



■ RESEARCH

Age-related effects on osteoclastic activities after orthodontic tooth movement

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Objectives

To elucidate the effects of age on the expression levels of the receptor activator of the nuclear factor- κ B ligand (RANKL) and osteoclasts in the periodontal ligament during orthodontic mechanical loading and post-orthodontic retention.

Materials and Methods

The study included 20 male Sprague-Dawley rats, ten in the young group (aged four to five weeks) and ten in the adult group (aged 18 to 20 weeks). In each rat, the upper-left first molar was subjected to a seven-day orthodontic force loading followed by a seven-day retention period. The upper-right first molar served as a control. The amount of orthodontic tooth movement was measured after seven-day force application and seven-day post-orthodontic retention. The expression levels of RANKL and the tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts were evaluated on day 7 (end of mechanical force loading) and day 14 (after seven days of post-orthodontic retention). Statistical analysis was performed using the *t*-test, and significance was set at $p < 0.05$.

Results

There was no significant difference between the amount of tooth movement in the young group (0.96, standard deviation (SD) 0.30mm) and that in the adult group (0.80mm, SD 0.28) ($p > 0.05$) after the seven-day force application. On the compression side, the expression of RANKL and TRAP-positive osteoclasts in both the young and the adult groups increased after the application of force for seven days, and then decreased at the end of the seven-day retention period. However, by the end of the period, the expression of RANKL on the compression side dropped to the control level in the young group ($p > 0.05$), while it was still higher than that on the control side in the adult group ($p < 0.05$). The expression of RANKL on the compression side did not show significant difference between the young and the adult groups after seven-day force application ($p > 0.05$), but it was significantly higher in the adult group than that in the young group after seven-day post-orthodontic retention ($p < 0.05$). Similarly, the decreasing trend of TRAP-positive osteoclasts during the retention period in the adult group was less obvious than that in the young group.

Conclusions

The bone-resorptive activity in the young rats was more dynamic than that in the adult rats. The expression of RANKL and the number of osteoclasts in adult rats did not drop to the control level during the post-orthodontic retention period while RANKL expression and the number of osteoclasts in young rats had returned to the baseline.

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Article focus

- Age effects on the activity of osteoclasts in response to orthodontic force stimuli during post-orthodontic retention.
- The model of orthodontic tooth movement (OTM) and retention was performed in rats.

Key messages

- The study showed the expression level of nuclear factor- κ B ligand and the number of osteoclasts in the periodontium during the OTM and post-orthodontic retention periods in young and adult rats.
- This article might explain why orthodontic procedures and retention take longer in the treatment of adult humans than in adolescents.

Strengths and limitations

- The findings of this study support the hypothesis that there are age effects on the activity of bone in response to orthodontic stimuli and post-orthodontic retention.
- Limitation: The experimental period was short.

Introduction

It has been widely reported that orthodontic tooth movement (OTM) is achieved via alveolar bone remodelling and the reaction of the periodontal ligament (PDL) to mechanical stimuli.¹⁻³ The combination of bone resorption on the compression side and bone deposition on the tension side aligns the teeth into normal occlusion.⁴ Research has indicated that osteoclasts are the multinucleated bone-resorbing cells responsible for bone resorption.⁵ There have been extensive studies of the regulatory mechanism of osteoclastogenesis, in which the receptor activator of the nuclear factor- κ B (RANK)/RANK ligand (RANKL)-mediated signaling pathway plays a critical role.⁶⁻⁸ RANKL, produced by osteoblasts and identified as a membrane-bound protein, binds to RANK to trigger the differentiation of osteoclast precursors and to regulate osteoclast activities.⁶⁻⁸

On the other hand, relapse often occurs after orthodontic tooth movement due to complicating factors such as craniofacial growth, dentoalveolar changes and muscular and PDL adaptation.⁹ Orthodontic relapse may even occur immediately after the teeth are released from the orthodontic force, undergoing the same process as with OTM.¹⁰ Hence, retention is regarded as a continuation of the orthodontic treatment, which is designed to create and maintain occlusion stability during the remodelling of the periodontium.

With an increasing number of adults requiring orthodontic treatment, it is suggested that under orthodontic force loading, remodelling of the alveolar bone and periodontal tissue in adults is much slower than in adolescents because of the reduced cellular activities and alveolar vascularity, altered bone composition and richer collagen in the tissue.¹¹⁻¹³ All of these factors indicate that orthodontic procedures may take longer in adults than in young patients. Melsen¹⁴ and Ren et al¹⁵ reported that the longer treatment duration in adults might be caused by a delay in the onset of tooth movement.

Only a few experimental studies on the relationship between ageing and osteoclastic activity during orthodontic

tooth movement have been performed. Some of them indicated no difference between young and old rats in number, size or activity of osteoclasts in mechanically stressed alveolar bone during orthodontic tooth movement.^{16,17} Others found that orthodontic forces induced faster osteoclast recruitment in young rats than in adult rats, and more osteoclasts were needed to achieve the same rate of tooth movement in adult rats as in young rats.¹⁸ Furthermore, it was recently demonstrated that the levels of mediators such as RANKL in gingival crevicular fluid (GCF) were different in adults and adolescents undergoing orthodontic treatment.^{19,20}

The effects of ageing on osteoclastic activities during orthodontic tooth movement have remained controversial. No study has been carried out to assess the age effects on osteoclastic activity during the post-orthodontic retention stage. Therefore, in this study, we investigated the expression level of RANKL and the number of osteoclasts in the periodontium during the OTM and post-orthodontic retention periods in rats, aiming to find out whether bone reaction to mechanical loads during and after orthodontic tooth movement is characterised by age-related effects.

Materials and Methods

Animals. A total of 20 male Sprague-Dawley rats were used for the experiment, of which ten were in the young group (aged four to five weeks; body weight 100 g to 200 g) and ten in the adult group (aged 18 to 20 weeks; body weight 350 g to 400 g). Both young and adult rats were randomly divided into two subgroups (five rats in each subgroup): the orthodontic group, in which rats were subjected to orthodontic force for seven days; and the retention group, in which rats received retention for seven days after a seven-day active orthodontic force loading. Male rats were chosen to avoid the hormonal changes associated with oestrus. The rats were kept under normal conditions in separate cages with a 12-hour 'circadian' cycle, and were fed a standard laboratory rodent diet (LabDiet, St. Louis, Missouri) and water ad libitum. Experimental procedures followed the guiding principles of the Animal Care and Use Committee in the Peking University.

Orthodontic tooth movement and retention. To manufacture the rat tooth movement model under ketamine (90 mg/kg, intraperitoneal) anaesthesia, the upper-left first molar in each rat was subjected to orthodontic force and moved mesially by a nickel-titanium closed-coil spring (Sentalloy, Tomy, Tokyo, Japan). One side of the spring was ligated to the upper-left first molar with a stainless steel ligature wire (TP Orthodontics, Inc., La Porte, Indiana). The other side of the coil spring was ligated (not cemented) to the upper-left incisor, which can prevent the effect of continuous incisor eruption of rats. The upper-right first molar served as control without

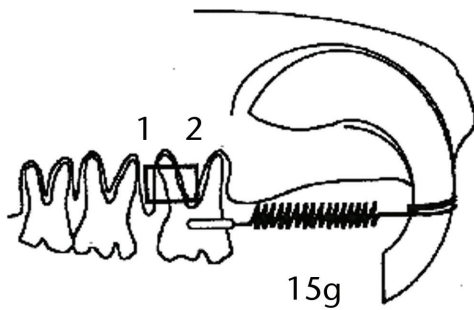


Fig. 1a

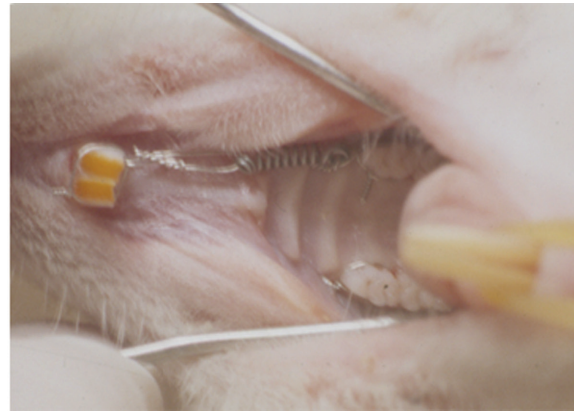


Fig. 1b

Design of the experimental tooth movement model: (a) a diagram of the orthodontic appliance design (1, tension side; 2, compression side); and (b) the upper left first molar was moved mesially by a closed coil spring with a force of 15 g.

treatment. The spring exerted a force of 15 g,²¹ measured by a force gauge. The force was exerted for seven days (Fig. 1).

The distance between the most mesial point of the upper first molar and the cemento-enamel junction of the ipsilateral incisor at the gingival level was measured with a digital calliper (Hu-Friedy Manufacturing Co., Chicago, Illinois) on the experimental and the control sides on day 7 (seven days of force loading) and day 14 (seven days of post-orthodontic retention after seven-day force loading). The amount of experimental tooth movement was calculated as the increments in the difference between distances on the experimental and the control sides.¹⁵ The same investigator performed all measurements, and every measurement was repeated three times. The measurement results were shown as mean and standard deviation (SD). The intra-examiner reliability revealed good agreement (intraclass correlation coefficient 0.954).

After the seven-day force loading, the spring was then replaced by a 0.010 inch stainless steel ligature (TP Orthodontics, Inc.), tied around the teeth with composite resin (3M ESPE, St. Paul, Minnesota), filling the interdental space for retention, and the retention lasted for another seven days.

Histological examination. A total of ten rats (five from the young and adult groups, respectively) were killed seven days after force application, and the remaining ten (five from the young and adult groups, respectively) were killed on day 14 after the seven-day post-orthodontic retention. After euthanasia by ether, the posterior maxillae were excised, including the first molar roots, and then fixed and stored in 10% neutral buffered formalin solution for 24 hours at 4°C. After rinsing with 0.1 mol/L phosphate-buffered saline (PBS) solution, the tissue blocks were demineralised in 10% ethylenediaminetetraacetic acid (EDTA) for four weeks at 4°C and then embedded in paraffin. The embedded specimens were serially sectioned into 30- μ m thick slices in the parasagittal plane

from 3.0 mm below the occlusal plane, and stained with haematoxylin and eosin.

RANKL immunohistochemical staining was performed to quantitatively evaluate the RANKL expression levels.²² Ultrathin slides were incubated overnight at 4°C with rabbit antiserum raised against RANKL (Dako Pty Ltd., Carpinteria, California), and then incubated in swine anti-rabbit Ig antibody (Dako Pty Ltd.). The slides were processed using the biotin-streptavidin-horseradish peroxidase method using a Histofine SAB-PO kit (Nichirei Co. Ltd, Tokyo, Japan).

To facilitate the identification of osteoclasts, the tartrate-resistant acid phosphatase (TRAP)-stained sections were assayed using a leukocyte acid-phosphatase kit (Sigma-Aldrich Corp., St Louis, Missouri). Cells with more than three nuclei were defined as TRAP-positive osteoclasts.

The immunohistochemistry (by percentage) and TRAP staining (by area) were measured using the Image-Pro Plus programme (version 6.0; Media Cybernetics, Inc., Rockville, Maryland). The ratio of the experimental sample to the control was calculated.

Statistical analysis. The amounts of RANKL and TRAP-positive osteoclasts were quantified at a magnification of $\times 180$ using a true-colour RGB computer-assisted image analysing system with a digital camera (Leica DC 300 V 2.0) and Leica Qwin version 2.4 software (Leica, Wetzlar, Germany). The measurement area was processed within a fixed measurement frame of 1044 \times 766 pixels. The same investigator performed all measurements, and every measurement was repeated three times.

The mean and standard deviation (SD) of each group were calculated using SPSS software (Statistical Product and Service Solutions 18.0, IBM Corporation, Armonk, New York). Two-tailed independent *t*-tests were performed to compare the values of the different parameters. Differences with a value of $p < 0.05$ were considered to be statistically significant.

Table 1. Amount of experimental tooth movement (mm).

	Young group		Adult group	
	Day 7	Day 14	Day 7	Day 14
n	10	5	10	5
Mean	0.96	0.94	0.80	0.79
Standard deviation	0.30	0.4	0.28	0.30

Results

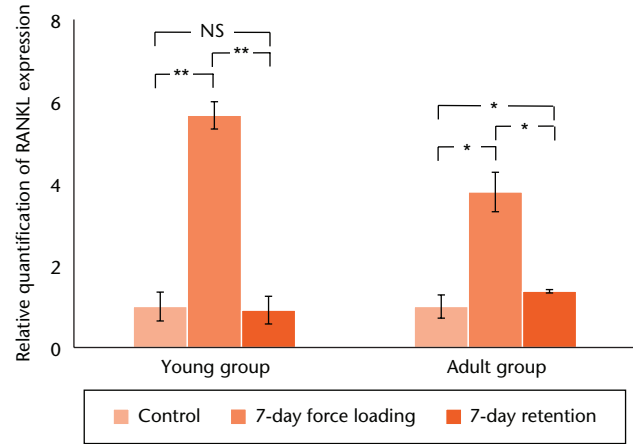
Amount of tooth movement. For both young and adult rats, a significant difference in the amount of tooth movement was found between experimental and control sides ($p < 0.05$). Experimental tooth movement was calculated as the increments in the difference between distances on the experimental and the control sides (Table 1). There was no significant difference between the amount of experimental tooth movement in the young group (0.96 mm; SD 0.30) and in the adult group (0.80 mm; SD 0.28; $p > 0.05$) after the seven-day force application. No significant difference in the amount of experimental tooth movement was found between day 7 (after seven-day orthodontic force loading) and day 14 (after the seven-day post-orthodontic retention) on the experiment side ($p > 0.05$) in both young and adult groups.

RANKL expression on the compression sides. The RANKL expression level on the compression sides in the young group (upper-left first molars: 17.29%; SD 5.76%) was significantly higher than that on the control sides (upper-right first molars: 3.05%; SD 1.07%) after seven days of force application ($p < 0.01$), while it decreased to 2.78% (SD 0.94%) which was almost the same level as the control sides ($p > 0.05$) after the seven-day post-orthodontic retention period (Figs 2 and 3).

In the adult group, the positive RANKL immunostaining on the compression sides (22.16%; SD 10.66%) was significantly higher than that on the control sides (5.84%; SD 1.66%) after seven days of force application ($p < 0.05$). At the end of the post-orthodontic retention period, moderate RANKL staining was still detected, although at a lower level than that under the application of force. Unlike in the young rats, the RANKL expression level in adult rats did not return to the control level after the seven-day retention period (8.02%; SD 0.33%), and the difference in RANKL expression levels between the experimental and control sides of the adult rats remained significant ($p < 0.05$) (Figs 2 and 4).

The expression of RANKL on the compression side did not show significant difference between the young and the adult groups after seven-day force application ($p > 0.05$), while it was significantly higher in the adult group than in the young group after seven-day post-orthodontic retention ($p < 0.05$).

TRAP-positive osteoclasts on the compression sides. In the young group, TRAP-positive osteoclasts were found both on the control sides and the compression sides after

**Fig. 2**

Comparison of RANKL expression on the compression sides during and after mechanical force loading in the young and adult groups ($*p < 0.05$; $**p < 0.01$).

the seven-day force application and retention periods. The number of osteoclasts was significantly higher on the compression sides under mechanical force loading than on the control sides ($p < 0.001$). During the retention period, the number of osteoclasts decreased significantly on the compression sides ($p < 0.001$), although it remained a little higher than the control sides ($p > 0.05$) (Figs 5 and 6).

TRAP-positive osteoclasts were also observed in the adult group after the seven-day force application and retention periods. However, the decreasing trend during the retention period was less obvious than in the young group (Figs 5 and 7).

The histogram revealed a much higher expression level of osteoclasts in the young rats after mechanical force loading (a ten-fold increase compared with the control group) and a faster decrease in the retention period (dropping to 1.2 times higher than the control group) compared with the adult rats, which presented a 1.8-fold increase under force loading, dropping to a 1.6-fold increase during retention.

RANKL expression on the tension sides. In the young group, there was stronger RANKL expression on the tension sides (16.62%, SD 6.29%) than on the control sides (2.06%; SD 1.29%; $p < 0.01$). Similar to the compression sides, the RANKL level on the tension sides had decreased to the same level as the control sides (2.44%; SD 1.59%; $p > 0.05$) after seven-day post-orthodontic retention (Fig. 8).

The adult group showed a similar pattern to the young group, with higher RANKL expression on the tension sides under force loading (24.78%; SD 16.93%) than on the control sides (5.37%; SD 0.54%; $p < 0.05$), followed by a downward trend in the retention period (5.41%; SD 0.36%) (Fig. 8).

The expression of RANKL on the tension side did not show significant difference between the young and the

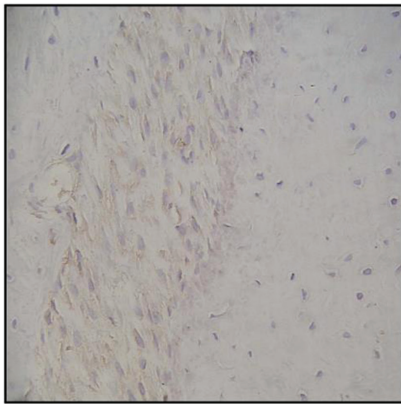


Fig. 3a

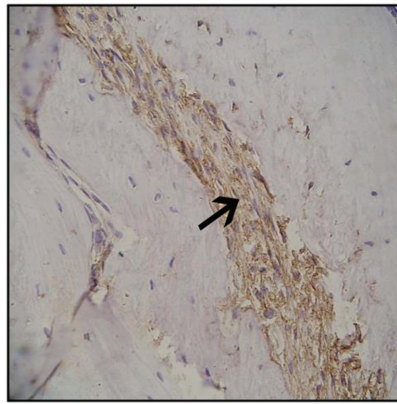


Fig. 3b

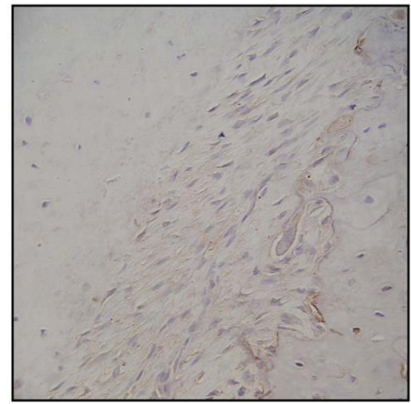


Fig. 3c

Immunostaining of RANKL expression on the compression sides during and after mechanical force loading in young rats: (a) the expression of RANKL was rarely observed on the control sides; (b) stronger RANKL expression (arrow) was found on the compression sides after seven-day force application; and (c) the level of RANKL expression decreased on the compression sides after seven-day post-orthodontic retention.

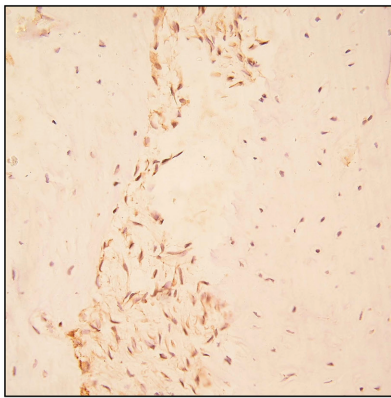


Fig. 4a

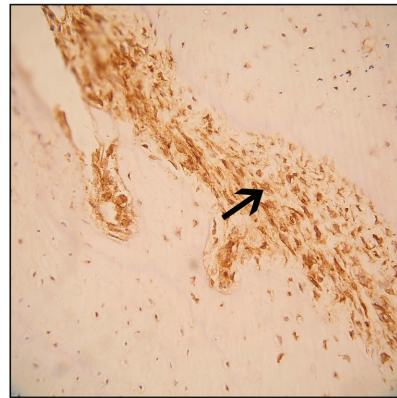


Fig. 4b

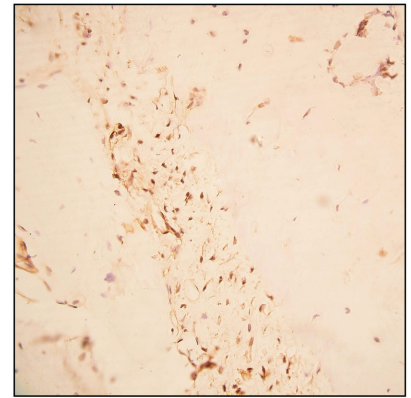


Fig. 4c

Immunostaining of RANKL expression on the compression sides during and after mechanical force loading in adult rats: (a) the expression of RANKL was observed on the control sides; (b) an intense immunoreaction for RANKL (arrow) was found on the compression sides after seven-day force application; (c) moderate RANKL staining was still found on the compression sides after seven-day post-orthodontic retention.

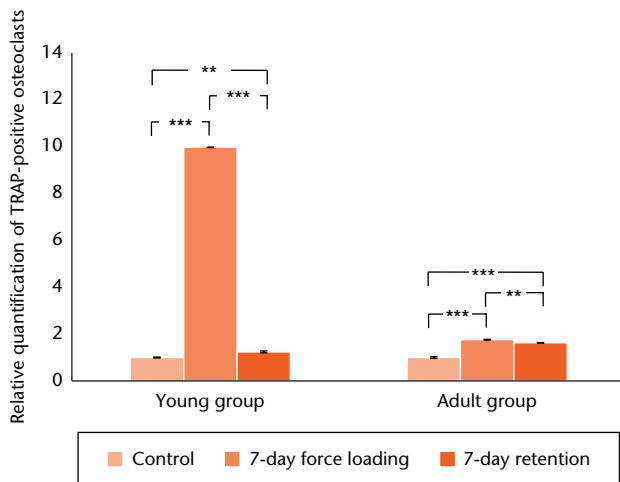


Fig. 5

Comparison of TRAP-positive osteoclasts on the compression sides during and after mechanical force loading in the young and adult groups (** $p < 0.01$; *** $p < 0.001$).

adult groups after seven-day force application ($p > 0.05$), while it was significantly higher in the adult group than in the young group after seven-day post-orthodontic retention ($p < 0.05$).

TRAP-positive osteoclasts on the tension sides. In both young and adult group, TRAP-positive osteoclasts were not found on the tension sides after the seven-day force application and seven-day retention periods. Histological reaction on the tension sides mainly presented as new bone build-up rather than bone resorption.

Discussion

Orthodontic tooth movement is induced by mechanical stimuli and facilitated by remodelling of the periodontal ligament and alveolar bone. Osteoclasts are the key participants in orthodontic bone remodelling and in the metabolic processes that regulate bone structure throughout the lifetime.⁶ RANKL, as a regulator of

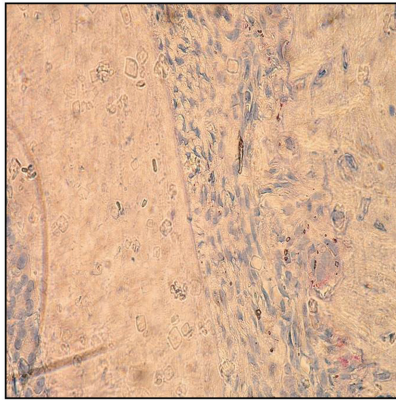


Fig. 6a

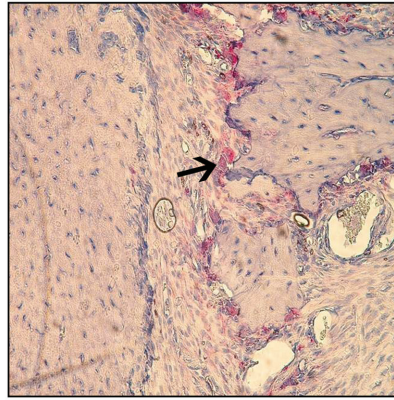


Fig. 6b

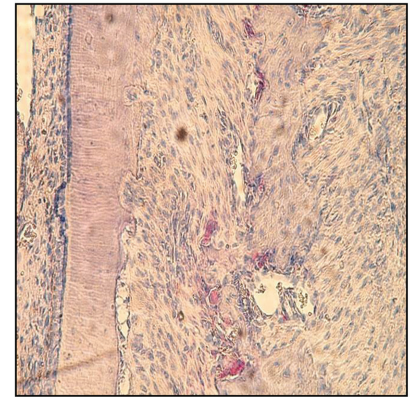


Fig. 6c

TRAP-positive staining of osteoclasts on the compression sides during and after mechanical force loading in young rats: (a) osteoclasts were barely observed on the control sides; (b) a large amount of osteoclasts (arrow) were seen on the compression sides after seven-day force application; and (c) a few osteoclasts were still found on the compression sides after seven-day post-orthodontic retention.

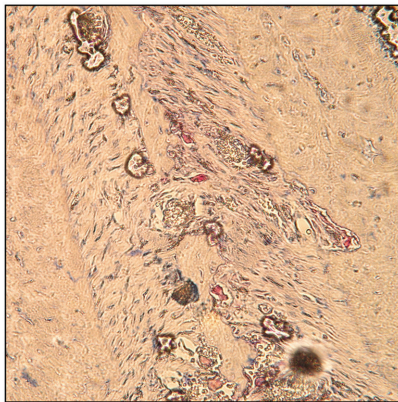


Fig. 7a

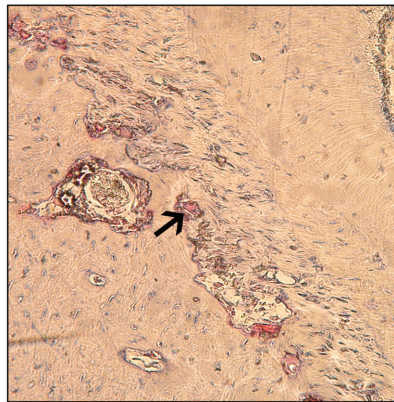


Fig. 7b

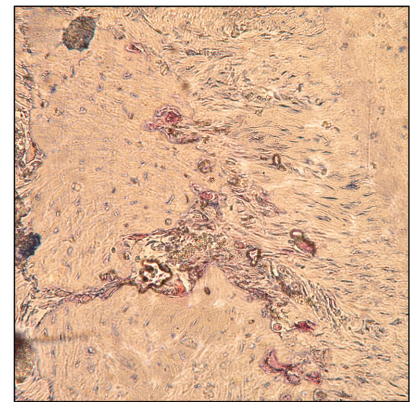


Fig. 7c

TRAP-positive staining of osteoclasts on the compression sides during and after mechanical force loading in adult rats: (a) a small number of osteoclasts were seen on the control sides; (b) numerous multinucleated osteoclasts (arrow) were observed on the compression sides after seven-day force application; and (c) a small amount of osteoclasts were still found on the compression sides after seven-day post-orthodontic retention.

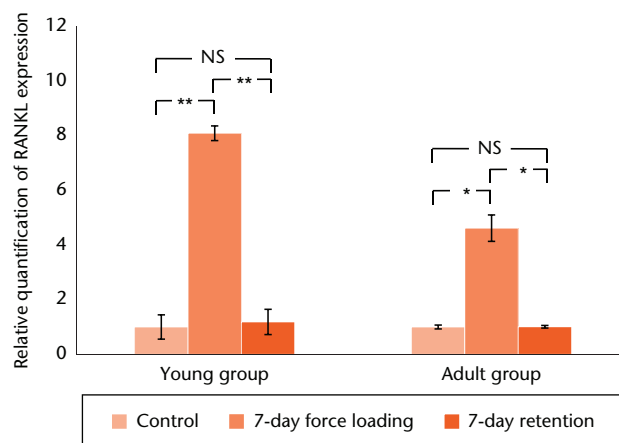


Fig. 8

Comparison of RANKL expression on the tension sides during and after mechanical force loading in the young and adult groups (* $p < 0.05$; ** $p < 0.01$).

osteoclast formation and activation, stimulates bone resorption.⁶⁻⁸ Osteoprotegerin (OPG) is a decoy receptor produced by osteoblasts and competes with RANK for RANKL binding, and thus preventing osteoclast differentiation and bone resorption.²³⁻²⁵ It has been found that the RANKL/RANK/OPG system plays an important role in orthodontic tooth movement.²⁶ Studies have shown that the levels of RANKL in GCF have been observed to increase during orthodontic tooth movement, while the levels of OPG decreased.²⁶ Concerning the age effect on GCF composition, there is evidence that the levels of RANKL and OPG in GCF respond differently to orthodontic force with age.¹⁹ Thus, the present study was to determine the number of osteoclasts and the expression level of RANKL during orthodontic tooth movement and post-orthodontic retention in young and adult rats.

Under normal physiological conditions, osteoclasts are rarely observed in young and adult rats. The number of osteoclasts on the compression sides in the young as well as the adult animals increased significantly after seven-day force application. Further, the number of osteoclasts on the compression sides in the young rats was higher than that in the adults after seven days of force application. This is in agreement with the study of Ren et al¹⁸ which has shown that orthodontic forces induce faster osteoclast recruitment in young than in adult rats. In addition, the number of osteoclasts in the adults did not decrease as remarkably as that in the young animals during the post-orthodontic retention period. It appeared that the alveolar bone of the young rats was more sensitive and active in response to mechanical stimuli than that of the adult rats, as confirmed by the TRAP staining of the osteoclasts. There is evidence that both the bone-resorptive activity and the bone-formative activity decrease with age.^{27,28} Therefore, orthodontic treatment in adolescent patients may be less time consuming, with more rapid and dynamic bone remodelling.

As the histological reaction on the tension side typically presents as new bone formation rather than bone resorption,⁴ we did not focus on the TRAP-positive osteoclast staining. Kobayashi et al also reported that osteoclasts on the tension sides disappeared through apoptosis during force-induced reversal from bone resorption to formation.²⁹ The number of osteoclasts on the compression sides showed a peak level in young and old animals after seven-day force application. At the same time, a positive level of RANKL immunostaining was observed on the compression sides in both the young and the adult rats. The trend between the number of osteoclasts and the expression of RANKL on the compression sides were the same in both the young and adult animals, which indicated that in response to mechanical stress, the periodontal ligament cells may induce osteoclastogenesis through upregulation of RANKL expression during orthodontic tooth movement.

In our previous study,³⁰ we found an age-related difference in OPG expression during orthodontic bone remodelling in Sprague-Dawley rats. There was escalated OPG expression in response to tensile force in young rats compared with adult rats whose OPG expression displayed no significant difference in response to pre-force loading. In the present study, RANKL expression on the tension sides in the young and adult rats was similar to that on the compression sides. RANKL expression increased significantly after seven days of force application and thereafter it tended to decrease. Taken together, our studies have demonstrated increasing expression of both OPG and RANKL on the tension sides of young rats, whereas only RANKL showed increased expression in adult rats, with OPG remaining unchanged. The above results suggest that young rats are more likely than adult

rats to achieve dynamic balance of bone remodelling regulated by RANKL/OPG on the tension sides.

It has been widely reported that rat molars migrate distally with ageing, and this physiological drift of the teeth is possibly caused by occlusal forces.^{31,32} Therefore, the retention is important to achieve a stable occlusion relationship and prevent relapse after removal of orthodontic force. In our study, orthodontic retention was performed by placing resin into the interdental space after removal of the spring between the molars in rats. It has been shown that restoration of the mechanical strength of the PDL occurs in accordance with the closure of the interdental space after removal of the orthodontic force.³³

During the retention period, RANKL expression and the number of osteoclasts should have dropped to around the normal physiological level, because orthodontic-related bone resorption would be expected to cease. In this study, RANKL expression in the PDL of young rats recovered to the level of the control while the number of osteoclasts was just slightly higher than the control after the seven-day retention period. In contrast, although RANKL expression and the number of osteoclasts showed a downward trend in the adult rats, they still remained much higher than that on the control sides. The results suggest that it may take longer for the alveolar bone in adult rats to recover to its normal physiological status following OTM. These findings help to explain why adult orthodontics should include a longer retention period than that required for young patients. The adolescent alveolar bone tends to recover more quickly once the orthodontic force is removed, probably due to its relatively dynamic bone remodelling ability. The results of this study support our hypothesis that there are age effects on the activity of bone in response to orthodontic force application, as suggested by other studies,^{15,18,26,27} and that there are also age effects on the activity of bone in response to post-orthodontic retention.

In conclusion, young rats showed more dynamic bone-resorptive activity than did adult rats, which might explain why adult orthodontic treatment is more time-consuming. The expression of RANKL and the number of osteoclasts in adult rats did not drop to the control level during the post-orthodontic retention period while RANKL expression and the number of osteoclasts in young rats had returned to the baseline, which suggests that orthodontic retention for adults may take longer than for adolescents in order to prevent relapse.

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ICMJE conflict of interest

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