NFATc1 and RUNX2 Expression on Orthodontic Tooth Movement with Gradually Increasing Force

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ABSTRACT

Background. Orthodontic tooth movement occurs due to bone resorption and apposition on the pressure and tension side of the PDL. The transcription factors associated with osteoclast differentiation are NFATc1 while osteoblast differentiation is associated with RUNX2. The optimum force of orthodontic tooth movement can move the teeth to the desired position, without causing discomfort and tissue damage to the patient.

Objective. This study aims to analyse the effect of gradually increasing force on orthodontic tooth movement (by evaluating the NFATc1 and RUNX2 expression) in rats.

Methods. This research is an *in vivo* experimental study with a post-test control group design. Twenty-eight healthy male adult Wistar rats *(Rattus novergicus)* aged 4-5 months with body weights 200-250 g rats were divided into seven study groups. Treatment groups in this study are given the force (by applying a closed coil spring between the maxillary central incisor and the maxillary first molar) of 5 g, 5-10 g, 10 g, and 10-20 g with the duration of treatment in 14 and 28 days. After the treatment day was finished, the alveolar bone tissue was isolated and investigated by immunohistochemical methods.

Results. Indicate a significant difference between the control and all treatment groups of NFATc1 (p=0.003; p=0.000; p:0.010; p=0.001; p=0.001; p=0.000) and RUNX2 with groups of 10 g/14 days, 10 g/28 days, 5 g/28 days, 10 g/14 days,10-20 g/28 days (p=0.001; p=0.000; p=0.000; p=0.017; p=0.014; p=0.000) values.

Conclusion. Gradually increasing force affects orthodontic tooth movement by inducing bone resorption (high expression of NFATc1) in the pressure area and bone apposition (high expression of RUNX2) in the tension area. Applying heavy force by initially applying light force could inhibit hyalinization.

Keywords: NFATc1, RUNX2, orthodontic tooth movement, gradually increasing force

eISSN 2094-9278 (Online)
Published: August 15, 2024
https://doi.org/10.47895/amp.vi0.6940

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INTRODUCTION

Orthodontic tooth movement is indicated by remodeling of the periodontal tissues, including the periodontal ligament (PDL), alveolar bone, and gingiva. It is understood and accepted that tooth movement occurs in response to mechanical force.[1] Orthodontic tooth movement can occur quickly or slowly depending on physical characteristics, force application, magnitude, and the biological response of PDL. Orthodontic force is defined as the application of force to the teeth to move the teeth.[2] The optimal orthodontic force moves the teeth to the desired position, without causing discomfort and tissue damage to the patient.[3] Applying orthodontic pressure will cause the formation on the pressure side and tension side on the periodontal ligament.[4] Alveolar bone resorption by osteoclasts on the pressure side and apposition of the alveolar bone on the tension side is carried

out by osteoblastst.[3,5] The transcription factors associated with osteoclast differentiation are NFATc1 while osteoblast differentiation is associated with RUNX2.[4]

NFATc1 expression is induced during osteoclast differentiation. Some transcription factors have been shown to bind to the NFATc1 promoter during osteoclastogenesis.[6] NFATc1 is the main regulator of osteoclast differentiation.[7] RUNX2 is a multifunctional transcription factor that controls skeletal development by regulating the differentiation of chondrocytes, osteoblasts, and the expression of many extracellular matrix protein genes during chondrocyte and osteoblast differentiation. During osteoblasts differentiation, RUNX2 regulates gene expression of bone matrix proteins.[7] Yamashiro et al. explained that RUNX2 is required for the formation of bones and teeth and its expression is particularly limited in the condensation of osteoblasts and mesenchyme in the formation of bone, cartilage, and teeth.[8]

Orthodontic force application below the optimal level produces no reaction in the PDL while heavy orthodontic force exceeding the optimal level blocks blood vessels and causes necrosis of the surrounding tissue and hyalinization which will inhibit tooth movement.[5] Tooth movement would thus be delayed until undermining resorption eliminates the necrotic tissue obstacle.[9] The research by Tomizuka et al. showed that the use of orthodontic force gradually resulted in a high number of TRAP in the pressure area.[10] However, research on the expression of NFATc1 and RUNX2 with gradually increasing force application on orthodontic tooth movement has not been done. This study aims to investigate the expression of NFATc1 and RUNX2 on orthodontic tooth movement with gradually increasing force.

MATERIAL AND METHODS

Animal and Experimental Procedures

Ethical clearance was obtained from the Dental Medicine and Health Research, Universitas Airlangga, number 066/HRECC.FODM/VI/2017. All experimental animals of the study were provided by pharmacology and anatomy pathology laboratory. All animals were adapted for seven days before treatment to lessen the stress brought by the new environment. Polycarbonate cages (0.9 x 0.6 x 0.6 m) were used to maintain each rat and were kept with a 12-hour light/dark cycle at a steady temperature of 25°C and controlled humidity of 50%. All experimental animals were fed with a standard pellet diet and tap *ad libitum* drinking water. Every day, the animal cages were examined for signs of food and drink consumption as well as faecal characteristics, and cage hygiene was maintained. Each weight of the animal was checked using a digital scale. There were 28 healthy male adult Wistar rats (*Rattus novergicus)* aged 4-5 months with body weights 200-250 g. The animals were categorized into seven groups: one control group and six treatment groups. Each group contained four animals. When the duration of treatment was completed, the rats were euthanized using ether. The treatment of each group is described in Table 1 and Figure 1.

The process of NiTi closed coil spring installation: rats were anaesthetized with a solution consisting of a mixture of ketamine (100 mg/ml, xylazine (20 mg/ml) and acepromazine (10 mg/ml) intramuscularly. Prior to installation, the strength of the NiTi closed coil spring was measured using a tension gauge to produce strengths of 5 g, 10 gr/mm^2, and 20 gr/mm^2. A closed coil spring was placed between the maxillary central incisor and the maxillary first molar to move the molars mesially (Figure 2). This appliance was fixed using a 0.007 stainless steel ligature wire which was attached around the maxillary central incisor through a hole made using a cylindrical bur on the distocervical side just above the gingival papilla. The posterior part of the appliance was also ligated with a 0.007 stainless steel ligature wire which was placed around the first molar. To increase retention, after ligation, glass ionomer cement was also applied.

Histological Preparation

Fourteen days and 28 days after the activation of the NiTi closed coil spring, euthanasia was performed on the animal study using ether and then stretched on a surgical board for premaxilla dissection. Tissue decalcification was performed using 14% EDTA (Ethylenediaminetetraacetic acid) for 21-90 days. The tissue is washed with PBS (Phosphate Buffered Saline) 3-5 times to remove contaminants. Samples were fixed using 10% formalin for 24 hours. Dehydration was performed using graduated sequence of alcohol before being embedded in paraffin. Embedding paraffin using hard paraffin in the mould for one day. Then, cutting was carried out using a microtome with a thickness of 5 microns with a rotary microtome. Mounting on a glass object with 5% gelatine. Deparaffinization was performed, block paraffin is immersed in xylol two times. Rehydration was performed using radiant graduated sequence of alcohol for five minutes. Then rinsed in H_2O for five minutes.

Table 1. Pre-treatment and Post-treatment Cephalometric Analysis

Group		Treatment	Duration of Treatment	
			14 Days	28 Days
Control		Without treatment*	–	–
A	*T1*	10 g/mm^2	✓	–
	T2	10 g/mm^2	–	✓
B	*T3*	5 g/mm^2	✓	–
	T4	5 g/mm^2-10 g/mm^2**	–	✓
C	*T5*	10 g/mm^2	✓	–
	T6	10 g/mm^2 - 20 g/mm^2***	–	✓

* *Control group was sacrificed in day 0*

** *Force given to rats was 5 g/mm^2 for 14 days. On the 15th day, 10 g/mm^2 of force was added.*

*** *The force given to rats was 10 g/mm^2 for 14 days. On the 15th day, a force of 20 g/mm^2 was added.*

Statistical Analysis

The results of the calculation of NFATc1 and RUNX2 levels were tabulated according to each sample group, and then statistically tested using the Kolmogorov Smirnov to check whether the data were normally distributed. Then to find out the difference in NFATc1 or RUNX2 levels between groups, a test was performed using one-way analysis of variance (ANOVA) and continued with the Tukey HSD test.

RESULTS

Table 2 shows the mean value of NFATc1 on the pressure side and the highest is in the T2 group, the group of rats given a force of 10 g/mm^2 seen on day 28. While the lowest mean value of NFATc1 on the pressure side was found in the control and then T3 group, the group of rats given a force of 5 mg/mm^2 seen on day 14. The positive expression of NFATc1 was detected in all experimental groups (Figure 3).

Table 2. Mean Value of NFATc1 and Osteoclast on Pressure Area (mesial of the molar)

Biomarker	Control	T1	T2	T3	T4	T5	T6
NFATc1	4.50	11.00	16.25	10.25	12.00	12.00	15.75
Osteoclast	6.75	13.00	17.00	12.50	13.75	14.70	17.50

The mean value results of osteoclasts and NFATc1 on the pressure side are directly proportional, which means that if the NFATc1 value shows a low value, then the number of osteoclasts is also low. If the NFATc1 value is high, the number of osteoclasts is also high. The histological section result shows that osteoclast found in all experimental groups (Figure 4).

Then, the Tukey HSD test was used to compare results between groups. The results in Table 3 show that several groups differed significantly ($p<0.05$) and several groups did not differ significantly ($p>0.05$). Comparisons between groups that differed significantly included Group C with T1, T2, T3, T4, T5, and T6. Group T1 with T2 and T6, Group T2 with T1 and T3, Group T3 with T2 and T6.

Table 4 shows the mean value of RUNX2 on the tension side, which is highest in the T6 group, by the force from 10 g/mm^2 to 20 g/mm^2 seen on day 28. While the lowest RUNX2 mean value is in the control group and T3, which was given a force of 5 g/mm^2 on day 14. The positive expression of RUNX2 is found in all experimental groups (Figure 5).

Based on the RUNX and osteoblast values results, that the mean of osteoblasts and RUNX2 value on the tension side

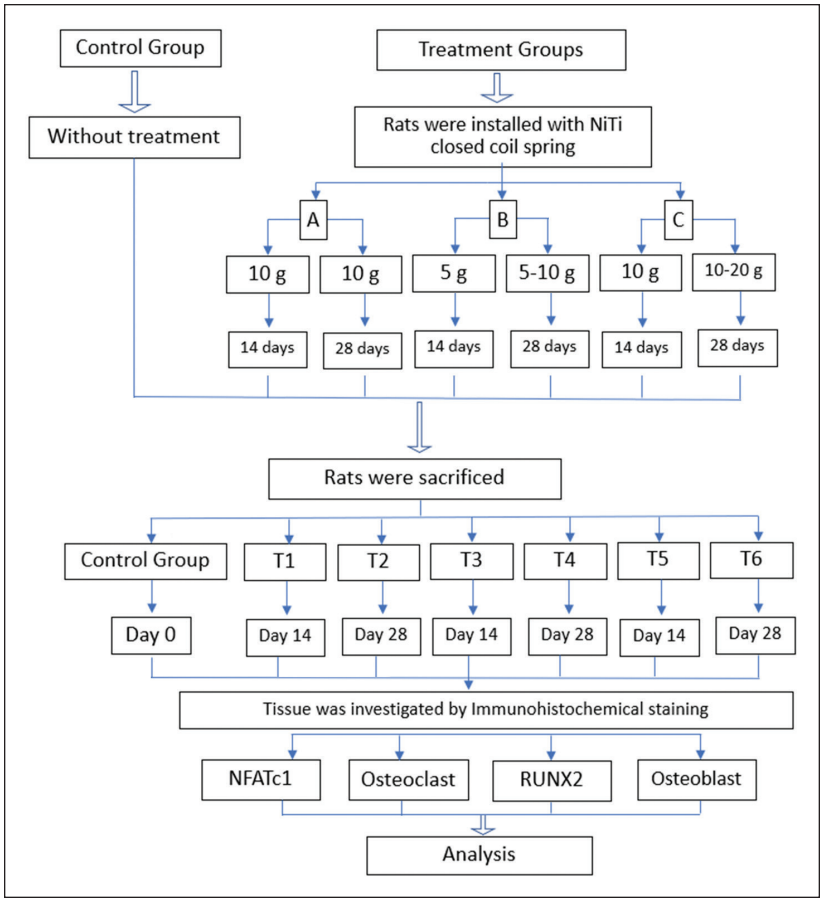

Figure 1. Schematic diagram of experimental procedure.

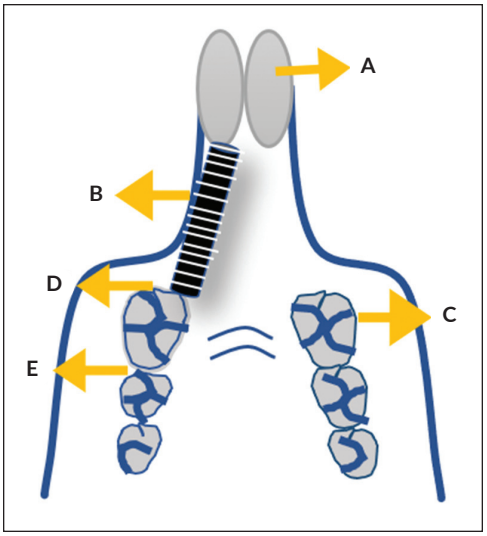

Figure 2. ***Installation of NiTi closed coil spring in rats.*** **(A)** incisive tooth; **(B)** closed coil spring; **(C)** molar tooth; **(D)** pressure area; **(E)** tension area.

Table 3. Tukey HSD NFATc1 Test Results in the Pressure Area (mesial of the molar)

Group	Comparison Group	p-Value
C	T1	0.003*
	T2	0.000*
	T3	0.010*
	T4	0.001*
	T5	0.001*
	T6	0.000*
T1	T2	0.022*
	T3	0.998
	T4	0.992
	T5	0.992
	T6	0.046*
T2	T3	0.007*
	T4	0.092
	T5	0.092
	T6	1.000
T3	T4	0.881
	T5	0.881
	T6	0.015*
T4	T5	1.000
	T6	0.175
T5	T6	0.175

**p<0.05, significant differences between the groups*

C (control); T1 (10 g/mm² 14 days); T2 (10 g/mm² 28 days); T3 (5 g/mm² 14 days); T4 (5 g/mm²-10 g/mm² 28 days; T5 (10 g/mm² 14 days); T6 (10 g/mm²-20 g/mm² 28 days)

are directly proportional, which means that if the RUNX2 value shows a low value, then the number of osteoblasts is also low. Likewise, if the RUNX2 value is high, the number of osteoblasts is also high. The histological section of osteoblast in all experimental groups is shown in Figure 6.

The results in Table 5 show that several groups differ significantly ($p<0.05$) and several groups do not differ significantly ($p>0.05$). Comparisons between groups that differed significantly included Group C with T1, T2, T4, T5, and T6; Group T1 with T2 and T6; Group T2 with T3 and T5; Group T3 with T4 and T6; Group T5 with Group T6.

DISCUSSION

The mechanical forces exerted on the teeth are transmitted to the surrounding alveolar bone via the periodontal ligament (PDL). Thus, providing this force will activate the process of bone remodelling, resorption and/or apposition that facilitates tooth movement within the alveolar bone. From a biomechanical point of view, it is clear that periodontal stress and/or strain, especially PDL plays an important

Table 4. Mean Value of RUNX2 and Osteoblast on Tension Area (distal of the molar)

Biomarker	Control	T1	T2	T3	T4	T5	T6
RUNX2	3.75	8.75	13.00	6.50	12.50	8.50	14.50
Osteoclast	3.75	7.75	14.25	9.50	14.50	11.25	17.00

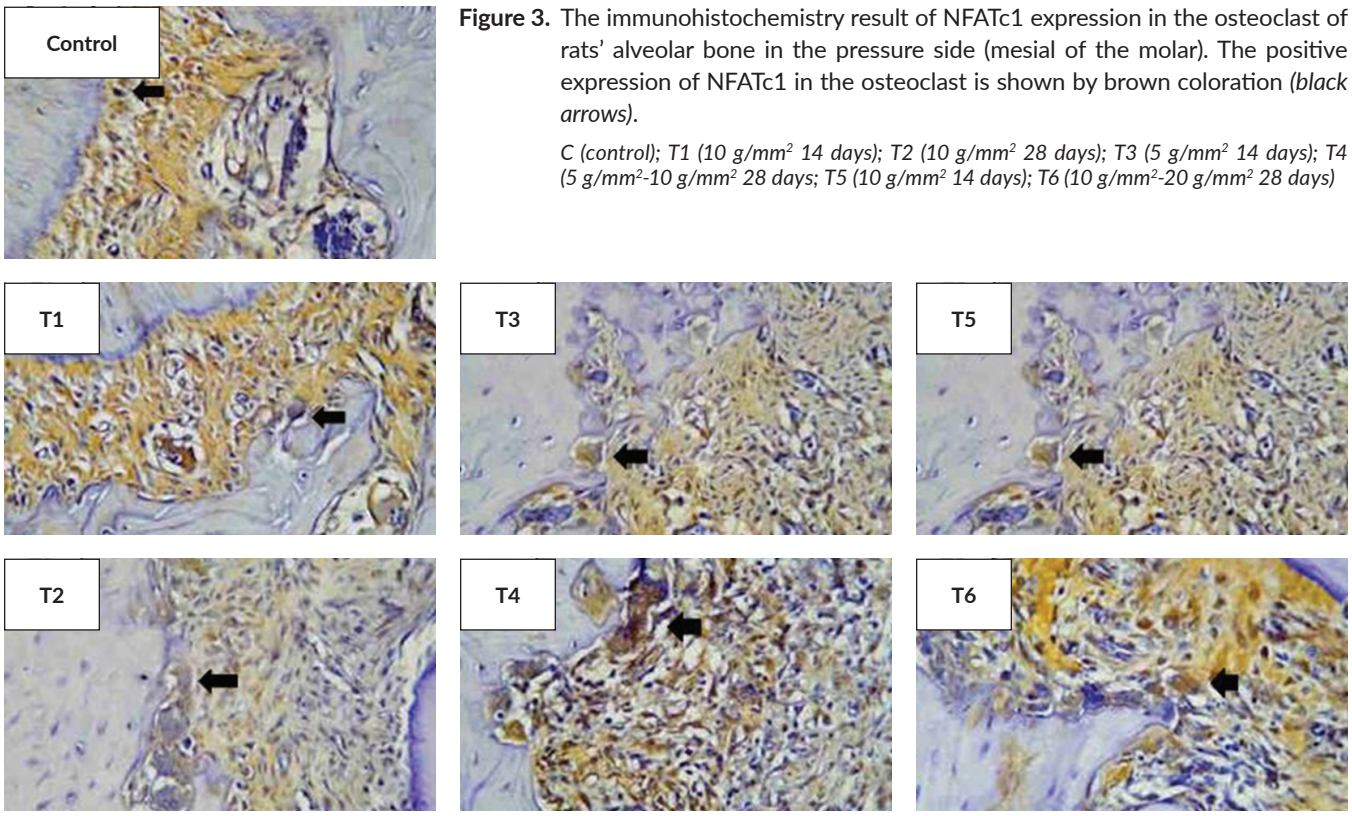

Figure 3. The immunohistochemistry result of NFATc1 expression in the osteoclast of rats' alveolar bone in the pressure side (mesial of the molar). The positive expression of NFATc1 in the osteoclast is shown by brown coloration *(black arrows)*.

C (control); T1 (10 g/mm² 14 days); T2 (10 g/mm² 28 days); T3 (5 g/mm² 14 days); T4 (5 g/mm²-10 g/mm² 28 days; T5 (10 g/mm² 14 days); T6 (10 g/mm²-20 g/mm² 28 days)

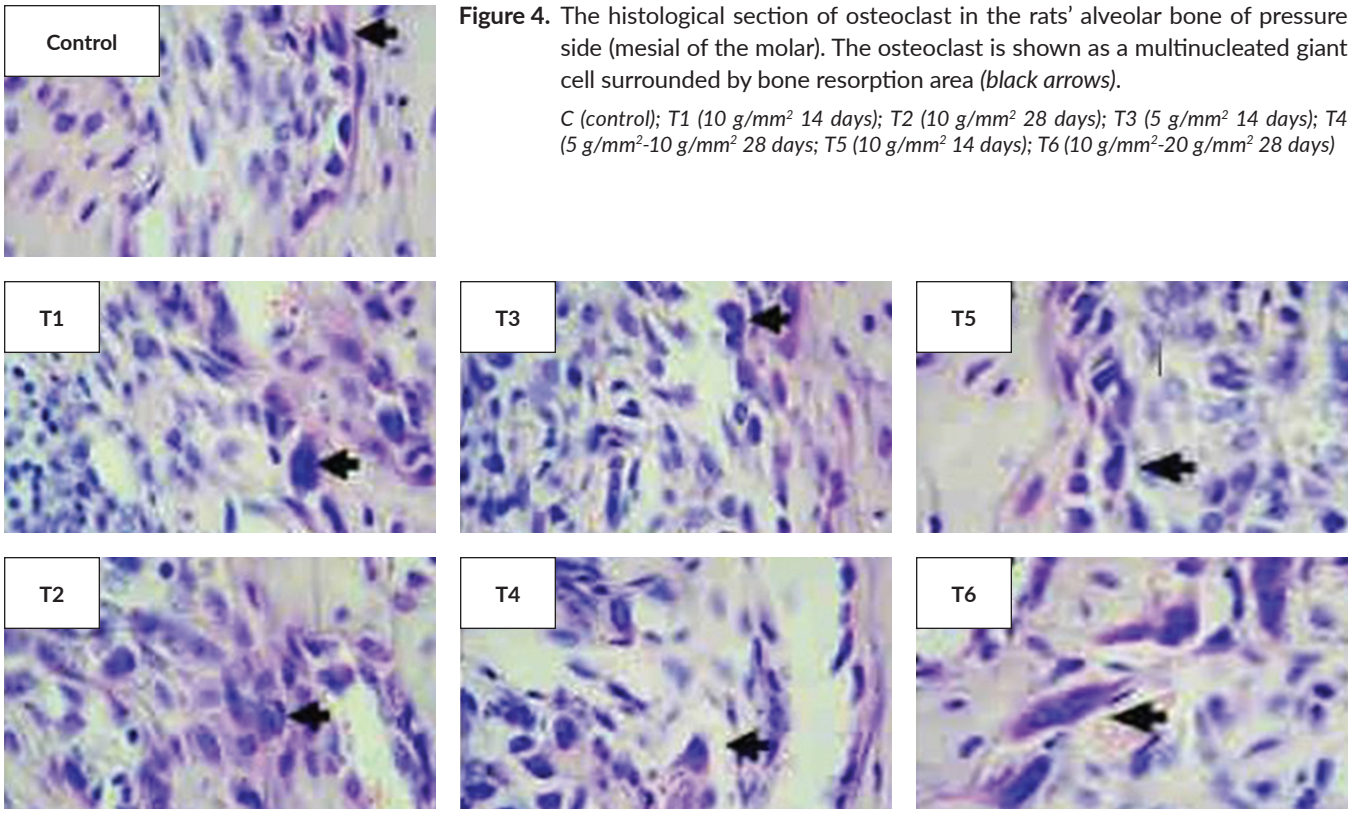

Figure 4. The histological section of osteoclast in the rats' alveolar bone of pressure side (mesial of the molar). The osteoclast is shown as a multinucleated giant cell surrounded by bone resorption area *(black arrows)*.

C (control); T1 (10 g/mm² 14 days); T2 (10 g/mm² 28 days); T3 (5 g/mm² 14 days); T4 (5 g/mm²-10 g/mm² 28 days; T5 (10 g/mm² 14 days); T6 (10 g/mm²-20 g/mm² 28 days)

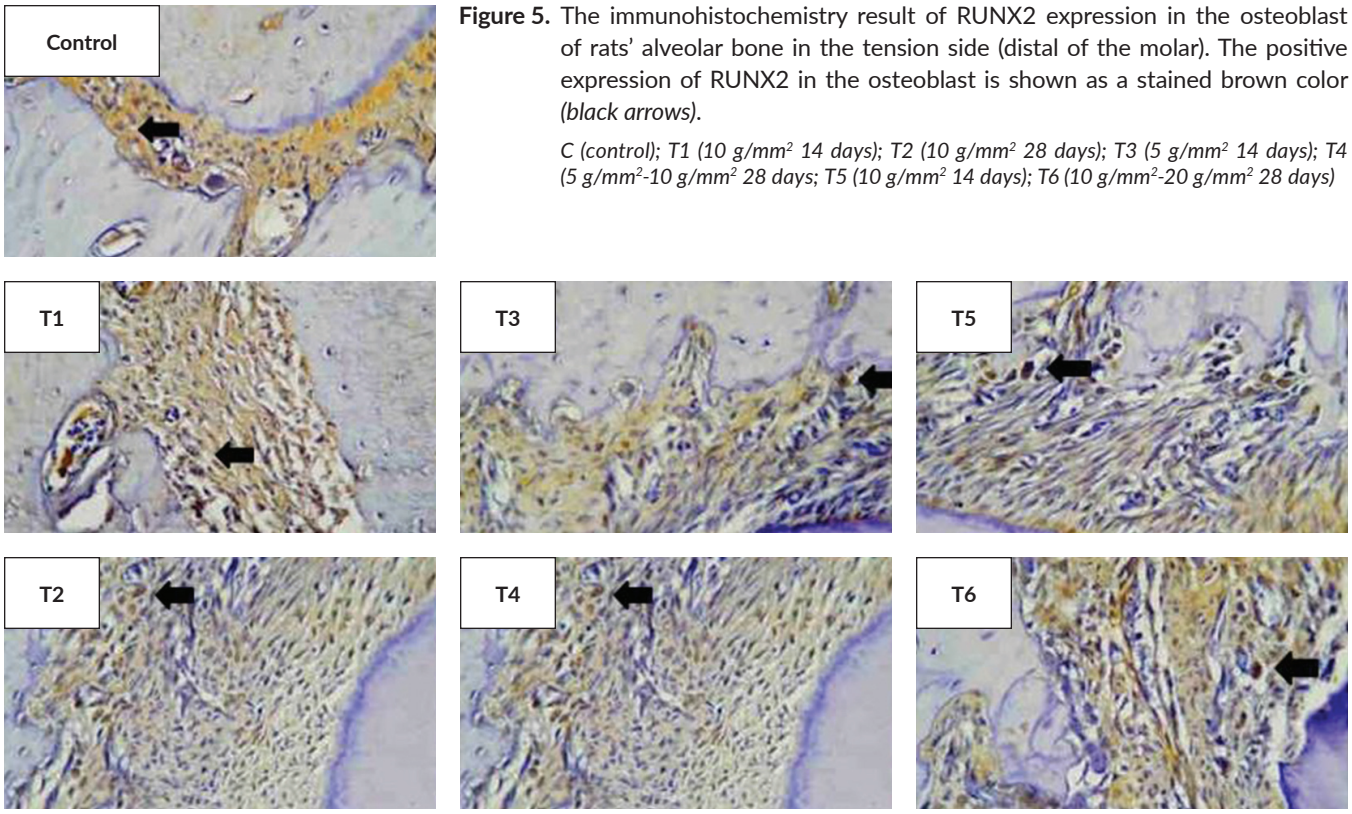

Figure 5. The immunohistochemistry result of RUNX2 expression in the osteoblast of rats' alveolar bone in the tension side (distal of the molar). The positive expression of RUNX2 in the osteoblast is shown as a stained brown color *(black arrows)*.

C (control); T1 (10 g/mm² 14 days); T2 (10 g/mm² 28 days); T3 (5 g/mm² 14 days); T4 (5 g/mm²-10 g/mm² 28 days; T5 (10 g/mm² 14 days); T6 (10 g/mm²-20 g/mm² 28 days)

Table 5. Tukey HSD RUNX2 Test Results in the Tension Area (distal of the molar)

Group	Comparison Group	p-Value
C	T1	0.011*
	T2	0.000*
	T3	0.352
	T4	0.000*
	T5	0.017*
	T6	0.000*
T1	T2	0.040*
	T3	0.578
	T4	0.090
	T5	1.000
	T6	0.003*
T2	T3	0.001*
	T4	1.000
	T5	0.027*
	T6	0.893
T3	T4	0.002*
	T5	0.697
	T6	0.000*
T4	T5	0.061
	T6	0.697
T5	T6	0.002*

**p<0.05, significant differences between the groups*

C (control); T1 (10 g/mm² 14 days); T2 (10 g/mm² 28 days); T3 (5 g/mm² 14 days); T4 (5 g/mm²-10 g/mm² 28 days; T5 (10 g/mm² 14 days); T6 (10 g/mm²-20 g/mm² 28 days)

role in tooth movement.[11] The initial phase of orthodontic tooth movement causes an acute inflammatory response and two mechanisms happened after the administration of the orthodontic force, the pressure side undergoes bone resorption by osteoclasts, whilst the tension side undergoes bone apposition by osteoblasts. The transcription factors associated with osteoclast differentiation are NFATc1 while osteoblast differentiation is associated with RUNX2. This process will result in a process of tooth movement.[12-14]

Based on the analysis of the data of this study, Table 3 shows a significant difference between NFATc1 expression in the control and all treatment groups. A high NFATc1 value indicates the occurrence of osteoclastogenesis in the pressure area. This was revealed by Takayanagi et al. who suggested that NFATc1 is a crucial transcription factor for osteoclast differentiation.[15] Mature osteoclasts become multinuclear giant cells through the fusion of mononuclear osteoclasts[16,17] (Figure 4). Meanwhile, Table 5 shows the significant difference between the RUNX2 expression in the control with the group of T1, T2, T4, T5 and T6. RUNX2 value indicates an increase in the process of osteoblast differentiation.

Analysis of the data in Tables 2 and 3 show that the group of 10 g/mm² has the highest expression of NFATc1 in the pressure area and RUNX2 in the tension area. It demonstrates that 10 g/mm² is an optimum force that leads to adequate bone remodelling that induces orthodontic tooth movement in this study. This result is in accordance with the study by Ren et al. which found that a force magnitude

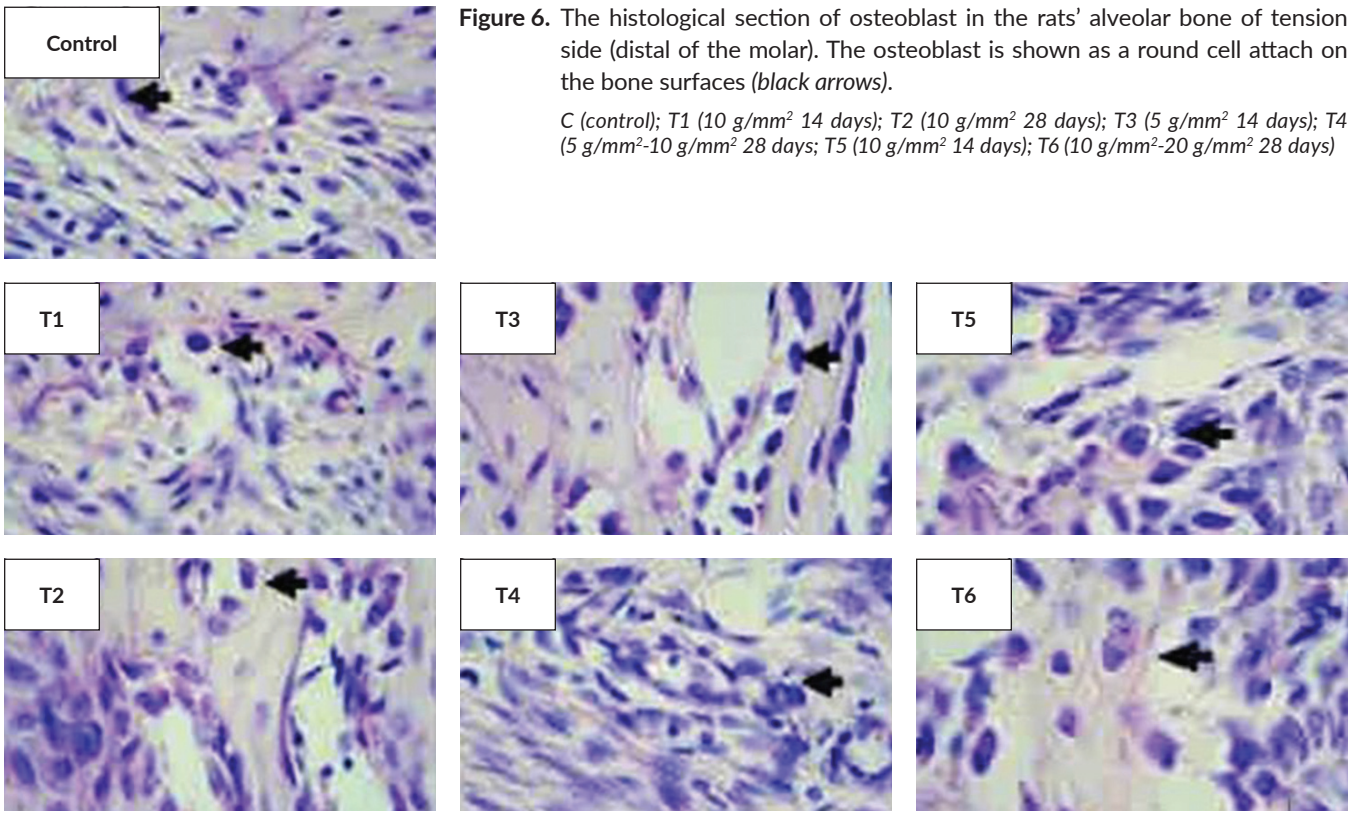

Figure 6. The histological section of osteoblast in the rats' alveolar bone of tension side (distal of the molar). The osteoblast is shown as a round cell attach on the bone surfaces *(black arrows)*.

C (control); T1 (10 g/mm² 14 days); T2 (10 g/mm² 28 days); T3 (5 g/mm² 14 days); T4 (5 g/mm²-10 g/mm² 28 days; T5 (10 g/mm² 14 days); T6 (10 g/mm²-20 g/mm² 28 days)

of 10 g proved to be stable and able to deliver a continuous and constant force in orthodontic tooth movement without interference in animal welfare.[18] Meanwhile, the group of 5 g/mm^2 force has the lowest expression of NFATc1 in the pressure area and has no significant difference of RUNX2 in the tension area compared to the control group (Table 5). It shows that the force of 5 g/mm^2 is a low force (below optimum force) which has inadequate bone remodelling that does not produce orthodontic tooth movement. According to Schwarz, forces below the optimal level do not cause reactions in the periodontal ligament.[17,18] Regarding the difference in duration of 14 days and 28 days, it shows that the highest expression of NFATc1 and RUNX2 is in the group of 28 days. It demonstrates that the duration of treatment of 28 days has more bone remodelling that induces more tooth movement compared to 14 days. This result is in accordance with the study by Gonzales, which found that 28 days of orthodontic treatment produced larger tooth movement.[19]

The T4 (5-10 g/mm^2) and T6 (10-20 g/mm^2) groups are the groups of gradually increasing force. Gradually increasing force in this study is defined as applying orthodontic light force initially, followed by the greater force. According to Schwarz, forces that exceed the optimum level will cause tissue necrosis and prevent frontal bone resorption. So that the tooth movement is delayed until undermining resorption removes inhibitory necrotic tissue.[17,18] In the T4 and T6 groups, the highest expression of NFATc1 and RUNX2 is the T6 group. This expression level is constant with the osteoclast amount. The higher expression of NFATc1, the higher the number of osteoclasts. The higher expression of RUNX2, the higher the number of osteoblasts. The formation of osteoclasts was lower in the group given strength from 5 g/mm^2 to 10 g/mm^2. This is because the strength of 5 g/mm^2 is a low force (under optimum force) and has not been able to activate adequate osteoclast formation for tooth movement. The formation of osteoclasts began to form when the force was increased to 10 g/mm^2. In contrast to the T4 group, the group with an increasing force of 10 g/mm^2 to 20 g/mm^2 showed higher osteoclast formation and osteoblast formation. This is because the T6 (10-20 g/mm^2) group was started with the light and optimum force (10 g/mm^2). Applying light force at an early stage and then increasing to a greater force can prevent the negative effects of applying heavy force. The balance-increasing number of osteoclasts on the pressure side and osteoblasts on the tension side demonstrates that tooth movement is still running and hyalinization does not occur.

Hyalinization of the periodontal tissue will inhibit tooth movement. Hyalinization not only inhibits osteoclast formation on the pressure side of frontal resorption but also induces undermining resorption.[20-22] This is consistent with the results of a previous study conducted by Tomizuka et al. which showed that applying light force at an early stage and gradually increasing its force induced the formation of more osteoclasts with less hyalinization and thus effective tooth movement.[10]

CONCLUSION

Gradually increasing force started by applying light force followed by greater force is an effective method for orthodontic tooth movement and inhibits hyalinization. Inducing a high expression of NFATc1 on the pressure side and RUNX2 on the tension side induces a high formation of osteoclasts on the pressure side and osteoblasts on the tension side.

Statement of Authorship

All authors certified fulfillment of ICMJE authorship criteria.

Author Disclosure

All authors declared no conflicts of interest.

Funding Source

This study was self-funded by the authors.

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