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The effects of dried root aqueous extract of *Salvia miltiorrhiza* and its major ingredient in acceleration of orthodontic tooth movement in rat

Li-qun Xiao [1], Hong-tao Wang [1], Yu-lan Li [1], Qing Zeng [1], E Zhou [1], Xia Ni [1], Zhong-ping Huan [1*]

[1] Department of Stomatology, The First College of Clinical Medical Science of China Three Gorges University, Yichang Central People's Hospital, Yichang 443003, China

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ABSTRACT

Objective(s): *Salvia miltiorrhiza* (SM) is a popular and classic herb in traditional Chineses medicines. The objective is to confirm the effects of aqueous extract of *S. miltiorrhiza* (ESM) and its main ingredient on the promotion of orthodontic tooth movement and healing of periodontal ligament in rat.

Materials and Methods: Male Sprague-Dawley rats (n= 150) were divided into five groups: model control group (0.5 ml/kg phosphate-buffered saline (PBS) injection), ESM group (0.75 g/kg/day of crude drugs) and Danshensu subgroups (250, 500, 750 mg/kg/day of body weight). All rats were administered intramuscularly into the buccal vestibular mucosa of first molar of left maxillary. The indicators such as the moving distance of orthodontic tooth, nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) expression and osteoclasts were tested.

Results: The expressions of RANKL and OPG in the treatment groups were obviously enhanced compared with control group (P<0.05). The increase rate of OPG expression was slower than that of RANKL. But, RANKL decreased conspicuously after no orthodontic pressure was applied, especially in the treatment groups (Danshengsu high dose group at day 30: 2.17 versus 3.47 of control, P<0.01). ESM groups promoted osteoclasts proliferation in the first 20 days.

Conclusion: There is a relationship between RANKL/OPG ratio and the number of osteoclasts. ESM might accelerate periodontal alteration of rat orthodontic tooth via producing more osteoclasts.

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Introduction

Orthodontic tooth treatment usually involves a long period of time for both patients and physicians due to the long treatment course and numerous subsequent visits. How to shorten orthodontic treatment is still a hot topic for both researchers in the lab and medical research and development institutions.

Many methods such as low-intensity pulsed ultrasound (1), low-level laser therapy (2), LED-mediated-photobiomodulation therapy (3), para-thyroid hormone (4), and many Chinese herbal medicines (i.e. puerarin (5), miltiorrhiza and drynaria (6)) have been tried to accelerate teeth movement. Most of them are still under animal studies, and there is no authoritative recommendation yet. However, Chinese herbal medicines are applied to rescue people from pain in eastern Asia for thousands of years. Considering that it is based on physiological modulation but not physical stimulus, it is believed that herbal medicine is safer and healthier that can provide benefit for patients.

Salvia miltiorrhiza (SM) Bunge (Lamiaceae) is a popular herb in traditional Chinese medicines used for promoting circulation and removing stasis. Its pharmacological activities on improving bone density (7-9), and accelerating the movement of orthodontic tooth were recently reported (10, 11). The main aqueous components of SM are Danshensu, protocatechuic aldehyde, protocatechuic acid, etc. The main fat-soluble ingredients are composed of hidden tanshinone, tanshinone I, tanshinone IIA, tanshinone IIB, etc. In this study, only Danshensu is considered and evaluated as it is the major water-soluble compound of SM. Therefore, the effects of ESM and its main ingredient Danshensu on the expressions of receptor activator for nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) in periodontal ligament of rat orthodontic tooth were warranted to be studied and discussed.

Materials and Methods

Animals

One hundred fifty male Sprague-Dawley rats at the age of 6 to 8 weeks with the body mass of 180 to 200 g

**Corresponding author:* Zhong-ping Huan, Department of Stomatology, The First College of Clinical Medical Science of China Three Gorges University, Yichang Central People's Hospital, Yichang 443003, China. email: huanzhongp@sina.com, Tel: +86-0717-6491347

were selected for this study. They were housed in natural light with laboratory temperature (25 ± 1˚C) and humidity of about 50% under ventilation condition. They were provided a fixed dose of food with free access to drinking water. After the adaptive feeding for 7 days, they were divided into five groups: control group of orthodontic force model (orthodontic force plus 0.5 ml/kg phosphate-buffered saline (PBS) injection), ESM group (orthodontic force plus injection with 0.5 ml/kg ESM, which was equivalent to 0.75 g/kg of crude drugs) and Danshensu subgroups (orthodontic force plus injection with 0.5 ml/kg Danshensu, which was equivalent to 250, 500, 750 mg/kg of body weight, respectively). Once orthodontic force model was established, rats in each group were sacrificed in batches at the time of 0, 5, 10, 20, 30 days by injecting an overdose of anesthesia, six for each batch.

Plant materials, extraction and isolation

The dried roots of SM were purchased from the Jiangxi Huiren Group. The aqueous extract method was referenced from Wang's report (11). In brief, 500 g of dried herbs was heated in 4 L double distilled water for 2 hr, this step was repeated for twice (2 × 4 l). The extract was concentrated, centrifuged and filtered. After that, the suspension was freeze-dried into powder and stored for use. The extract powder was tested by HPLC for fingerprint. The related peaks were checked with each pure compound, and components were basically matched with previous study (12). The final concentration of water solution prepared for use was equivalent to 0.75 g/kg of crude drugs.

Drugs and chemicals

The drugs and reagents used in this study were as follows: Danshensu (content>98%; Nanjing Zelang pharmaceutical Technology Co., Ltd., Jiangsu, China; No.: ZL201104162; structure was shown in Figure 1); anti-RANKL (N-19) antibody [sc-7628] (Santa Cruz Biotechnology, Inc., Texas, USA); anti-OPG (N-20) antibody [sc-8468] (Santa Cruz Biotechnology, Inc., Texas, USA), ultra-sensitive two-step immunohistochemical detection reagents (Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) and 3, 3-diaminobenzidine (DAB) kit (Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China).

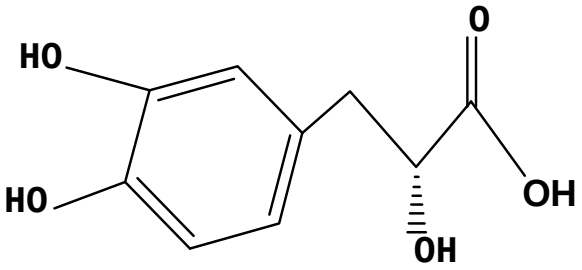

Figure 1. The molecular structure of Danshensu

Rat model of orthodontic tooth

The rat model of orthodontic tooth was referenced from Ren's report (13). The ligation of a nickel-titanium helical tension spring between the first molar of left maxillary and the upper incisors was performed with the pulling force of 40 g, and dragging the tilt movement of the first molar (Figure 2). The orthodontic force was reinforced per 5 day, ensuring stable pulling force of 40 g. The orthodontic force was no longer carried out 20 days later. In this model, the mandibular first molar was ground to reduce the bite force suffered by first molar of left maxillary. The occlusal trauma caused by tilting movement of first molar of left maxillary would be prevented.

Since the date of the rat model established, about 0.1 ml of different drugs (0.5 ml/kg) were injected into the buccal vestibular mucosa of first molar of left maxillary for different groups including PBS for the model control group, ESM (0.75 g/kg/day of crude drugs) for ESM group and Danshensu (250, 500, 750 mg/kg/day of body weight) for Danshensu subgroups. All animals were supplemented with soft diet in the first two days of model established.

Measurement of moving distance of orthodontic tooth

A stereo microscope was used to measure the ditch distance near tongue between the first and second molar occlusal surfaces of the model. Three repeated measurements for each sample were conducted. The mean value was regarded as the movement distance after adding pulling force in rat model.

Semi-quantitative measurement of RANKL and OPG expression

After conventional embedding, sectioning, and dewaxing in water, the tissue was sealed in 3% H_2O_2 for 10 min. Then, it was washed with PBS (3×3 min), mixed with the first antibody at 4ºC overnight, and then washed again with PBS (3×3 min). The second antibody was dripped at 37ºC and incubated for 20 min. Then, after washing with PBS (3×3 min) and dripping with DAB, it was colored under microscope.

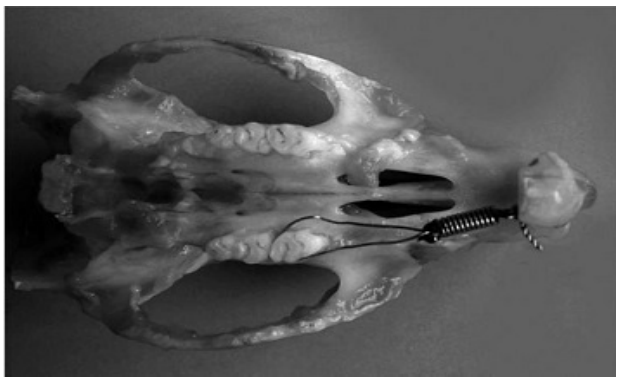

Figure 2. Skull of rats after the decollement of soft tissue

After PBS (2 × 3 min) washing, hematoxylin was used for counterstaining for 2 min and washed with tap water. After the differentiation of 1% hydrochloric acid and alcohol for 10 sec, it was washed by water, dehydrated, made transparent, and finally mounted.

At the positions of 1/3 root collar of periodontal ligament on the pressure side of rat orthodontic tooth (14), 10 visions (×400) of each slice were randomly selected for the measurement of the mean value of optical density (OD), which was tested by using image-pro plus 6.0 image analysis system to analyze the immunohistochemical staining intensity of RANKL, and OPG in the peripheral tissues of rat teeth. The test for each group was repeated in triplicate, and the results showed in the tables were the average value of three times.

Osteoclasts counting by tartrate-resistant acid phosphatase (TRAP) staining

After conventional embedding, sectioning, and dewaxing in water, the tissue was fixed in the fixative buffer for 30 sec, rinsed with deionized water, and heated in water bath to 37ºC with TRAP staining in dark for 1 hr. Then it was washed with deionized water, and counterstained with hematoxylin for 2 min. After wards, the tap water washing, dehydrating, making transparencies, and mounting was performed. On each slice, 10 visions (×400) were selected for osteoclasts counting with the TRAP staining positive color of wine red.

Statistical analysis

All data were expressed in the form of mean±standard deviation ($\bar{x}$ ±s). One-way ANOVA followed by Tukey's *post hoc* tests two-way analysis of variance as well as Bonferroni correction were executed with SPSS 21.0 analysis software. Compared with model control group, **P*<0.05 indicated a significant difference, and ***P*<0.01 represented a highly significant difference.

Results

The movement distance of orthodontic tooth

As shown in Figure 3, from the beginning of day 10, the movement distances of orthodontic tooth in ESM group (Equivalent to 0.75 g/kg/day of crude drugs), and Danshensu subgroups (250, 500, 750 mg/kg/day) except low-dose group were significantly longer than that in the model control group (0.5 ml/kg PBS, *P*<0.01). From the beginning of day 20, the movement distance of orthodontic tooth in Danshensu low-dose group was also significantly longer than that of the model control group (*P*<0.05).

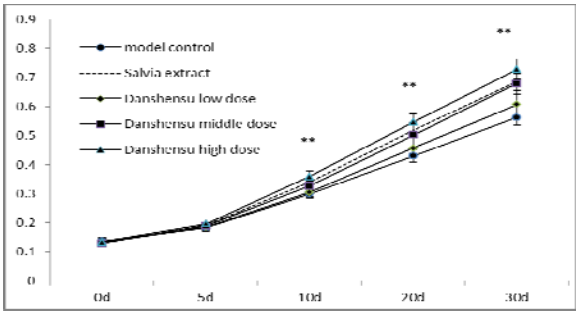

Figure 3. The movement distance of rat left maxillary first molar in each group ($\bar{x}$ ±s, n=6)

The OD value of RANKL staining

The average OD value of rat periodontal ligament after RANKL staining under orthodontic tooth pressure increased significantly (Figure 4 and 5). From the beginning of day 5, the OD values of RANKL staining in ESM group (Equivalent to 0.75 g/kg/day of crude drugs) and Danshensu subgroups (250, 500, 750 mg/kg/day) except low-dose group were significantly longer than that in the model control group (0.5 ml/kg PBS, *P*<0.05). From the beginning of day 10, the OD value of RANKL staining in Danshensu low-dose group was also significantly longer than that in the model control group (*P*<0.05). Since no orthodontic tooth pressure performed anymore during day 20 to 30, the mean OD value of RANKL staining in each group was significantly reduced.

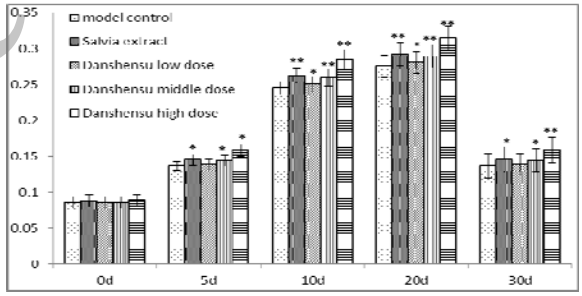

Figure 4. The mean optical density value of nuclear factor κB ligand staining in each group ($\bar{x}$ ±s, n=6)

The OD value of OPG staining

The average OD value of rat periodontal ligament after OPG staining under orthodontic tooth pressure increased very slowly (Figure 6 and 7). From the beginning of day 10, the OD values of OPG staining in ESM group (Equivalent to 0.75 g/kg/day of crude drugs) and Danshensu subgroups (250, 500, 750 mg/kg/day) except low-dose group were significantly longer than that in the model control group (0.5 ml/kg PBS, *P*<0.05). There was no significant effect shown in Danshensu low-dose group totally. Since no orthodontic tooth pressure performed anymore during day 20 to 30, the mean OD value of OPG staining in each group continued to increase, showing its relatively slow reaction in the rat model of orthodontic tooth.

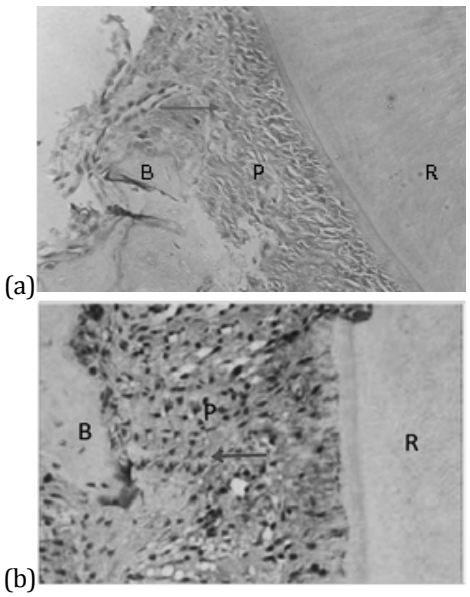

Figure 5. The nuclear factor κB ligand staining image (×400) on the pressure side of maxillary first molar in rat model at day 5 under (a) aqueous extract of *Salvia miltiorrhiza* intervention and (b) control group (B: alveolar bone; P: periodontal ligament; R: tooth root; Arrow: positive periodontal ligament fibroblasts in staining)

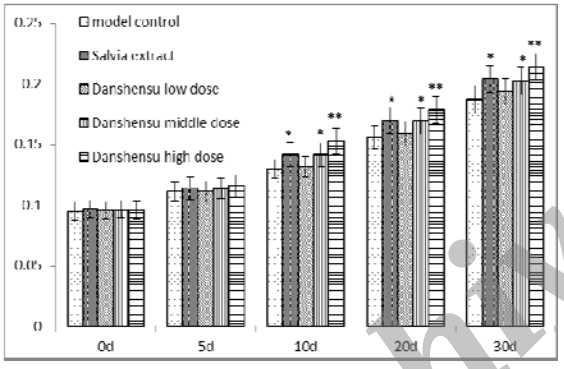

Figure 6. The mean optical density value of osteoprotegerin staining in each group ($\bar{x}$ ±s, n=6)

Osteoclasts counting

The osteoclasts in the rat periodontal ligament of orthodontic tooth significantly increased (Figure 8 and 9). From the beginning of day 5, the numbers of osteoclasts in ESM group (Equivalent to 0.75 g/kg/day of crude drugs) and Danshensu subgroups (250, 500, 750 mg/kg/day) except low-dose group were significantly higher than that in model control group (0.5 ml/kg PBS, P<0.05). From the beginning of day 10, the number of osteoclasts in Danshensu low-dose group was also significantly higher compared with model control group (P<0.05). Since no orthodontic tooth pressure reinforced anymore during day 20 to 30, the number of osteoclasts in each group was significantly low. In particular, osteoclast number in each treatment group was significantly low compared with the model control group, and extremely different from those under rat model of orthodontic tooth. It can be assumed that SM promoted the proliferation of osteoclasts in the first 20 days.

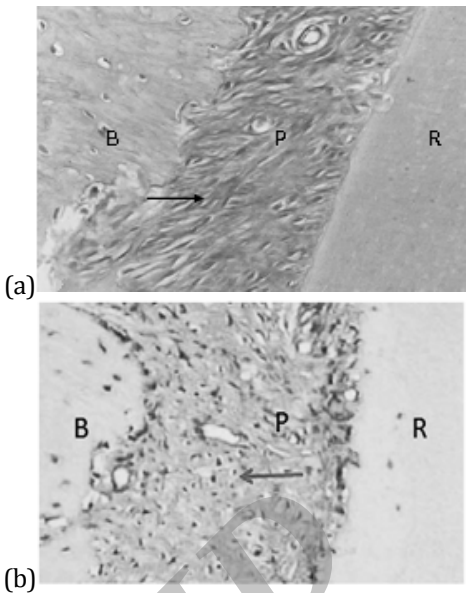

Figure 7. The osteoprotegerin staining image (×400) on the pressure side of maxillary first molar in rat model at day 5 under (a) aqueous extract of *Salvia miltiorrhiza* intervention and (b) control group. (B: alveolar bone; P: periodontal ligament; R: tooth root; Arrow: positive periodontal ligament fibroblasts in staining)

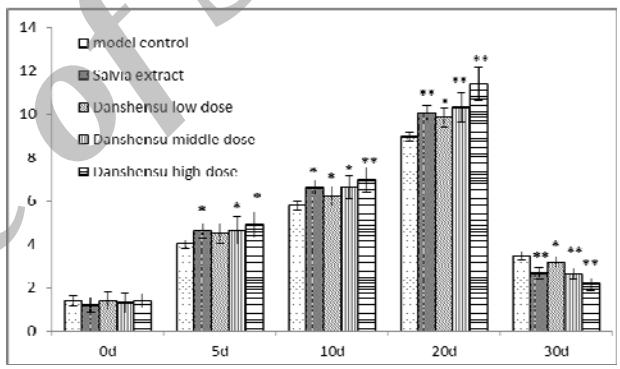

Figure 8. The osteoclasts counting in each group ($\bar{x}$ ±s, n=6)

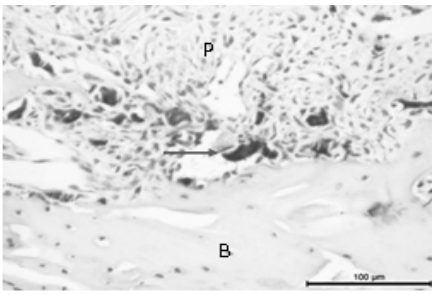

Figure 9. The tartrate-resistant acid phosphatase staining image (×400) on the pressure side of maxillary first molar in rat modeling at day 5 under aqueous extract of *Salvia miltiorrhiza* intervention (B: alveolar bone; P: periodontal ligament; Arrow: positive osteoclasts in staining)

Discussion

The movement of orthodontic tooth can be divided into three periods of initial, plateau and fast-moving period (13, 16). This classic three-period pattern was demonstrated in each group of this experiment. Plateau

period was due to periodontal fiber remodeling under orthodontic tooth pressure, which led to blood flow blocked and hyaline degeneration in the periodontal ligament, and finally blocked tooth movement. Only when the necrotic tissues were removed and adjacent alveolar bone was absorbed, the further movement of orthodontic tooth occurred. In this process, phagocytic cells recruited from bone marrow or adjacent intact periodontal ligament (17). In figure 3, it is shown that SM intervention spent shorter time during the plateau period. So, it was hypothesized that maybe SM can improve local microcirculation of periodontal ligament, leading to the re-opening of blood vessels with structural disorder and atresia in the periodontal ligament of orthodontic tooth, which might make it faster and more capable of recruiting phagocytic cells to remove necrotic tissue and shorten the time of the plateau period to accelerate movement of orthodontic tooth. The same situation and mechanism was also existed in fast-moving period, making the movement distances of orthodontic tooth in SM treatment groups longer than in the model control group.

RANKL, one of the tumor necrosis factor superfamily, can regulate the formation and function of osteoclasts (18-21). The expression of RANKL from osteoblasts was induced by many hormones and cytokines that promoted the bone resorption (22, 23). OPG, a secreted protein, belonged to tumor necrosis factor receptor-associated factor that regulates bone mass and bone mineral density, and inhibits pathological bone resorption (24-26). OPG is a kind of pseudo-receptor (or decoy receptor) that can bind to RANKL, and thus blocks RANKL signaling (27).

It was reported that tanshinone IIA reduced the expression of c-fos and NFATc1, which were the downstream signaling pathways of RANKL to inhibit the differentiation of osteoclasts (28,29). In this study, the associated pharmacological effects of ESM and Danshensu were also confirmed. The effect of SM on RANKL expression was faster and more significant since day 5. The expression of OPG increased remarkably till day 10 with an obvious time delay after orthodontic force removed.

Plenty of cytokines are involved in the process of osteoclast increase. OPG competes with RANKL in binding to osteoclast to inhibit osteoclastogenesis (30). And it was learnt that upregulating the expression of RANKL or inhibiting OPG expression would conduce to osteoclasts generation and related osteoclastic activities (31), which was proved in this study. It is indicated that SM promoted the proliferation of osteoclasts in the first 20 days and provided benefit for the acceleration of orthodontic tooth movement.

Conclusion

A handful of conclusions could be made here: (1) both ESM or pure Danshensu promoted the movement of orthodontic tooth, and shortened the course of plateau period during orthodontic movement, which might be related to its role on promoting microcirculation; (2) SM accelerated periodontal alterations by inducing more osteoclasts, but decreased osteoclasts immediately after releasing from orthodontic force, which might be related to its effect of inducing the apoptosis of osteoclasts via modulating OPG (15); (3) SM upregulated OPG expression but could not make it reach the peak ahead of time, finally leading to bone mass maintained along with the movement accelerating of orthodontic tooth. Meanwhile, it might also maintain bone mass gradually after the movement of orthodontic tooth and accelerated bone healing.

Declaration of interest

There is none of conflict of interest.

Acknowledgment

The dried roots of SM were identified by Dr. Zhong-ping Huan on October 2012. Thanks for other staff at Three Gorges University in China.

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