, including killing, were performed according to the National Institutes of Health Guidelines for the Use of Laboratory Animals and the protocol (Permit number: 201302056) were approved by the Institutional Animal Care and Use committee of Shandong University.

Icariin was obtained from Winherb Medical Science Co., Ltd with purity (HPLC) 98%, (Shanghai, China), and was dissolved into 10 mg/ml

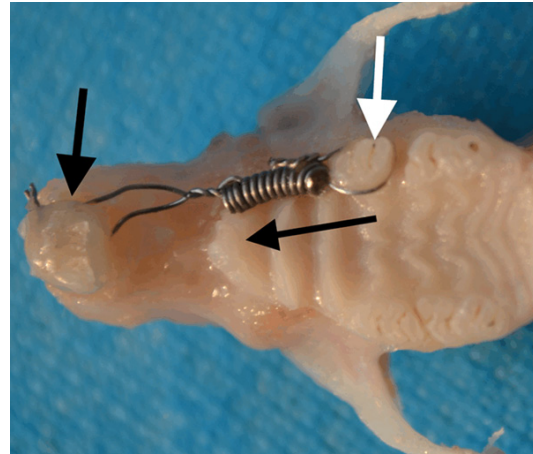


Figure 2. Isolated maxilla of a rat model for orthodontic tooth movement. The appliance was between the upper incisor (short black arrow) and the left first molar of the rat (white arrow). As the first molar moved mesially (long black arrow), a space between the first and second molars was created.

icariin solution, reserved at 4 in dark until used [11]. Naphthol AS-BI phosphate for tartrate-resistant acid phosphatase (TRAP) staining was purchased from Sigma-aldrich (N-2125-100, St Louis, MO, USA).

Methods

48 SD rats were randomly and evenly divided into two groups: the untreated control group and the icariin-treated experimental group. Each group included four subgroup of 6 rats each: 7 days, 14 days, 21 days and 28 days group. Establish of experiment movement model were made on the start of the study [21]. Anesthesia was induced with an intraperitoneal injection 3.3 ml/kg 10% chloral hydrate in both control and experimental groups. After anesthesia, the rats were fixed on board with belly up and tongue pulled out. A Ni-Ti closed coil spring (4 mm in length, 0.12 inch in diameter, Grikin Advanced Materials Co. Ltd, Peking, PR China) was put between their maxillary first molar and incisors, the two head of Ni-Ti coil were fixed with a 0.20 mm stainless wire around teeth. In addition, about 0.5 mm-deep shallow grooves were prepared around the dental neck of the maxillary central incisors to prevent the appliance dislodging from the incisors. The stainless wire in the groove of the neck of the incisors using light curing composite resin (Charisma, Heraeus Kulzer, Germany). And

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Table 1. Amount of orthodontic tooth movement (mm)

	7 d	14 d	21 d	28 d
Control (mm)	0.23±0.01	0.51±0.02	0.77±0.04	1.08±0.04
Icariin (mm)	0.36±0.02*	0.69±0.02*	0.86±0.04*	1.26±0.04*

*P<0.05; n=6 rats per group.

besides that, the two central incisors were bonded together as a whole to enhance the anchorage when drawing the maxillary first molar mesially, so the anchorage loss was neglected. **Figure 2** shows the orthodontic appliance adopted in the present study, and the force of the coil spring after activation is approximately 40 g. As the first molar moved mesially, a space between the first and second molars was created. All efforts were made to minimize rat suffering during the operation. The rats were kept warm by lighting in order to maintain their body temperature and the vital sign were closely observed until the rats were fully awake.

The rats of experimental group was given icariin solution by intragastric administration 20 mg/kg/day respectively from the day of the appliance was activated [22], and the control group was injected the same volume solvent everyday. Intragastric feedings were administered with a ball-tipped lavage apparatus. Once the appliance were activation, their food were replaced with powder to reduce the expulsion rate of orthodontic appliance.

The rats of each subgroups were killed on the 7th, 14th, 21st and 28th days after appliance activation respectively. The rats were anesthetized with excess chloral hydrate, myocardial perfusion with 4% paraformaldehyde solution. The isolated maxilla of rats were soaked in 4% paraformaldehyde solution. The space between the first and second molars created by tooth movement was measured three times with a vernier caliper (Accuracy of 0.02 mm, shanghai, PR China). To ensure the consistency of our measuring technique, all measures were performed three times a day during three consecutive days by the same operator, the data from the same specimen, among which there was no significant differences were obtained. The specimens were decalcified by 10% EDTA solution at 4°C. 0.5 mm-thick sections were obtained by slicer (Germany, LEICA, G1150H) and stained with hematoxylin and eosin (HE) for descriptive histological observation. In addition, TRAP staining was performed to identify

and quantify osteoclasts. Osteoclasts were defined as TRAP-positive multinuclear cell on the bone surface. Observation of these slices and images capture depended on a light microscope (DM4000B, LEICA, German) with

an image acquisition system (QwinV3, LEICA, German).

Statistical analysis

All statistical analysis were performed by using SPSS software version 16.0 for Windows (SPSS Inc, USA). The Graph-Pad Prism version 5.0 software was used. The statistical significances between the experiment group and the control group at each different time point were calculated by analysis of non-paired Student's t-test. Results are reported as means ± SME. P<0.05 was considered statistically significant.

Results

Effects of icariin on OTM

Table 1 illustrates the differences of distance created by OTM between the two groups at four different point time. Compared with the groups, OTM in the experimental groups was promoted by icariin significantly at four different point time. Icariin increased OTM (P<0.05) by 65.2%, 35.3%, 11.7% and 16.7% on day 7, 14, 21, 28 respectively.

Effects of icariin on bone resorption on the pressure side

Figure 3 shows the histological changes in the periodontium of the pressure side of the two groups. Compared with the control group (**Figure 3A**), icariin induced more bone resorption lacunae at the surface of the alveolar bone on day 7 (**Figure 3B**). And the differences between the two groups are not apparent on other time points.

Figure 4 shows TRAP positive osteoclasts in the periodontium of the two groups on day 7, 14, 21 and 28 after application of the orthodontic appliance respectively. After counting osteoclasts on the pressure side (**Figure 5**), we found that on day 7, the number of osteoclasts

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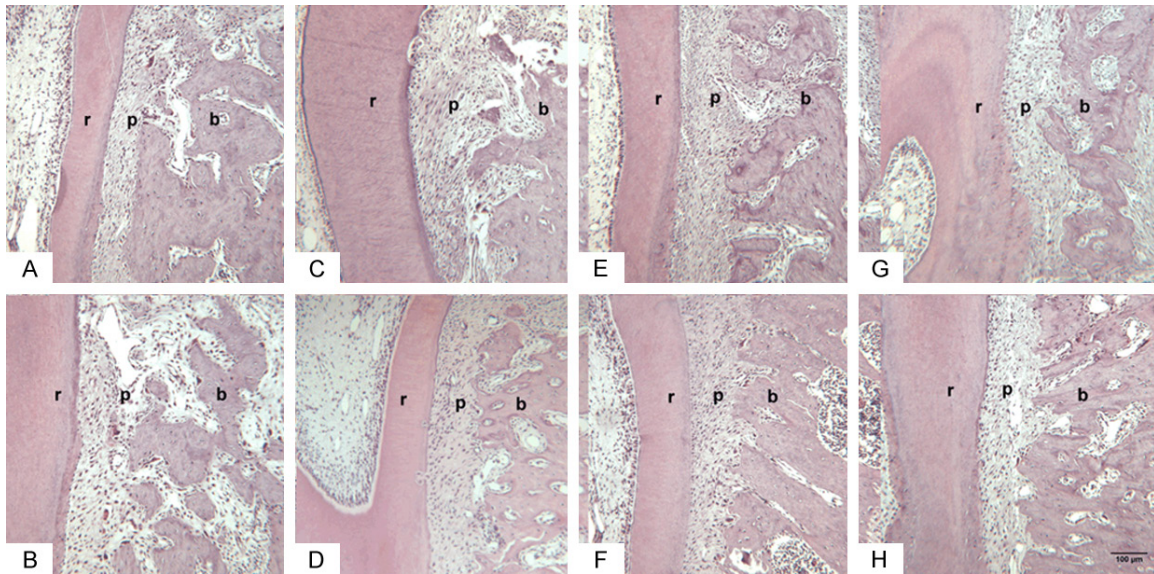


Figure 3. Shows the bone resorption of the pressure side of the control group (A, C, E, G) and the icariin group (B, D, F, H) on day 7, 14, 21 and 28 respectively after application of the orthodontic appliance histologically. r, root; b, alveolar bone; p, periodontium. Bar =100 µm.

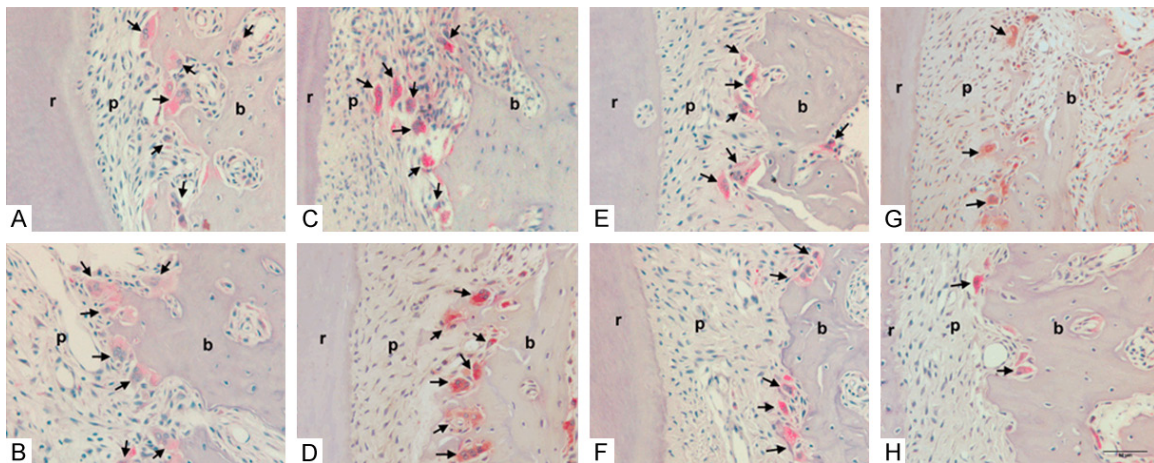


Figure 4. TRAP positive osteoclasts in the periodontium. TRAP positive osteoclasts (black arrow) of the pressure side of the control group (A, C, E, G) and the icariin group (B, D, F, H) on day 7, 14, 21 and 28 after application of the orthodontic appliance respectively. r, root; b, alveolar bone; p, periodontal ligament. Bar =50 µm.

was much more than that of the control group (20.33 ± 1.21 ; 9.83 ± 1.47 ; $P < 0.05$), which peaked among the four icariin groups. And subsequently the number of osteoclasts decreased to the baseline level on day 14, where there was no significant differences between the two groups. Further more, the decline of the number of osteoclasts in the icariin group went on, few osteoclasts could be found until day 28, which was less than the control group obviously (1.33 ± 0.82 ; 4.50 ± 1.05 ; $P < 0.05$).

Effects of icariin on bone formation on the tension side

Figure 6 shows the histological changes in the periodontium of the tension side on 7, 14, 21 and 28 day in the two groups. Different levels of new bone formation were observed. The periodontal membrane was stretched and telangiectasia. New bone formation were apparent in the icariin groups, and more than the control group. New bone deposition was incremental

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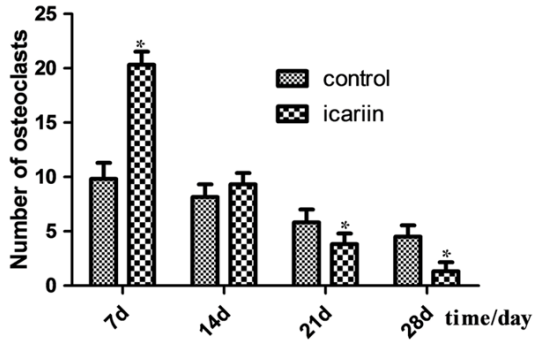


Figure 5. The number of osteoclasts on the pressure side of the periodontium of the first molar during orthodontic tooth movement in the control group (dotted bars) and icariin group (hatched bars). The number of osteoclasts on the pressure side per section was quantified as described in the text. The data were expressed as the mean \pm SME, $n=6$. *Significantly different from the control group * $P<0.05$ by Student's t-test.

as times gone. Additionally, osteoblasts lined on the bone surface in the icariin group were much more than the control group from the histological observation.

Discussion

In the present study, we evaluated the effects of icariin on OTM in rat models histologically and explored the possible mechanism involved. The results suggested that icariin could accelerate OTM in rats through promoting bone remodeling of alveolar bone because of its well-balanced effects on bone formation and bone resorption.

The technique of establishing experimental tooth movement model has been proved to be effective and feasible repeatedly [3, 21, 23, 24]. Previous studies have demonstrated that a 40-60 g level of force stimulated substantial molar tooth movement in rats [24], the force of the coil spring after activation in our experiment is approximately 40 g. Application of a single force to the crown of the first molar to make it move mesially in our study also created a rotation around a point approximately half-way down the root. Thus, pressure is exerted at the distal root apex and at the mesial crest of the alveolar bone, tensile force is applied to the mesial root apex and at the distal crest of the alveolar bone [25]. To ensure the comparability among the groups, the region we chose to measure and compare was the cervical part of the

alveolar bone around the maxillary first distal-palatal root, 1 mm long from the crest along the root.

As mentioned above, Herba Epimedii has been a commonly used in Chinese medicine for treatment of osteoporosis in China [9]. Icariin, as the major pharmacologically active flavonol diglucoside of HEP [9, 26]. In the present study, icariin was used to evaluate its effect on tooth movement when systematic administration in rats. The drug-delivery way we chose was intragastric administration, making the possible result closer to clinical situation, because the main way of intake icariin when used to treat osteoporosis clinically was oral administration of compound preparation [9]. Our chosen dose was $20 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, proven efficient when used in a pharmacokinetic study [11], and in accord with that in treating osteoporosis in rats [22].

In the present study, OTM in rats was induced by icariin generally. The result agreed with the current literature [7]. The latter studies showed three kinds of traditional Chinese medicine (TCM) were beneficial to the alveolar bone remodeling by promoting osteoclast differentiation, and accelerate OTM.

As described above, for a tooth to move, osteoclasts must be formed so that they can remove bone from the area adjacent to the compressed part of the PDL. Osteoblasts also are needed to form new bone on the tension side and remodel absorbed areas on the pressure side [3, 25]. Osteoclast was multinucleated giant cell which was thought to be originated from hematopoietic stem cell of the mononuclear phagocyte system [27]. The origin of osteoclasts in the process of bone remodeling after orthodontic appliance activation has not very clear, but the present data demonstrate that at the initial stage, osteoclasts are originated from the local PDL or the bone marrow of the alveolar bone, and recruitment of precursor cells from hematopoietic tissues is involved at the later stage [3]. Rygh et al [28] reported an increase in the vascular activity of the periodontal ligament and an increase of blood supply to areas of osteoclastic activity on the pressure side during OTM. Although systematic delivery of icariin, it would make effect on the adjacent periodontium of the moved tooth through blood circulation.

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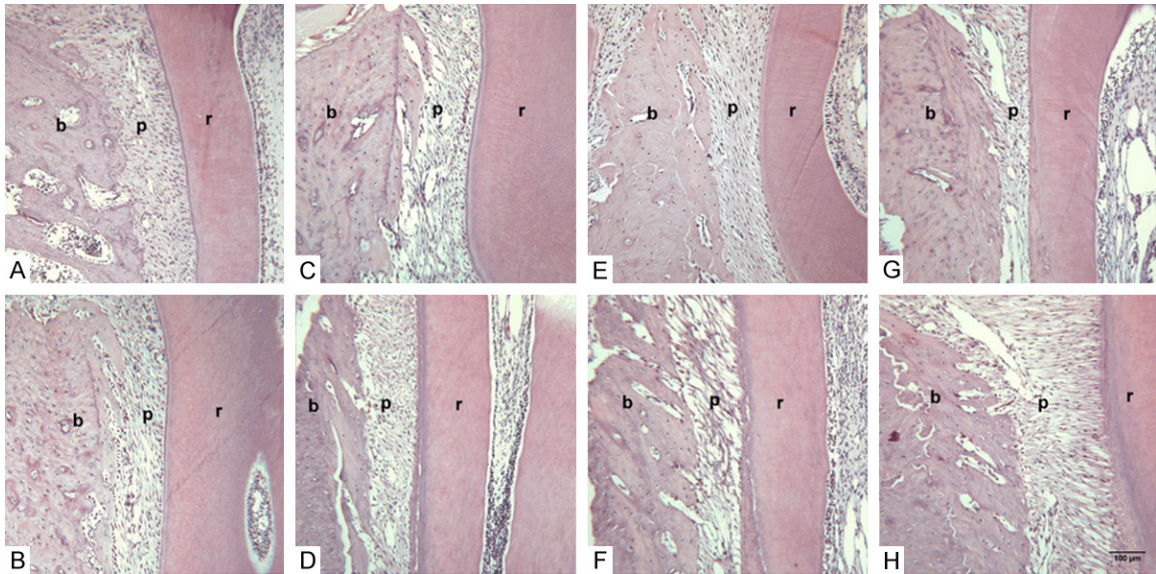


Figure 6. Shows the histological changes in the periodontium of the tension side of the control group (A, C, E, G) and the icariin group (B, D, F, H) on 7th, 14th, 21st and 28th day after application of the orthodontic appliance. Different levels of new bone formation were observed. r, root; b, alveolar bone; p, periodontium. Bar =100 µm.

Osteoclastogenesis was mainly regulated by two different cytokines, macrophage colony-stimulating factors (M-CSF) and receptor activator of nuclear factor-kappa ligand (RANKL) [27]. As reviewed by Proff [29], RANKL was a member of the TNF gene family, which was predominantly expressed on the surface of osteoblast/stromal cells. When RANKL binded to its RANK that exists as a surface receptor on the membrane of pre-osteoclasts, and the osteoclast differentiation was accomplished. Osteoprotegerin (OPG), another cytokine produced by osteoblasts, acts as decoy receptor for RANKL, thereby, inhibited osteoclast differentiation. RANK/RANKL/OPG signaling pathway, as the main access regulate osteoclast function in orthodontics [30]. So, the proportion of RANKL and OPG are critical in the regulation of the differentiation and activation of osteoclasts.

What we observed from the result demonstrated a transient but sudden increase in the number of osteoclasts on the first 7 days of the daily delivery of icariin, but a persistent decrease after that. The number of osteoclasts was less than that of the control group on day 21 and 28, and the osteoclastic bone resorption was also inhibited. From this point, the effect of icariin on osteoclast was inhibition. It agreed with the previously reported studies

[18, 19, 31], in which demonstrated icariin could inhibit osteoclastic differentiation and activities in vitro [31] and suppressed osteoclast formation induced by RANKL and M-CSF in mouse bone marrow cultures [18]. Meantime, further studies demonstrated the direct effects of icariin on osteoblastic cell proliferation and differentiation in vitro [17, 31-33]. These studies suggested that icariin might prevent bone loss by modulating the process of bone remodeling. Zhao et al. [32] demonstrated the induction of osteogenic differentiation in pre-osteoblasts by icariin might be mediated by Runx 2 as well as BMP signaling pathways. [17] showed the possible mechanism that icariin induced osteoblast proliferation, differentiation and mineralization through estrogen receptor-mediated signal activation. [34] also demonstrated in vitro icariin may exert its osteogenic effects through the induction of BMP-2/Smad and nitric oxide (NO) synthesis. In our study, a consistent result was observed, that was the bone formation in the icariin group was more apparent than that in the control group at four time points.

Our histological photos showed that on day 7, there was remarkable increase of the number of TRAP-positive osteoclasts in the icariin group, compared with the control group (Figure 5). Correspondingly, the amount of OTM in the

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icariin group was more than that of the control group on day 7. In the study of [34], with icariin, the RANKL gene expression of osteoblasts was found to have increased significantly on the 7th day. The reason may be that over-expression of Cbfa-1/Runx-2 mRNA in the cells of the osteoblastic lineage does express a positive effect on osteoclast differentiation in vitro and can dramatically enhance bone resorption in vivo through increased RANKL expression [35]. Therefore, icariin might stimulate osteoclasts through up-regulated RANKL and down-regulated OPG at the early stage. The increased RANKL/OPG can promote activation of osteoclasts and differentiation of preosteoclast through RANK/RANKL/OPG signaling pathway [30, 36, 37]. Then a mass of osteoclasts were recruited to the bone surface of the pressure side and the initial bone resorption stimulated by icariin occurred. Bone resorption and formation were closely related with each other. Thus, a pharmacologic agent used to accelerate tooth movement via enhancement of alveolar bone resorption must not disturb this coupling between resorption and formation, such as 1, 25-DHCC, the active form of Vitamin D and PGE2 [38].

Our results declared the OTM in the two groups have made a vast increase during day 21 to 28. The possible reason included two points below, firstly, the smooth progression of tooth movement with light force may be an unattainable ideal. In practice, even with light forces, small avascular areas are likely to develop in the periodontal ligament and tooth movement usually proceeds in a more stepwise fashion because of the inevitable areas of undermining resorption. Tooth movement will be delayed until these can be removed by undermining resorption, resulting in a rapid stage of tooth movement [25]. Secondly, with icariin osteoclasts in the alveolar bone were activated at the early stage just as we observed, resulting in more activated bone remodeling. This may explain the change in the study.

In conclusion, icariin could influence bone resorption through its three successive steps, just as reviewed by [39]. Icariin could inhibit the formation of osteoclast progenitors in hematopoietic tissues and the resorption function of activated osteoclasts directly. Moreover, icariin may make advanced effects on osteoclasts

through osteoblasts indirectly. Icariin took advantages of coupling of bone formation and resorption in vivo, to promote the activity of osteoclasts through RANK/RANKL/OPG signaling pathway, resulting in accelerating tooth movement.

Our results suggested that icariin could promote bone remodeling of alveolar bone and accelerate OTM in rats. However, the exact mechanism involved in OTM has not been unknown, and further in vivo and in vitro studies are needed to elucidate it.

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Disclosure of conflict of interest

None.

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