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Inhibitory effect of baicalin on orthodontically induced inflammatory root resorption in rats

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Abstract

Objective: This study investigated the inhibitory effect of baicalin on orthodontically induced inflammatory root resorption in rats.

Methods: Forty-five male Wistar rats were randomly divided into three groups of 15 rats each. Fifty grams of force was used to establish an orthodontic tooth movement model. Baicalin (40 mg/kg) was locally injected into rats in the baicalin group at 3-day intervals; concurrently, normal saline was injected into rats in the negative control group. On the 21st day after orthodontic treatment, the tooth movement distance and root resorption area ratio were measured. Histomorphology changes were observed by hematoxylin and eosin staining and immunohistochemistry.

Results: There was no significant difference in tooth movement distance between groups. The root resorption area ratio was significantly lower in the baicalin group than in the negative control group. Runx-2 expression was significantly higher in the baicalin group than in the negative control group, while tumor necrosis factor (TNF)-α expression was significantly lower in the baicalin group than in the negative control group.

Conclusions: Baicalin inhibits orthodontically induced inflammatory root resorption by enhancing the expression of Runx-2 and reducing the expression of TNF-α, but does not affect tooth movement distance.

Keywords

Baicalin, orthodontics, root resorption, Runx-2, tumor necrosis factor-α, tooth movement, flavonoid, inflammation

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Introduction

Orthodontically induced inflammatory root resorption (OIIRR) is one of the most common side effects of orthodontic treatment, which has attracted considerable attention from clinicians and researchers. This unpredictable process can occasionally cause severe root shortening that threatens the health of the tooth.[1] Thus, there is an urgent need to avoid onset of OIIRR.

To the best of our knowledge, the development of root resorption can only be checked regularly by radiographs taken during treatment; there is no method for assessment of resorption risk before orthodontic treatment. Several pharmacological agents (e.g., prostaglandin E2, zoledronate, alendronate, and fluoride) may prevent root resorption; however, the clinical applications of these agents are limited because they have adverse effects on orthodontic tooth movement.[2–4] Previous studies have shown that low-intensity pulsed ultrasound reduces the severity of OIIRR and promotes cementum repair by enhancing alkaline phosphatase activity, collagen-I synthesis, and runt-related transcription factor 2 (Runx-2) protein levels in cementoblasts.[5,6] However, extensive exposure to low-intensity pulsed ultrasound can cause occasional degeneration and fibrosis.[7]

Flavonoids, a group of naturally occurring compounds that are commonly found in various vegetables and herbal medicines, have been extensively investigated regarding their abilities to affect bone metabolism. Baicalin (7-glucuronic acid, 5, 6-dihydroxyflavone) is the major flavonoid isolated from dry roots of *Scutellaria baicalensis* Georgi.[8,9] Baicalin has multiple biologic effects, including anti-inflammatory,[10,11] anti-tumor,[12] anti-bacterial,[13] anti-viral,[14] and antioxidant functions.[15] Baicalin has been shown to improve cell viability, enhance osteoblast activity, and elevate levels of Runx2 and osteocalcin expression in primary osteoblasts through activation of Wnt/β-catenin and mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathways.[16] Moreover, baicalin has been shown to increase osteoclast maturation and function, which may aid in treatment of bone fracture.[17] Baicalin can regulate the expression of osteoprotegerin in osteoblasts; thus, it might influence the differentiation of osteoblasts and osteoclasts.[18] Baicalin may also be a potential therapeutic agent for the treatment of periodontitis;[19] animal studies have demonstrated its protective effects on periodontal tissue in periodontitis.[20] Baicalin can induce significant expression of type I collagen (at both mRNA and protein levels) in periodontal ligament cells.[21] Therefore, baicalin is presumed to have an inhibitory effect on OIIRR. The present study aimed to investigate whether baicalin exhibits an inhibitory effect on OIIRR, thereby providing relevant information for clinical treatment.

Materials and methods

Experimental rats

Animal studies were approved by the Animal Ethics Committee of Shandong University. For this study, 45 specific pathogen-free male Wistar rats were purchased from the Shandong University Medical Laboratory Animal Center. The rats weighed 200 ± 10 g and were aged 8 weeks; all had normal periodontal and dental characteristics (i.e., no periodontitis, dental caries, or defects in dentition). Rats were fed and housed in the animal laboratory; the laboratory environment included temperature of 20°C to 25°C, balanced humidity of 45% to 60%, noise <50 decibels, and 12-hour light/dark circle. Food and water were provided ad libitum.

Main reagents and materials

The following reagents, materials, and equipment were used in this study:

Baicalin (99% purity; Nanjing Jingzhu Biotechnology Co., Ltd., Nanjing, China); Runx2 antibody (sc-390351, Santa Cruz Biotechnology, Inc., Dallas, TX, USA); tumor necrosis factor-α (TNF-α) antibody (sc-28318, Santa Cruz Biotechnology, Inc.); SP Kit (Solarbio Science & Technology, Beijing, China); DAB Substrate kit (Solarbio Science & Technology); orthodontic materials (Hangzhou Xinya Dental Materials Co., Ltd., Hangzhou, China); electronic vernier caliper (Mitutoyo Corporation, Kawasaki, Japan); scanning electron microscope (Olympus Corporation, Tokyo, Japan); alginate impression material (Hangzhou Xinya Dental Materials Co., Ltd.); and dental gypsum (Hangzhou Xinya Dental Materials Co., Ltd.).

Experimental procedures

Forty-five male Wistar rats (8 weeks old) were divided into a baicalin group (n=15), a negative control group (n=15), and a blank group (n=15). All models were established after rats had been fed for 1 week. Rats were placed in the supine position and anesthetized with 2% sodium pentobarbital. The orthodontic appliance consisted of a nickel–titanium closed coil spring, which was placed between the right maxillary first molar and incisor. The spring was ligated to the right maxillary first molar and incisors by means of 0.010-in. steel ligature wires. To secure the appliance in place, a cervical groove was prepared on the incisor for ligature wire seating; the ligature wire was also covered with composite resin. The force exerted by this appliance was approximately 50 g (Figure 1). With rats under anesthesia, baicalin (40 mg/kg) was locally injected into the mucoperiosteum around the right maxillary first molar of rats in the baicalin group at 3-day intervals; equal volumes of normal saline were injected into rats in the negative control group in the same manner, with the same intervals. On the 21st day after insertion of orthodontic appliances, all rats were sacrificed by overdose with 2% sodium pentobarbital. All experimental tissues were fixed in 4% paraformaldehyde for 12 hours.

Assessment of tooth movement

Tooth movement was assessed two times (before the experiment and on the 21st day after insertion of orthodontic appliances) using an electronic vernier caliper (accuracy of 0.02 mm), from the distal contact area of the first molar to the mesial contact area of the second molar. All rats had tight contacts between molars at the beginning of the experiment. An additional silicone impression was taken and poured

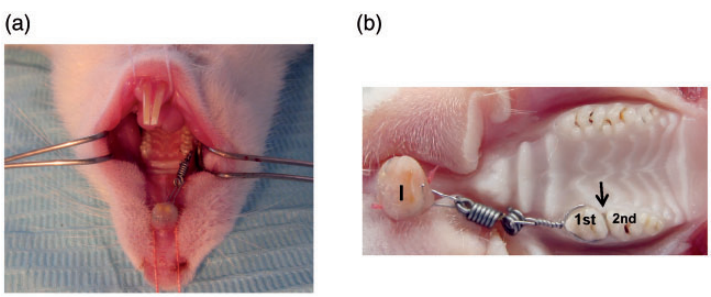

Figure 1. Photographs of experimental setup. (a) Animal model. (b) Tissues assessed in this experiment. Arrow indicates tooth movement.
Abbreviations: I, Incisor; 1st, first molar; 2nd, second molar.

with dental gypsum to ensure reproducible measurements of tooth movement (Figure 2a). All measurements were repeated three times by the same investigator to ensure consistency.

Scanning electron microscopy

Extracted rat teeth were evaluated to determine resorptive changes on the molar root surface. Rat maxillae were incubated for 12 hours in 5% sodium hypochlorite solution, beginning immediately after extraction; first molars were then carefully extracted. The right maxillary first molar root was then stripped and residual periodontal tissue was removed; the other roots were ground off of the tooth. Scanning electron microscopy examinations were performed on the mesial surfaces of the right maxillary first molar root; measurements were processed with Mimics 10.0 software (Materialise Corporation, Leuven, Belgium).

Histological study

To evaluate histological changes in bone and tissue surrounding each examined tooth, rats' posterior maxillae (i.e., three molar teeth, bone, and soft tissue) were dissected and immersed in 4% paraformaldehyde for 48 hours. Samples were rinsed with water and placed in 10% ethylenediaminetetraacetic acid solution for 2 to 3 months to soften the bone. Samples were seated in paraffin and 5-mm-thick mesiodistal sections were cut; every fifth section was stained with hematoxylin and eosin.

Immunohistochemical staining

Sections were prepared as described in the Histological study section above; they were then dewaxed and rehydrated. Endogenous peroxidase activity was inhibited by treatment with 3% H_2O_2 for 10 minutes at 25°C; sections were then incubated with goat serum (SL038, Solarbio Science & Technology) for 20 minutes at 37°C, incubated with polyclonal Runx-2 or TNF-α antibodies (each at a dilution of 1:100 in phosphate-buffered saline) at 37°C for 1 hour, and then incubated overnight at 4°C with the same antibodies. The secondary antibody (rabbit anti-goat IgG; SE238, Solarbio Science & Technology) was added for 15 minutes at a dilution of 1:100 in phosphate-buffered saline; sections were then incubated with the SP Kit for

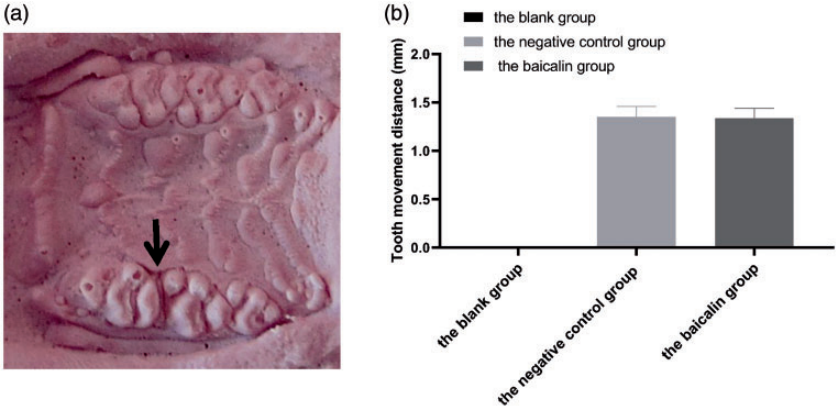

Figure 2. Tooth movement distance measurement. (a) Gypsum model of tissues assessed in this experiment. Arrow indicates tooth movement distance. (b) Distances of tooth movement in each group (n = 15). Data are expressed as mean ± standard deviation (**$P < 0.05$).

15 minutes. Finally, sections were developed with the DAB Substrate Kit for 2 minutes. Counterstaining was performed by incubation with hematoxylin for 30 s. Immunohistochemical controls comprised sections processed with normal non-immune serum, rather than primary antibodies. Sections were examined by light microscopy (Carl Zeiss, Oberkochen, Germany). The average optical density (AOD) in periodontal tissues was measured using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA) to determine the staining intensities corresponding to expression of Runx-2 and TNF-α.

Statistical analysis

IBM SPSS Statistics, version 20.0 (IBM Corp., Armonk, NY, USA) was used for statistical analyses. Measurement data were expressed as mean ± standard deviation; data were compared using one-way analysis of variance and post hoc least significant difference test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Tooth movement distance measurements

Following 21 days of experimental force application, all rats had substantial spaces between the first and second right maxillary molars. The average distance measured was slightly lower in the baicalin group (1.339 ± 0.098 mm) than in the negative control group (1.352 ± 0.105 mm); however, this difference was not statistically significant (Figure 2b).

Scanning electron microscopy findings and root resorption ratios

Root resorption craters with different forms and rough cementum areas were observed in the baicalin and negative control groups after the experiment, mainly in the cervical and middle thirds of the root; no obvious root resorption was observed in the blank group. Large numbers of root resorption lacunae were found in the negative control group, whereas small numbers of root resorption lacunae were found in the baicalin group (Figure 3). The mean root resorption area ratio was significantly lower in the baicalin group (8.73 ± 1.45%) than in the negative control group (29.55 ± 4.61%, $P < 0.05$; Figure 3).

Histological staining results

Hematoxylin and eosin staining was used to observe the reconstruction of periodontal tissues. In the blank group, periodontal ligament specimens were composed of relatively dense connective tissue fibers and fibroblasts that exhibited regular arrangements in a horizontal direction from the root cementum toward the alveolar bone. In the negative control group, many resorption lacunae with multinucleate osteoclasts were observed on the alveolar bone surface; large numbers of root resorption lacunae with multinucleate odontoclasts were observed on the root surface. In the baicalin group, small numbers of osteoclasts and root resorption lacunae were found in the periodontal ligament (Figure 4).

Expression levels of Runx-2 and TNF-α in periodontal tissues

To study the effects of baicalin on osteoblast and osteoclast differentiation during orthodontic tooth movement, the expression levels of Runx-2 and TNF-α were evaluated. Runx-2 expression was mainly observed in osteocytes, osteoblasts, and fibroblasts in periodontal tissues (Figure 5). The mean AOD of the Runx-2-positive signal indicated substantial expression in the baicalin group on the tension side in periodontal tissues

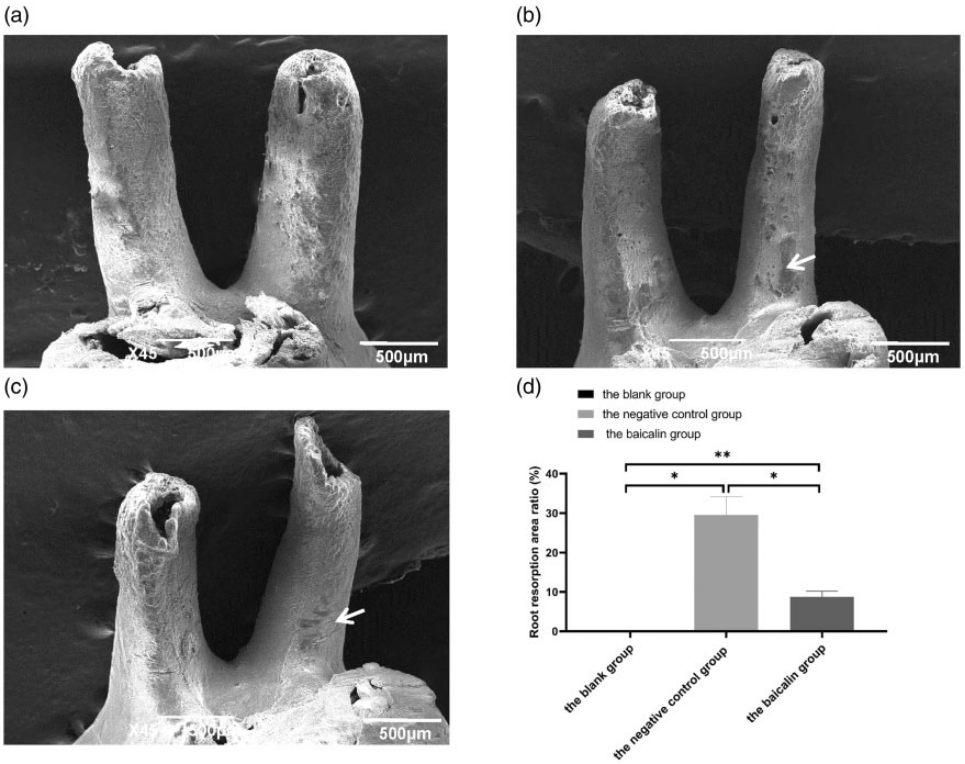

Figure 3. Micrographs of roots, taken by scanning electron microscopy. (a) Blank group. (b) Negative control group. (c) Baicalin group. Arrow indicates root resorption lacunae. (a–c) Bars indicate 500 μm. (d) Mean root resorption area ratio in each group (%). Data are expressed as mean ± standard deviation (*P<0.05, **P<0.05).

(0.127 ± 0.010). In the negative control group, the mean AOD value was 0.081 ± 0.009; in the blank group, the mean AOD value was 0.026 ± 0.004. Runx-2 expression levels were significantly higher in both the baicalin and negative control groups than in the blank group ($P < 0.05$). Moreover, the Runx-2 expression level on the tension side was significantly higher in the baicalin group than in the negative control group ($P < 0.05$; Figure 5).

TNF-α expression was mainly observed in osteoclasts and fibroblasts in periodontal tissues. Some osteocytes scattered in alveolar bone adjacent to resorbing surfaces also exhibited TNF-α expression (Figure 6). The mean AOD of the TNF-α-positive signal indicated considerable expression in the negative control group on the pressure side in periodontal tissues (0.139 ± 0.013). In the baicalin group, the mean AOD value was 0.086 ± 0.011; in the blank group, the mean AOD value was 0.037 ± 0.006. TNF-α expression levels were significantly higher in both the negative control and baicalin groups than in the blank group ($P < 0.05$). Moreover, the TNF-α expression levels on the pressure side was significantly lower in the baicalin group than in the negative control group ($P < 0.05$; Figure 6).

Discussion

OIIRR is a common complication of orthodontic treatment,[22] which can be induced

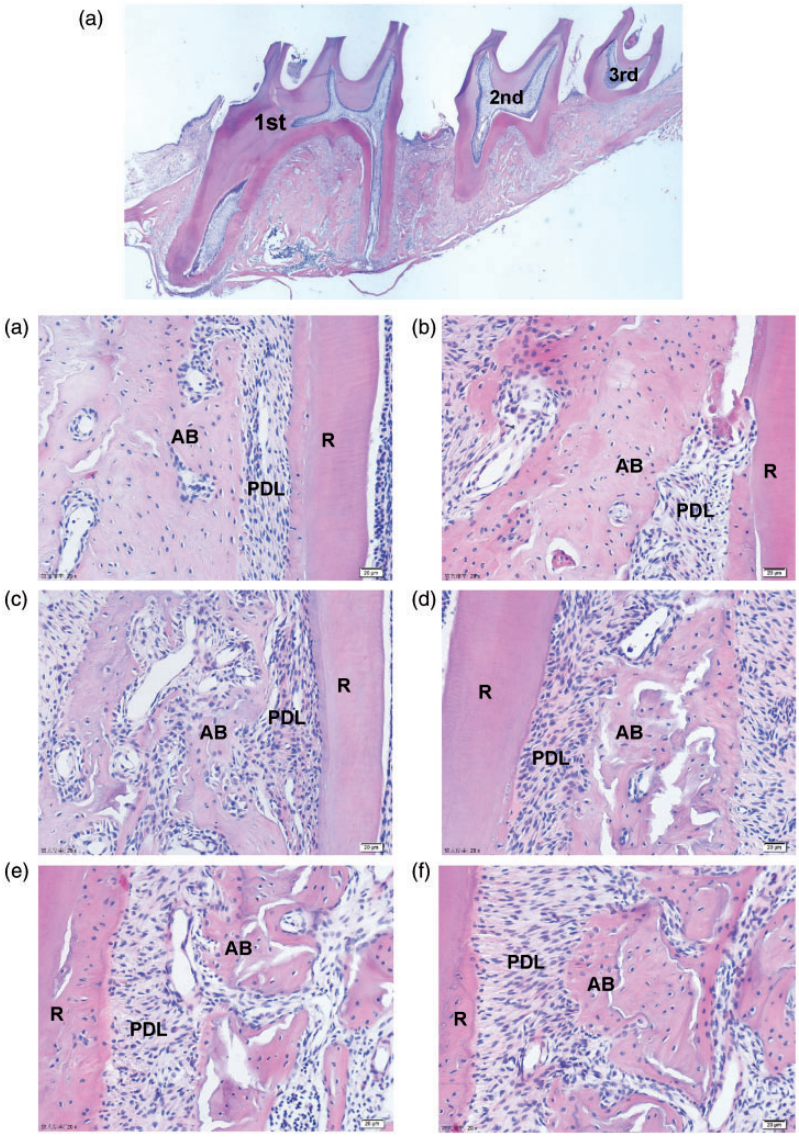

Figure 4. Histological changes in periodontal tissues. (a) Rat tooth subjected to orthodontic treatment (hematoxylin and eosin staining). (b, c, and d) Pressure side of periodontal tissues. (e, f, and g) Tension side of periodontal tissues. (b and e) Blank group. (c and f) Negative control group. (d and g) Baicalin group (hematoxylin and eosin staining). (a) Bar indicates 500 μm. (b–g) Bars indicate 20 μm
Abbreviations: 1st, first molar; 2nd, second molar; 3rd, third molar; AB, alveolar bone; PDL, periodontal ligament; R, root.

by the application of heavy orthodontic force, injury to moving teeth, metabolic diseases, traumatic occlusion,[23] and other causes (e.g., systemic conditions,[24] malocclusion type,[25] or treatment period[26]). OIIRR is an inflammatory response characterized by the periodontal accumulation of many proinflammatory

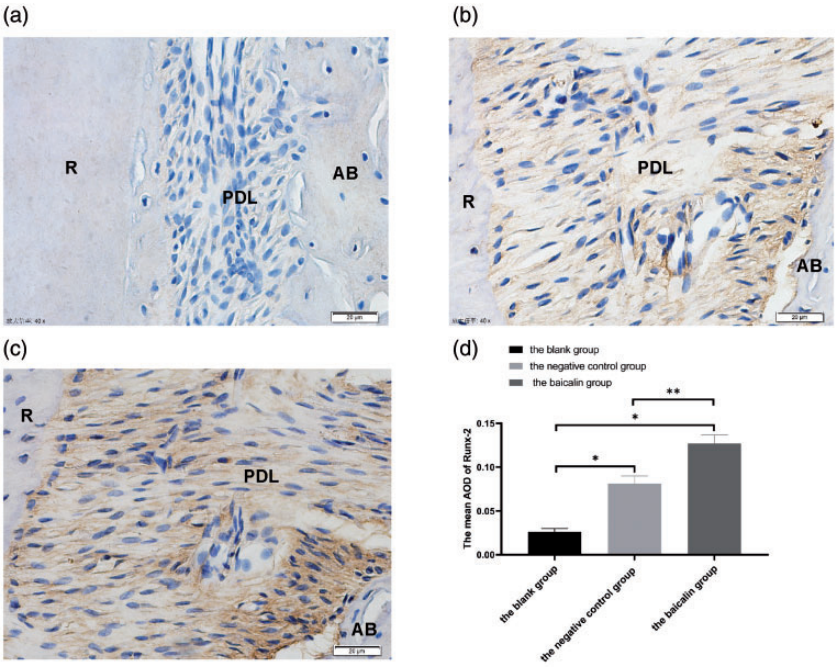

Figure 5. Immunohistochemical staining of Runx-2 on the tension side. (a) Blank group. (b) Negative control group. (c) Baicalin group. (a–c) Bars indicate 20 μm. (d) Mean AOD of Runx-2. Data are expressed as mean ± standard deviation (*$P<0.05$, **$P<0.05$).
Abbreviations: AB, alveolar bone; AOD, average optical density; PDL, periodontal ligament; R, root.

cytokines released from migrated cells and paradental resident cells, including TNF-α, interleukin-1β, interleukin-6, interleukin-7, interleukin-8, prostaglandin E2, and cyclooxygenase-2.[27] Therefore, inhibition of early acute inflammation of periodontal tissue and promotion of periodontal tissue regeneration could be a feasible approach for avoidance of OIIRR.

Baicalin is a natural molecule found in the Baical skullcap root (*Scutellaria baicalensis* Georgi).[9] Baicalin has a variety of pleiotropic properties including anti-inflammatory,[28,29] antitumor,[30] antiviral,[31] and antibacterial effects,[13] which have been attracted widespread attention. There is considerable evidence that baicalin might promote osteogenic differentiation. Wang et al.[16] demonstrated that baicalin accelerated osteogenic differentiation of osteoblasts through elevation of Wnt/β-catenin and MEK/ERK pathways. Baicalin triggers activation of Wnt/β-catenin and MEK/ERK pathways by means of miR-217 inhibition. Aya et al.[32] confirmed that baicalin enhanced osteogenic differentiation of human cementoblast cells through the Wnt/β-catenin signaling pathway, which may be useful for promotion of periodontal tissue regeneration. Wang et al.[33] showed that baicalin is able to enhance bone mineralization. Notably, baicalin modulates the Ca^{2+} homeostasis pathway, which is crucial for controlling secondary metabolism via protein kinase C, as well as for skeletal anabolism (i.e., hydroxyapatite formation). Baicalin might be useful as a component of nutraceuticals for osteoporosis prevention, or in bone implants. Moreover, Li et al.[17] revealed that baicalin

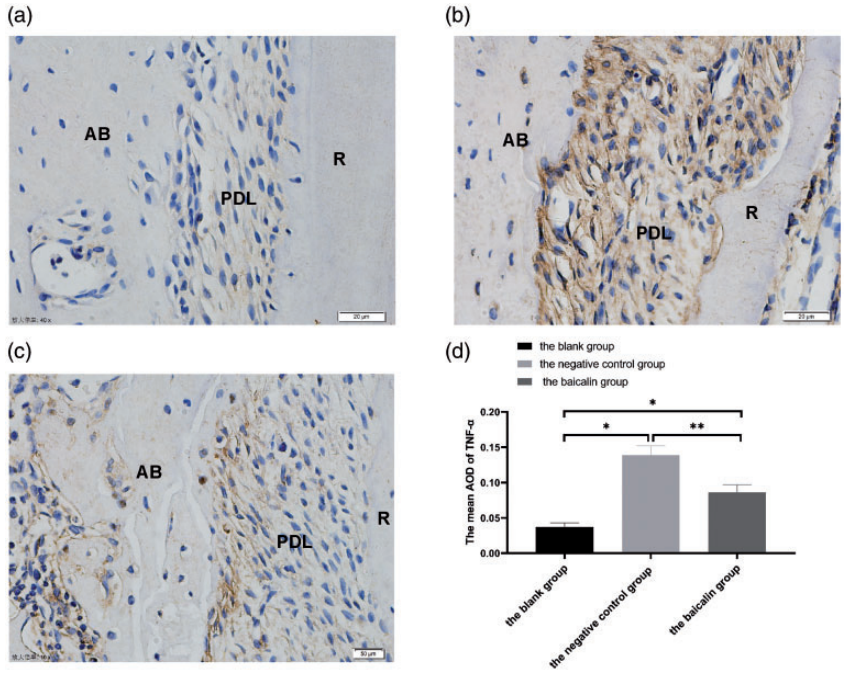

Figure 6. Immunohistochemical staining of TNF-α on the pressure side. (a) Blank group. (b) Negative control group. (c) Baicalin group. (a–c) Bars indicate 20 μm. (d) Mean AOD of TNF-α. Data are expressed as mean ± standard deviation (*P<0.05, **P<0.05).
Abbreviations: AB, alveolar bone; AOD, average optical density; PDL, periodontal ligament; R, root.

promoted osteoclast maturation and function via p-ERK/Mitf signaling. Thus, baicalin can potentially be used as a natural product for the treatment of bone fracture.

In this study, the average tooth movement distance was slightly lower in the baicalin group than in the negative control group; however, this difference was not statistically significant. Therefore, baicalin does not significantly influence tooth movement distance during orthodontic treatment. Scanning electron microscopy observation revealed large numbers of root resorption lacunae in the negative control group, whereas small numbers of root resorption lacunae were observed in the baicalin group. The mean root resorption area ratio was significantly lower in the baicalin group than in the negative control group. This finding indicated that baicalin was able to reduce root resorption in rats.

Runx2 is considered a crucial transcription factor during osteoblast differentiation, which plays a vital role in bone formation.[34] In addition, TNF-α plays an important role in compressive-force-induced odontoclast formation and root resorption during orthodontic tooth movement.[35] Therefore, Runx-2 and TNF-α are suitable markers for osteoblasts and osteoclasts, respectively.[36] Immunohistochemical staining showed that the Runx-2 expression level on the tension side was significantly higher in the baicalin group than in the negative control group, while the TNF-α expression level on the pressure side was significantly lower in the baicalin group than in the negative control group. Thus, baicalin may play a role in

prevention of OIIRR by promoting the expression of Runx2 on the tension side and reducing the expression of TNF-α on the pressure side in periodontal tissue surrounding teeth subjected to orthodontic treatment.

There were some limitations in this study. First, it only examined the short-term effect of baicalin on rats subjected to orthodontic treatment. Second, it is unclear whether the findings in this study can be applied to patients in clinical practice. Therefore, future studies should evaluate the long-term effects of baicalin during orthodontic treatment and explore the mechanism by which baicalin inhibits root absorption. Furthermore, clinical trials are needed to confirm whether our findings are generalizable to clinical applications.

Conclusion

The findings in this study confirmed that baicalin can inhibit OIIRR by promoting the expression of Runx-2 and reducing the expression of TNF-α, although it does not significantly influence tooth movement distance during orthodontic treatment.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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