Original Article

Osteoprotegerin and Ligand of Receptor Activator of Nuclear Factor *kappaB Expression in Ovariectomized Rats during Tooth Movement*

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ABSTRACT

Objective: To test the null hypothesis that increased tooth displacement in ovariectomized rats is not related to differential expressions of OPG and RANKL in the periodontium.

Materials and Methods: Eighty-four 12-week female rats were used; half were ovariectomized and half were not. Three months later, the maxillary first molar was moved mesially. Groups of rats were sacrificed at days 0, 1, 3, 5, 7, 10, and 14 after activation. Tooth movement was measured at each time point. OPG and RANKL expressions were examined through immunohistochemistry.

Results: Ovariectomized and nonovariectomized rats showed three-phase tooth movement. In both groups, OPG expression increased at the tension area and RANKL increased at the pressure area. The OPG/RANKL ratio coincided with tooth movement, especially in the linear phase from 7 to 14 days.

Conclusions: The null hypothesis is rejected. The increased rate of tooth movement in ovariectomized rats was related to differential expressions of OPG and RANKL. (*Angle Orthod.* 2009; 79:292–298.)

KEY WORDS: Tooth movement; RANKL; OPG; Ovariectomy; Periodontal ligament

INTRODUCTION

Orthodontic tooth movement results from remodeling of the periodontal ligament (PDL) and alveolar bone. Remodeling of alveolar bone consists of an interaction of bone resorption by osteoclasts with bone formation by osteoblasts.[1] Previous studies have shown that the efficiency of tooth movement is related to the number and activity of osteoclasts.[2,3] Initiation of bone resorption involves recruitment of new osteoclasts and activation of existing osteoclasts.[4] In addition, in both processes, the OPG(Osteoprotegerin)/RANK(Receptor Activator of Nuclear Factor KappaB)/RANKL(Rank Ligand) signal pathway plays an essential role.[5] In this molecular triad, RANKL binds and activates the TNF-related protein RANK on osteoclast precursors[6,7]; OPG acts as a decoy receptor by competing with RANKL binding to its receptor RANK.[8] RANKL and OPG coordinate to regulate the activation of the RANK signal, that is, the differentiation and activation of osteoclasts. As a highly specialized connective tissue, the PDL consists of a heterogeneous cell population with distinct functions.[9] The PDL cells express OPG and RANKL in vitro[10,11] and in vivo during experimental tooth movement.[12,13]

Osteoporosis is defined as a skeletal disorder characterized by low bone mass and microarchitectural deterioration of bone tissue.[14] Decreased estrogen is the most important risk factor for osteoporosis.[15] Ovariectomy is a common method of inducing estrogen deficiency. Significant bone loss in ovariectomized rabbits, especially of the trabecular bone, and reduction in total and trabecular bone mineral density have been reported previously.[14,16,17] In rats, estrogen deficiency af-

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Accepted: April 2008. Submitted: March 2008.

ter ovariectomy resulted in significantly faster tooth movement,[18] and it influenced the expression of many growth factors, including OPG and RANKL.[19] However, the relationship between faster tooth movement and expressions of OPG and RANKL has not been studied.

The aim of the present study was to investigate whether OPG and RANKL showed differential expression with time related to accelerated tooth movement in ovariectomized rats. We hypothesized that compared with nonovariectomized rats, significantly faster tooth movement in ovariectomized rats is related to the differential regulation of OPG and RANKL in the PDL.

MATERIALS AND METHODS

Experimental Animals

Eighty-four 12-week-old virgin female SD rats were used. Ethical approval was obtained from Sichuan University, China. A total of 84 rats were used; 42 rats were ovariectomized (the OVX group) according to a well-established method.[20] Briefly, the rats were anesthetized through an intramuscular injection of ketamine hydrochloride and Xylazine (Haokang, Chongqing, China) at doses of 50 mg/kg and 10 mg/kg body weight, respectively. The rats were ovariectomized bilaterally from a dorsal approach. The other 42 rats (the non-OVX group) were subjected to a sham surgery during which the ovaries were exteriorized. After the surgery, both groups were kept for 3 months without intervention. During this period, the animals were restricted in feeding to minimize the increase in body weight.[21]

Experimental Tooth Movement

The rats were anesthetized by the method described above and were mounted on a head-holding device. A unilateral appliance was placed to move one of the maxillary first molars mesially. The appliance consisted of a 6-mm length of closed-coil spring (3M, Monrovia, Calif) ligatured with 0.010-inch steel ligature (Shangchi, Shanghai, China). The maxillary incisors were used as the anchorage and were fixed with the coil spring by a ligature. A 0.5-mm groove was drilled along the gingival margin of the incisors to improve the attachment of the ligature. The initial force magnitude was 20 cN. No reactivation of the appliance was performed during the experiment. In all, six rats from each group were sacrificed at due time points (0, 1, 3, 5, 7, 10, and 14 days) through the application of an overdosed anesthesia.

Before sacrifice, the distance from the most mesial point of the rat maxillary molar to the enamel-cementum junction of the incisors at the gingival level was measured. The amount of tooth movement was calculated as the difference between the final measurement and the initial measurement at day 0. All measurements were taken by one observer, and each was repeated three times. Intraobservation agreement was verified by the intraclass correlation coefficient (.97).

Immunohistochemistry

After sacrifice, the maxillae were dissected into halves. The success of ovariectomy was confirmed by failure to detect the ovary, and marked uterus atrophy was seen on naked eye examination. The hemimaxillae were fixed in 4% paraformaldehyde (Sigma, St Louis, Mo) and then were decalcified in 15% EDTA (Sigma). The samples were embedded in paraffin, and 5-μm-thick sagittal sections were cut. These sections were placed in a tris-hydroxy methyl-aminomethane buffered saline solution (TBS) at pH 7.4 for 10 minutes. Endogenous peroxidase activity was blocked in 3% H_2O_2 for 15 minutes. Antigen was retrieved with 1% trypsin at 37°C for 40 minutes. Sections first were preincubated in TBS/BSA for 30 minutes; this was followed by the application of the following polyclonal primary antibodies: SC-8468 for OPG, and SC-7628 for RANKL (Santa Cruz, Calif) in 1:50 solution (4 μg/ml) of TBS/BSA at 37°C for 30 minutes and then at 4°C overnight.

The slides were incubated with a second antibody—rabbit anti-goat immunoglobulin (Zhongshan, Beijing, China)—for 30 minutes at 37°C. The second antibody was diluted to 1:150 as a working solution. Incubation was stopped in TBS before the DAB-complex (Zhongshan, Beijing, China) was administered. To confirm the specificity of the immunostaining, control straining was performed by replacement of the primary antibody with goat serum at the same dilution in PBS.

Image Analysis

As the molar moved mesially, the mesial side was the pressure area, and the distal side was the tension area. The coronal one-third of the palatal root of the maxillary first molar was selected as the study area under 400× magnification with Nikon E600 microscopy (Nikon, Japan). Because the size of the study area at each micrograph varied, OPG and RANKL protein expressions were measured by the mean optical density (MOD). Briefly, the whole study area and positive staining within the study area were selected manually. Both the size of the study area and the integrated optical density (IOD) of the positive stains were measured by Image-Plus Pro 5.0 (Media Cybernetics, Bethesda, Md). MOD was calculated as follows: MOD = IOD/study area.

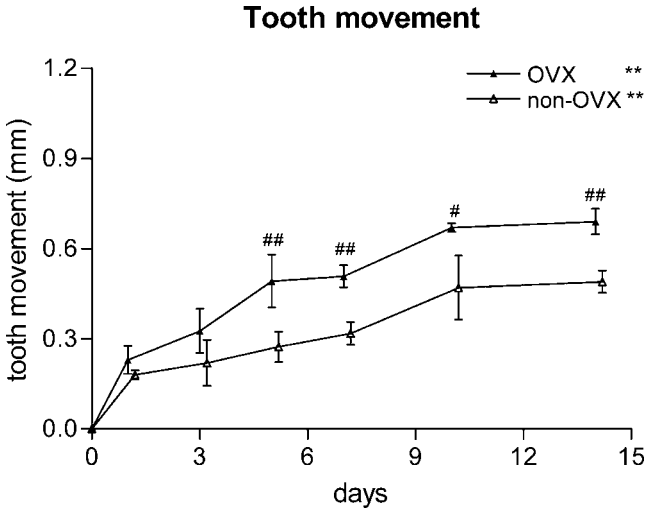

Figure 1. Experimental tooth movement. A three-phase tooth movement pattern was shown in both groups. * and ** indicate comparisons across time within the group; # and ##, comparisons between groups at each time point; * and #, $P < .05$; ** and ##, $P < .01$. The same applies in Figures 3 and 4.

Statistics

All data were distributed normally. Mean IOD values and standard errors of the mean were calculated for RANKL and OPG, respectively. The ratio of OPG and RANKL protein levels was calculated at each time point. Analysis of variance (ANOVA) followed by post hoc tests was performed to examine the differences in tooth movement velocity and in RANKL and OPG expressions across time. Intergroup comparisons were performed with an independent samples *t*-test. Differences were considered significant when $P < .05$.

RESULTS

Both OVX and non-OVX rats showed a significant increase in tooth movement over time ($P < .01$) (Figure 1). A three-phase tooth movement pattern was shown in both groups: the initial fast movement phase (days 0–3), the slowing-down phase (days 3–7), and the linear phase (days 7–14). The amount of tooth movement in the OVX group was significantly greater than in the non-OVX group from day 5 onward ($P < .01$ at days 5, 7, and 14; $P < .05$ at day 10).

The immunohistology of OPG and RANKL showed different intensities at tension and pressure areas. At the tension side, the PDL cells showed strong staining of OPG (Figure 2a) and weak staining of RANKL (Figure 2b); at the pressure side, RANKL staining was strong, especially in the osteoclasts along the alveolar surface (Figure 2d), and OPG staining was very weak (Figure 2c).

Quantitative data showed that at the tension side, OPG expressions increased over time in both groups ($P < .01$) with differences between the two groups noted only in the linear phase ($P < .05$ at days 7 and 14; $P < .01$ at day 10; Figure 3a). RANKL expression showed a nonsignificant decrease over time. Differences between the two groups were observed at day 0 and day 1 ($P < .05$; Figure 3c); at the pressure side, OPG showed a nonsignificant tendency to decrease in both groups (Figure 3d). RANKL expressions increased over time only in the OVX group ($P < .01$), with significant higher expression seen in OVX rats at day 1 ($P < .05$), day 10, and day 14 ($P < .01$; Figure 3d).

The OPG/RANKL ratio at the tension side (Figure 4a) increased over time in both groups ($P < .01$); this increase was more pronounced in the non-OVX group. Significant differences were noted between the two groups in the linear phase ($P < .05$ at days 7 and 14; $P < .01$ at day 10). The ratio at the pressure side (Figure 4b) decreased over time in both groups, mainly in the linear phase ($P < .01$).

DISCUSSION

The present study showed a time-related differential expression of OPG and RANKL in ovariectomized rats during orthodontic tooth movement. The OPG/RANKL ratio increased at tension areas and decreased at pressure areas. These results supported our hypothesis that faster tooth movement in ovariectomized rats was related to the differential regulation of OPG and RANKL in the PDL. The present study focused on the effects of ovariectomy on tooth movement and OPG and RANKL expressions. Therefore, nonovariectomized rats were used as the control instead of the commonly used nonappliance (split-mouth) group.

Our results confirmed the findings of a previous study that ovariectomy accelerated tooth movement.[18] The mechanism could be that ovariectomy resulted in unbalance of the hormone system, which increased osteoclastic activity. This induced significant bone loss and reduction of the stiffness and elastic modulus of the alveolar bone.[22] During the initial phase, tooth movement in OVX rats tended to be faster. However,

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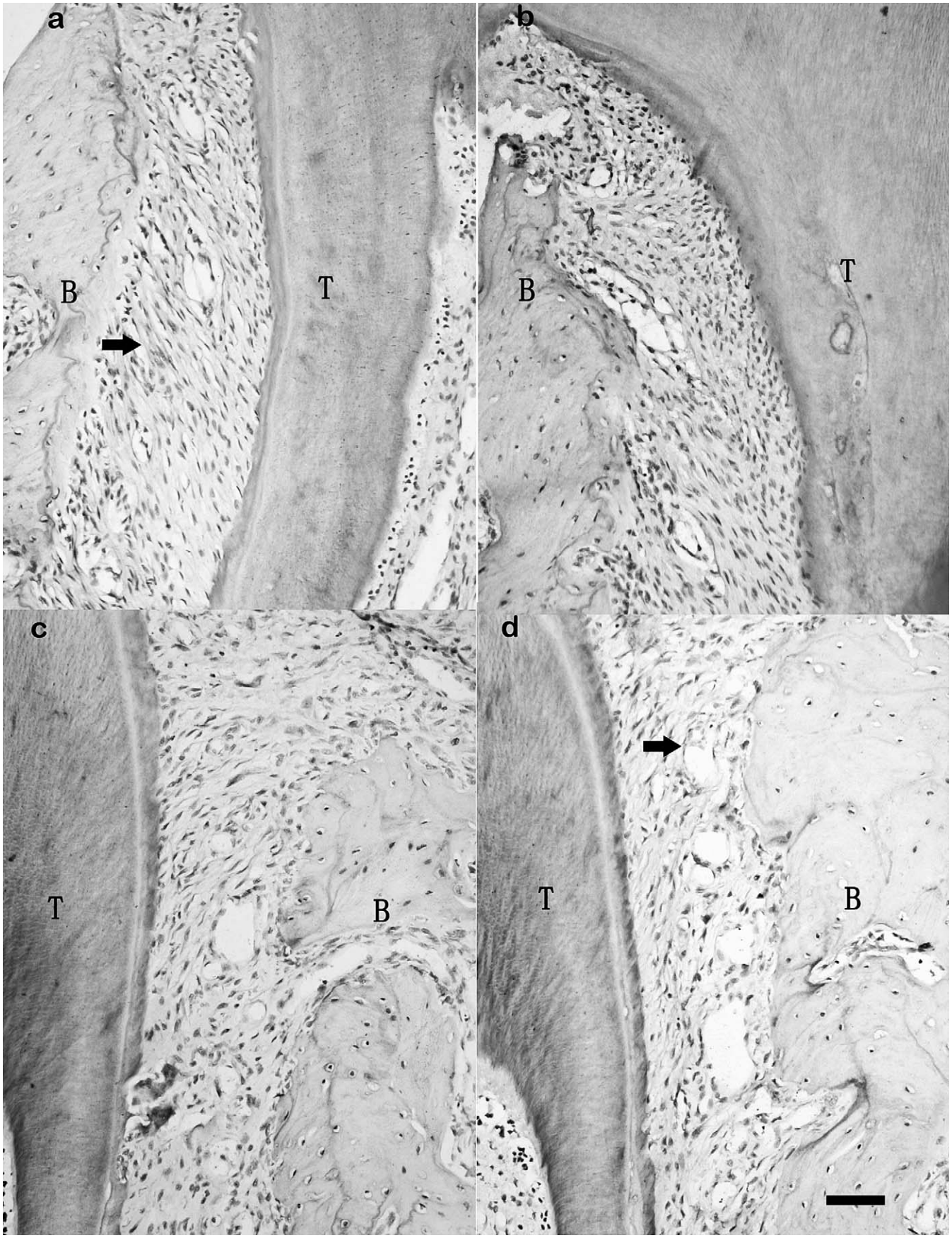
Figure 2. Immunohistochemical staining of OPG and RANKL in the PDL. OPG showed strong expression in PDL cells (a, arrow) at the tension side, and RANKL showed weak expression (b) on day 10; at the pressure side, the expression of OPG was weak (c), and RANKL expression on the cell membrane is obvious (d, arrow). Teeth were moved to the right side. B indicates alveolar bone; T, tooth.



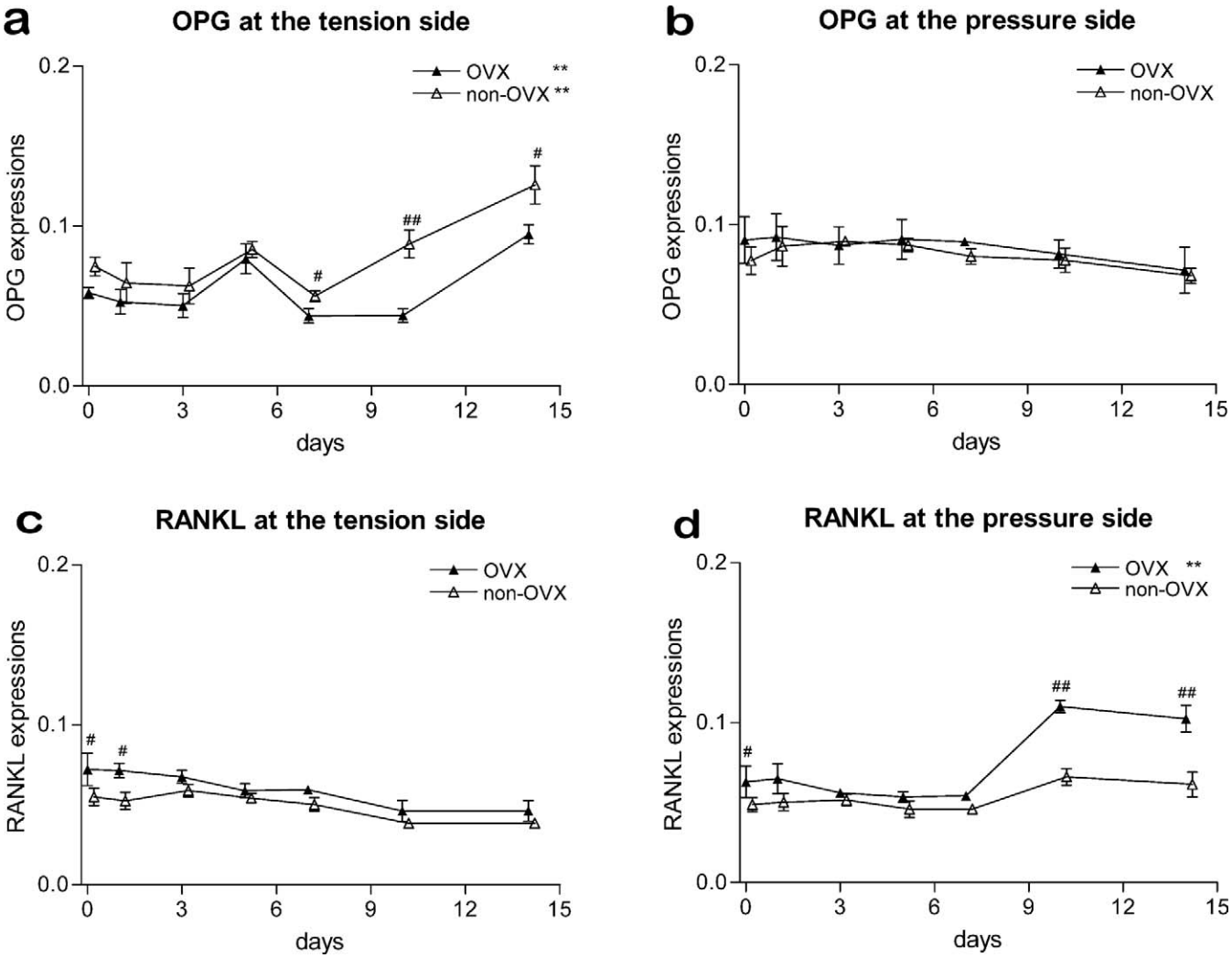


Figure 3. The mean optical density of OPG and RANKL in the PDL (mean ± SD). (a and b) OPG at the tension and pressure sides; (c and d) RANKL at the pressure side at tension and pressure sides.

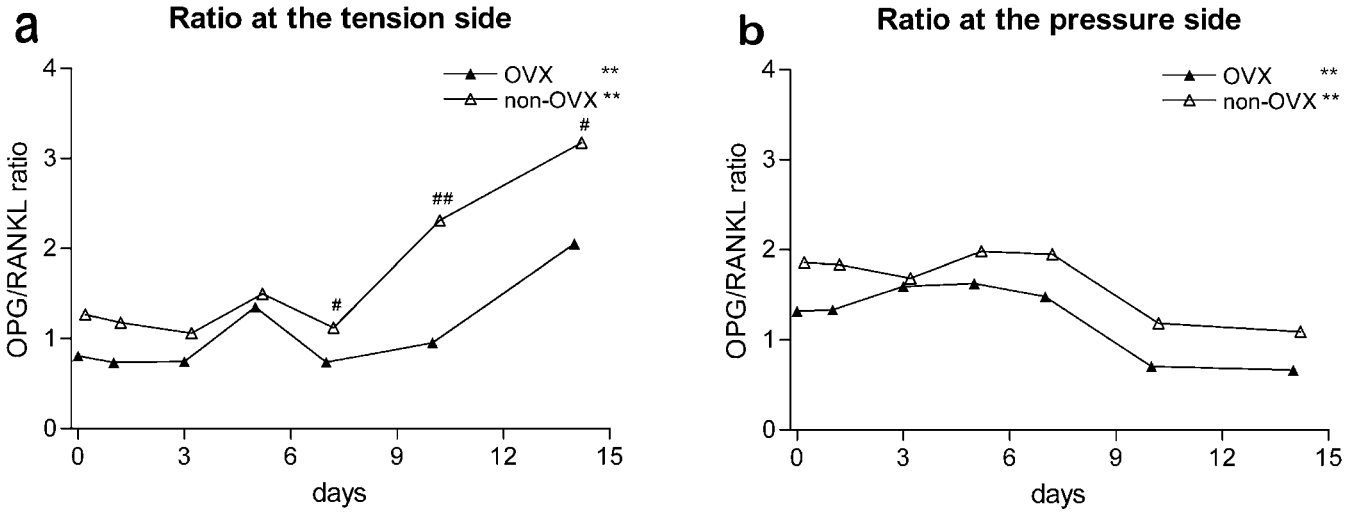


Figure 4. The ratio of OPG and RANKL expressions over time (mean ± SD). (a) At the tension side. (b) At the pressure side.

it was not different from that in the non-OVX group. We did not find some statistical difference between OVX and non-OVX groups during the initial phase.

During the second phase, tooth movement slowed down in both groups. This may have been related to the initiation of hyalinization, which retards tooth movement.[18] In the present study, differences in tooth movement were observed between the two groups from day 5 onward, but in a previous study, tooth movement started to accelerate only in the linear phase.[18] The difference may be related to the different timing of experimental tooth movement. In the present study, tooth movement was initiated 12 weeks after ovariectomy was performed. This is supported by the findings of several previous studies, which showed that changes in the mechanical property of trabecular bone were evident only 8 weeks after ovariectomy and were significant 12 weeks after the procedure.[16,17,23] In the other study, however, tooth movement was started 2 weeks after ovariectomy.

Ovariectomy results in a gradual reduction in estrogen levels. Such a decrease triggers an increase in osteoblast and osteoclast precursors.[24] Osteoblast precursors further stimulate the recruitment and differentiation of osteoclasts,[24] during which OPG and RANKL are two important molecules.[5] However, it remains unclear how estrogen deficiency affects OPG and RANKL expressions. Evidence has suggested that the effect of estrogen deficiency was regulated by a network of cytokines.[25,26] Another line of evidence showed that decreased estrogen levels upregulated PGE_2 production, which subsequently activated prostaglandin receptors in osteoblasts and upregulated the expression of RANKL[27]; it also upregulated the expression of bone-resorbing cytokines such as interleukin (IL)-1, IL-6, and transforming growth factor (TGF)-α; these cytokines may have caused a decreased ratio of OPG/RANKL through osteoblasts and stromal cells.[26,28] Our results showed that before tooth movement was initiated (day 0), the RANKL expression was upregulated in ovariectomized rats at pressure and tension areas of the PDL. This may have stimulated osteoclastogenesis and osteoclastic activity, which subsequently reduced bone mass and destroyed the microarchitecture of bone tissues. Our results did not agree with those of a previous study, which showed decreased OPG expression after ovariectomy.[19] The reason could be that this study measured OPG expressions in homogenate bone tissues and not in PDL cells.

Theoretically, the ratio of OPG/RANKL indicates the activity of osteoclast. An increased ratio could be related to a reduction in recruitment and activation of osteoclasts because a high affinity of OPG to RANKL blocks the functional binding of RANKL to RANK; a decreased ratio, on the other hand, may indicate an increased inducement of osteoclast progenitors to mature and active osteoclasts, as well as an extended osteoclast life span.[5] The change in OPG/RANKL ratio dictated the rate of bone resorption in a number of pathologic states.[29] Our results showed that the coupling of bone resorption and bone formation during accelerated tooth movement in ovariectomized rats is related to the differential change in OPG/RANKL ratio at pressure and tension areas of the PDL.

Results from the present study support the use of light force in older and osteoporotic patients in orthodontic practice. Osteoporotic patients are more sensitive to mechanical force during orthodontic treatment because of the decreased mechanical property of alveolar bone and increased reaction to mechanical force. Therefore, clinicians should treat these patients with caution to avoid unexpected damage to the periodontal tissues.

CONCLUSION

- A three-phase tooth movement pattern was shown in both groups. The amount of tooth movement in ovariectomized rats was significantly greater than that in nonovariectomized rats.
- Accelerated tooth movement in ovariectomized rats was related to the differential expression of OPG, RANKL and the differential change in OPG/RANKL ratio in the PDL.
- Light force should be applied to osteoporotic patients to prevent insufficient tissue reconstruction caused by increased tooth movement.

ACKNOWLEDGMENT

This work was supported by a grant from the Youth Technological Foundation of Sichuan, China (03ZQ026-040).

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