

## Short-term effects of nicotine on orthodontically induced root resorption in rats

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### ABSTRACT

**Objective:** To investigate the effect of nicotine exposure on root resorption in an in vivo rat model of orthodontic tooth movement (OTM), and its association with odontoclastogenesis and receptor activator of nuclear factor-kappa B ligand (RANKL) expression.

**Materials and Methods:** Forty-eight 10-week-old male Wistar rats were divided into three groups. The negative control group was untreated. The left maxillary first molars in the nicotine-treated group and the positive control group received OTM with an initial force of 0.6 N in the mesial direction. Also, the nicotine-treated group received intraperitoneal injection of nicotine at 7 mg/kg per day. After 21 days, the rats were humanely killed. Eight rats from each group were randomly chosen for crater volume analysis by micro-computed tomography. For the remaining eight rats in each group, specimen slices were generated for histologic examination to determine the odontoclast number and the mean optical density value of RANKL.

**Results:** The resorption volumes in the nicotine-treated group were significantly larger than those in the control groups. Also, the nicotine-treated group displayed significantly higher number of odontoclasts and elevated RANKL expression compared to the control groups.

**Conclusions:** In an in vivo rat model, nicotine exposure promotes odontoclastogenesis and RANKL expression, evoking aggravated root resorption during OTM. (*Angle Orthod.* 2016;86:199–205.)

**KEY WORDS:** Nicotine; Root resorption; Micro-computed tomography; RANKL; Odontoclasts

### INTRODUCTION

Tobacco smoking has been suggested to be an important risk factor throughout the body; accounting for about 5.4 million premature deaths annually worldwide.<sup>1</sup> It has been reported that the percentage of smoking among patients who are under orthodontic treatment is not negligible,<sup>2</sup> and nicotine has been

demonstrated to be the major toxic component in tobacco smoke.<sup>3</sup>

A number of in vivo and in vitro studies have suggested that nicotine can affect bone metabolism. It was suggested that nicotine aggravated alveolar bone loss in experimental periodontitis.<sup>4</sup> Several other studies in animals have suggested that nicotine may not only adversely affect fracture healing,<sup>5</sup> but also accelerate orthodontic tooth movement (OTM) in a dose-dependent manner.<sup>6</sup> All of this evidence indicates that nicotine may play an important role in the process of bone resorption and bone formation.

To our knowledge, bone undergoes constant remodeling with bone resorption and the bone formation process, which is mainly induced by osteoclasts and osteoblasts. Previous in vitro studies reported that nicotine stimulated the formation and differentiation of osteoclasts,<sup>7,8</sup> suppressed osteogenesis in osteoblasts, directly stimulated osteoclast precursors, and induced an imbalance of osteoblasts and osteoclasts in vitro.<sup>9,10</sup> Rothen et al.<sup>11</sup> suggested that nicotine affected osteoblast cell proliferation in a biphasic manner, including toxic and antiproliferative effects at high levels of nicotine and stimulatory effects at low

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levels.<sup>11</sup> In a word, the cellular effects of nicotine are still controversial.

The effects of nicotine on osteogenic and osteolytic mediators have been demonstrated in many *in vitro* studies. Nicotine was found to upregulate the expression of various osteolytic mediators, such as interleukin (IL)-1, IL-8, receptor activator of nuclear factor-kappa B/RANK ligand (RANKL), matrix metalloproteinase, and tissue-type plasminogen activator, and downregulate the expression of osteoprotegerin.<sup>12–14</sup>

Most clinicians regard orthodontically induced root resorption as an inevitable sequela of OTM. The odontoclasts were reported to be responsible for the process of root resorption and share many features with osteoclasts, such as the expression of H<sup>+</sup>-ATPase, cathepsin K, matrix metalloproteinase-9, and so on.<sup>15</sup> Furthermore, the processes of primary root resorption and bone remodeling involve the same receptor ligand system known as RANK/RANKL. So, it may indicate that there is a common mechanism in cellular resorption of mineralized tissues such as bone and teeth.

Considering the similarity between bone resorption caused by osteoclasts and root resorption induced by odontoclasts, it is conceivable to observe that nicotine exposure affects root resorption by altering odontoclastogenesis and the expression of relevant cytokines. To our knowledge, there have been no reports on the effects of nicotine on root resorption. Therefore, our study was conducted.

## MATERIALS AND METHODS

### Experimental Animals and Groups

Forty-eight 10-week-old male Wistar rats were used as experimental animals and randomly assigned into three groups of 16 rats each: two control groups (positive and negative controls) and a nicotine-treated group. Nicotine (Sigma Chemical, St Louis, Mo) was diluted in saline at a concentration of 1 mg/mL and injected intraperitoneally at a dose of 7 mg/kg per day for 21 days in the nicotine-treated group. The positive control group received OTM and saline according to the same protocol. The negative control group received only saline. Eight rats in each group were used for microcomputed tomography (micro-CT) scanning to observe the root resorption area quantitatively and the remaining eight were used for histologic examination (hematoxylin and eosin staining, tartrate-resistant acid phosphate staining, and immunohistochemical staining). During the experimental period, the rats were fed a power diet and water *ad libitum*. The study was conducted

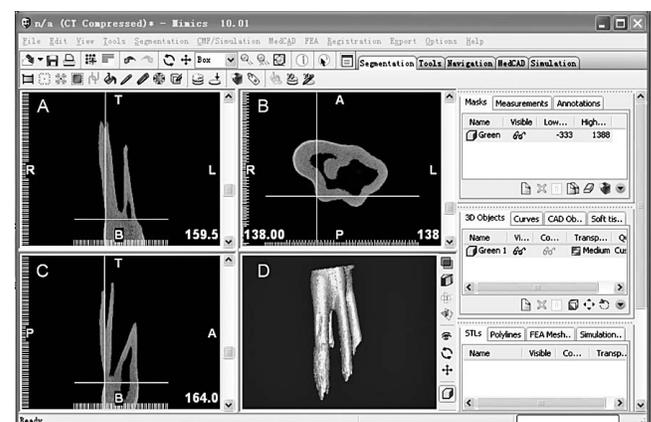
under approval from the Animal Welfare Committee of Shandong University.

### Animal Model Establishment

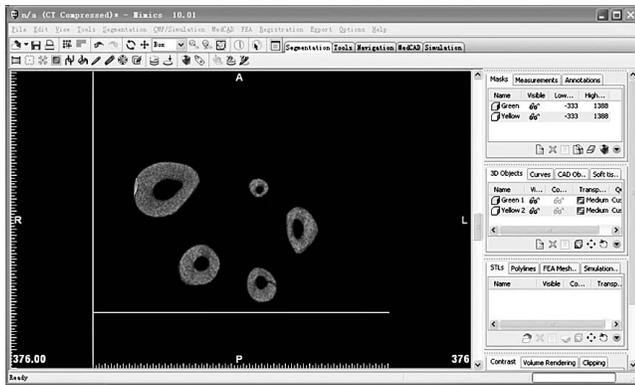
The rats were generally anesthetized, and a Ni-Ti closed coil spring (0.012-inch in diameter, IMD, Shanghai, China) was placed between the maxillary incisors and left maxillary first molar by steel ligatures as reported by Sodagar et al.<sup>6</sup> The force magnitude of the coil spring was set approximately 0.6 N and was measured when the appliance was mounted and at the end of the experiments with an ergometer (Changsha Tiantian Dental Co Ltd, Changsha, China). The stability of the ligature on the incisors was secured with a prepared cervical groove and the dental adhesive agent (Tianjin Synthetic Material Research Institute, Tianjin, China).

### Micro-CT

After 21 days of force application, eight rats of each group were randomly chosen to be humanely killed by neck break. The left maxillary first molars were carefully separated from the periodontal tissue, cleared by 1% sodium hypochlorite, and then stored in 10% formalin. The samples were scanned by a SkyScan1172 (SkyScan, Kontich, Belgium) micro-CT at the condition of 80 kV and 100  $\mu$ A with an exposure time of 744 milliseconds and an image pixel size of 4.83  $\mu$ m. NRecon software provided by the SkyScan1172 was used to transfer the raw data to two-dimensional (2D) axial cross-sections. The 3D reconstructed images were achieved by juxtaposition of 2D adjacent cross-sections with the Mimics 10.01 software (Materialise, Leuven, Belgium) (Figure 1).



**Figure 1.** Three-dimensional reconstruction of the maxillary first molar using Mimics 10.01 software. (A, B, C) Two-dimensional sections of the molar from three different section views. (D) Three-dimensional image of the reconstructed molar.



**Figure 2.** Three-dimensional reconstruction of the resorption lacunae: the yellow part represents a measured lacunae area in the cross-section view.

The mesial roots of the upper first molars were chosen to analyze. The resorption craters scattered on the mesial roots were established from micro-CT images in cross-sections by using the edit marks tool in the software (Figure 2), and then they were 3D reconstructed. The location, number, and volume of resorption craters were investigated after reconstruction, and the volume information was calculated using the Mimics analysis software.

### Tissue Preparation

At the end of the experiments, the remaining eight rats in each group were generally anesthetized and perfused transcardially with 4% paraformaldehyde (pH 7.2–7.6), and then the maxillae, including first and second molars, were dissected. The acquired specimens were immersed in 4% paraformaldehyde overnight at 4°C, decalcified with 10% EDTA at 4°C for 8 weeks, dehydrated with alcohol, rendered transparent by xylene, and embedded in paraffin wax. Serial sections of 5- $\mu$ m thickness were cut mesiodistally at the midpoint of the mesial root and prepared for hematoxylin and eosin, tartrate-resistant acid phosphatase (TRAP), and immunohistochemical staining.

### TRAP Staining

For TRAP staining, a reagent kit (Sigma-Aldrich) was used, and all procedures were finished under the guide of the manufacturer's instructions. Counterstaining was conducted with hematoxylin. The TRAP-positive multinuclear cells with burgundy red cytoplasm located on the surface of alveolar bone and root were identified as osteoclasts and odontoclasts, respectively. Areas (400 $\times$ ) around the mesial side of the cervical and middle one-third of the root of the maxillary first molar were used. The results were shown as mean  $\pm$  SD.

### Immunohistochemical Staining

For immunologic staining, RANKL polyclonal antibodies (Santa Cruz, Calif), the streptavidin-peroxidase (S-P) immunohistochemical reagent kit, and the diaminobenzidine developing box (Beijing Boisynthesis Biotechnology Co Ltd, Beijing, China) were used, and all operations were completed according to the manufacturers' instructions. The sections were counterstained with hematoxylin and mounted. Areas around the mesial side of the cervical and middle one-third of the root of the maxillary first molar were examined by light microscopy. The mean optical density (MOD) value was measured by Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA) under 400 $\times$  magnification.

### Statistical Analysis

One-way analysis of variance (ANOVA) was performed with SPSS statistics version 17.0 analysis software (SPSS Inc, Chicago, Ill), and  $P < .05$  was taken as the minimum level of significance.

## RESULTS

### Micro-CT

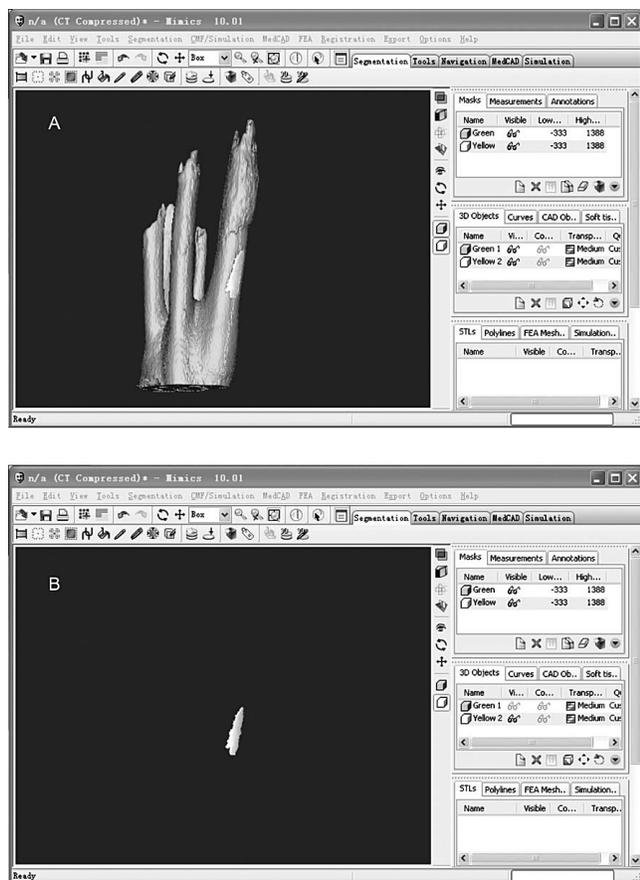
The reconstructed 3D images of the tooth and the resorption lacunae are shown in Figure 3. The samples in the groups with orthodontic tooth movement displayed more root resorption pits compared with the negative control group. A greater number of resorption craters were found on the root surfaces of teeth in the nicotine-treated group compared with the positive control group, and there were significant ( $P < .05$ ) differences between the two groups in the volume of orthodontically induced root resorption (OIRR) (Figure 4).

### Histologic Observations by Hematoxylin and Eosin Staining

No obvious resorption craters were observed on the surface of alveolar bone and root in the negative control group (Figure 5A). While both nicotine-treated group and positive control group showed pronounced resorption lacunae on the surface of alveolar bone and root (Figure 5B,C), the extent of the resorption was more severe in the nicotine-treated group than the control group, which was embodied in the larger and deeper craters in the resorption area.

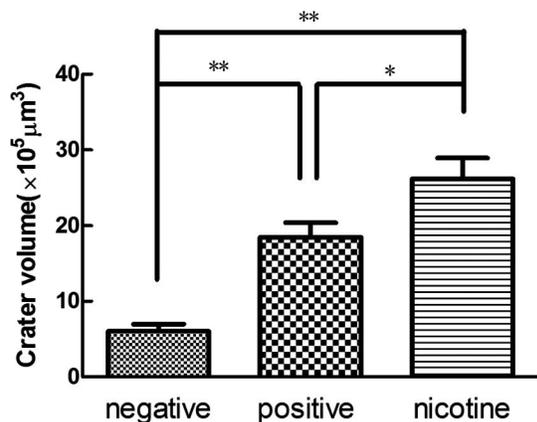
### TRAP Staining

The TRAP positive cells were observed lining on the resorption craters of both the root and the alveolar bone in the positive control and nicotine-treated group. Few TRAP positive cells were detected in the negative

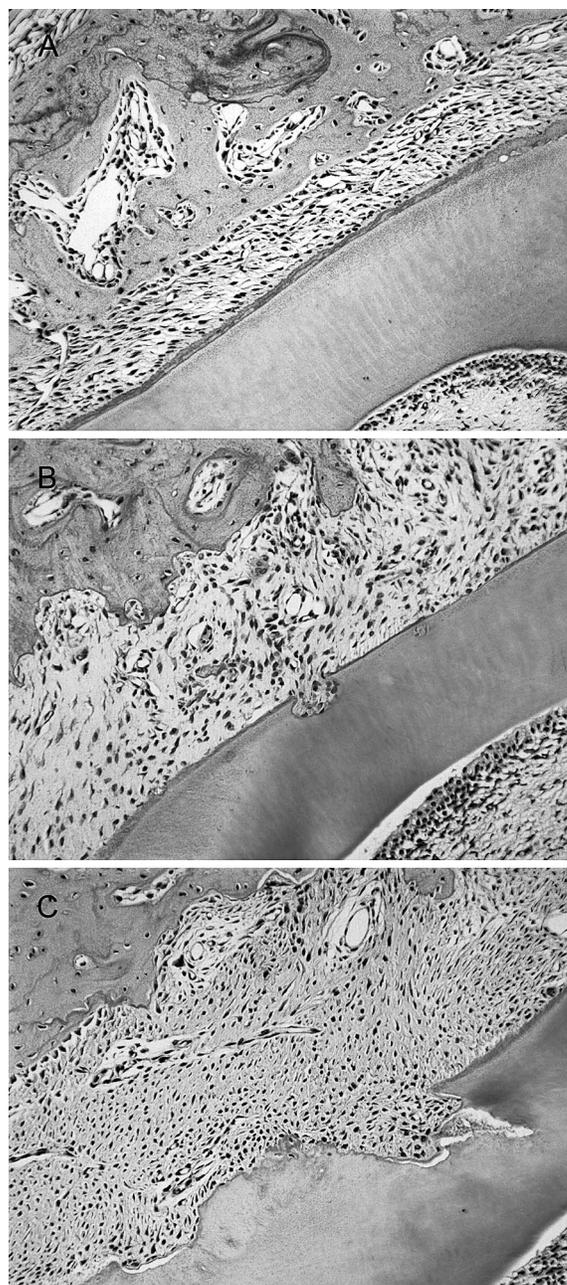


**Figure 3.** The finished reconstructed images of the tooth and the resorption lacunae. (A) The combined image of tooth and resorption lacunae after reconstruction. The yellow area shows a reconstructed resorption crater. (B) The isolated image of the crater after reconstruction.

control group (Figure 6). The number of odontoclasts counted in the positive control group was fewer than that in the nicotine-treated groups. The difference between the two group was statistically significant ( $P < .01$ ) (Figure 7).



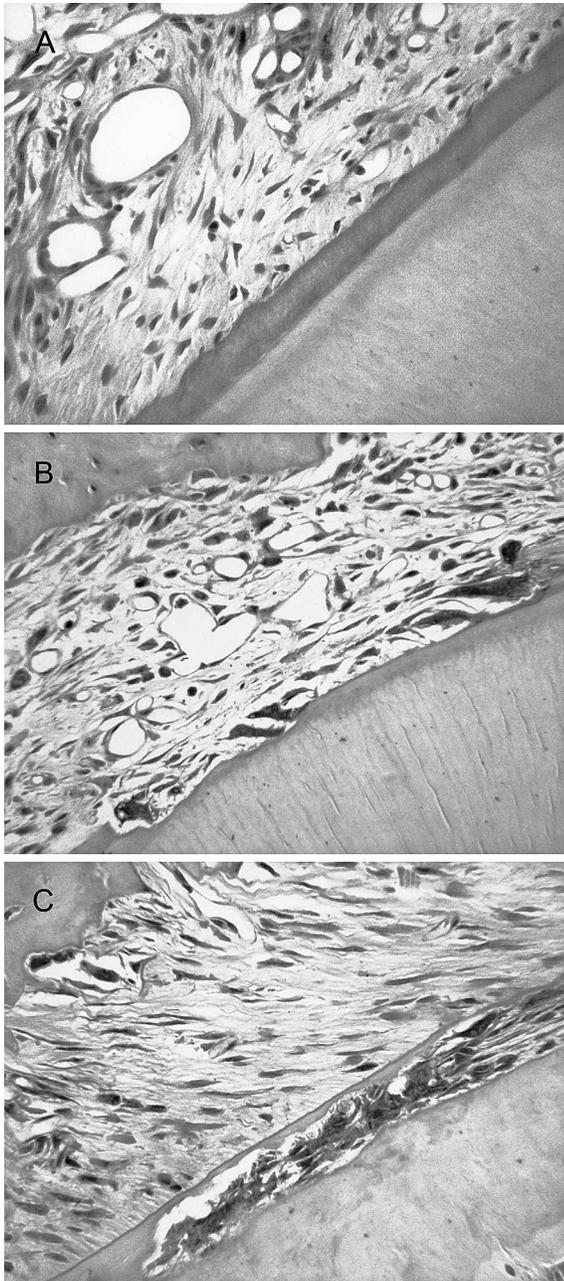
**Figure 4.** The volume of the resorption craters. Data are presented as mean  $\pm$  SD (\*  $P < .05$ ; \*\*  $P < .01$ ).



**Figure 5.** Histologic observation of the molars on the compression side. Few resorption lacunae occurred in the (A) negative control group. More resorption lacunae and wider resorption area were observed in the (C) nicotine-treated group compared with the (B) positive control group (hematoxylin and eosin staining, magnification 200 $\times$ ).

**Immunohistochemical Examination**

The positive RANKL expression with yellow-brown cytoplasm stained was mainly located in the periodontal membrane, odontoclasts, and osteoclasts in the resorption craters. The negative control group showed the weakest positive signals. More intense positive signals were apparently observed around the resorption craters

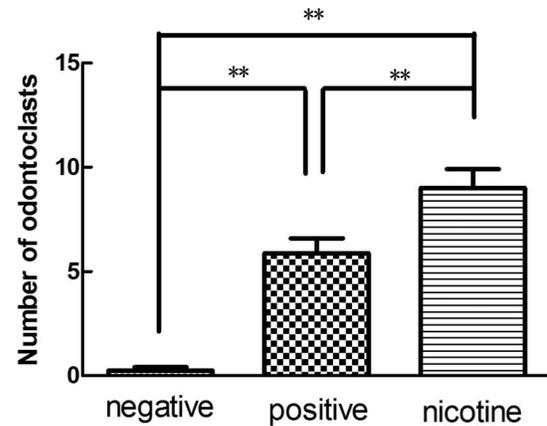


**Figure 6.** Histologic sections from TRAP staining of the molars. Few odontoclasts were observed in the (A) negative control group. The number of odontoclasts was more in the (C) nicotine-treated group compared to the (B) positive control group (magnification 400 $\times$ ).

in the nicotine-treated group than the positive control group (Figure 8). The MOD value of RANKL expression in the nicotine-treated group was higher than that in the positive control group, and the difference was statistically significant ( $P < .01$ ) (Figure 9).

## DISCUSSION

In our rat model, the force application on their maxillary first molars was started at week 8, and root

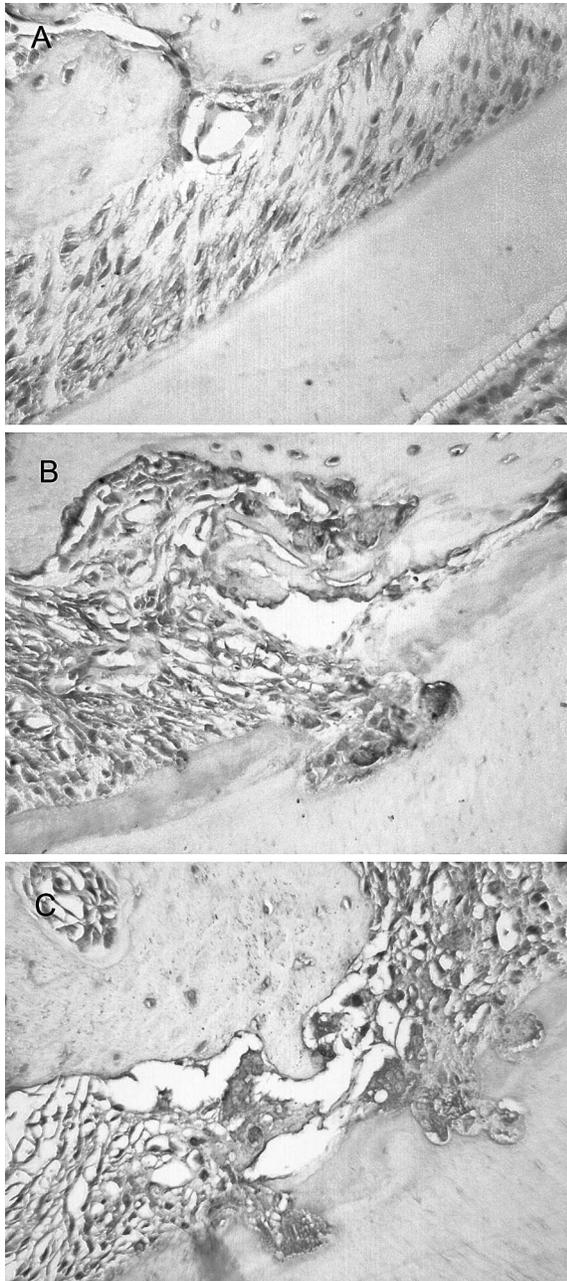


**Figure 7.** The number of odontoclasts. Data are presented as mean  $\pm$  SD (\*\*  $P < .01$ ).

formation of the molars should have finished as suggested by Matias et al.<sup>16</sup> The nicotine dose chosen in this study was based on the research of Hapidin et al.<sup>17</sup> about the effect of nicotine on bone metabolism, and it was similar to that of individuals who smoke 10–20 cigarettes per day.<sup>18</sup> Helsing and Hammarstrom<sup>19</sup> found definitive resorption sites forming within a week of appliance placement using a scanning electron microscope, so our 3-week experimental period was adequate to produce resorption sites to analyze.

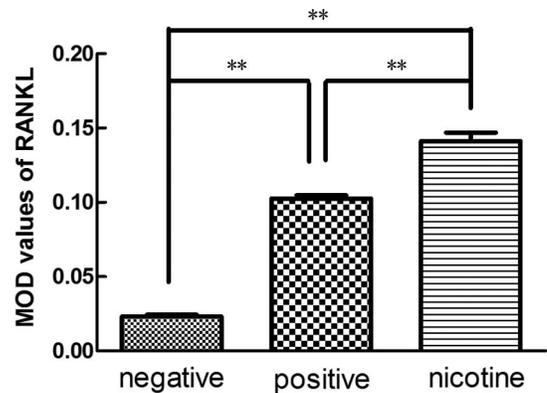
Odontoclasts, which were reported to mediate root resorption, are similar to osteoclasts in morphologies and biologic characteristics.<sup>15</sup> In the present study, more TRAP-positive osteoclasts and odontoclasts, which may contribute to more severe root resorption, were seen in the group treated with nicotine than in the control groups. This observation is in accordance with the study by Henemyre et al.<sup>8</sup> In their research, nicotine was found to stimulate osteoclast differentiation, and the number of osteoclasts increased in a linear relationship to the increasing nicotine concentrations. Similarly, several lines of evidence suggested that nicotine favors osteoclastogenesis in human periodontal ligament cells co-cultured with CD4<sup>+</sup> T cells<sup>14</sup> and stimulates the formation of osteoclast-like cells via an increase in macrophage colony-stimulating factor and prostaglandin E2 production.<sup>7</sup>

However, some other controversial conclusions regarding the effect of nicotine on bone cells have been reported. Tanaka et al.<sup>9</sup> found that nicotine induces osteoclasts with the small number of nuclei, but reduces the number of osteoclasts with many nuclei at the same time. Some investigations on the influence of nicotine on the proliferation of osteoblasts came to the adverse conclusion that one study reported that nicotine decreases cell proliferation in rat osteoblastic osteosarcoma cells,<sup>20</sup> while another suggested the stimulation



**Figure 8.** Histologic sections from RANKL immunohistochemical staining of the molars. (A) Negative control group shows weaker immunoreactivity for RANKL compared to the other two groups. The immunoreactivity for RANKL was stronger in the (C) nicotine-treated group compared to the (B) positive control group on the compressed side (magnification 400 $\times$ ).

effect of nicotine in murine clonal osteoblast-like osteogenic MC3T3-E1 cells.<sup>21</sup> The discrepancy among the above mentioned reports may be related to the differences in species, cell types, and conditions for cell culture. Although the effects of nicotine on bone cells are still controversial, it can be concluded that nicotine



**Figure 9.** The mean optical density value of RANKL. Data are presented as mean  $\pm$  SD (\*\*  $P < .01$ ).

can modulate bone metabolism by regulating osteoclastogenesis and osteoblastogenesis.

Root resorption is a frequent iatrogenic consequence of OTM, and the mechanisms of it were reported to be similar to those of bone resorption.<sup>15</sup> In this study, the volume of root resorption craters in the nicotine-treated group was significantly greater than the volume measured in the control groups. This result indicated that nicotine can lead to root resorption, strengthening our conclusion about the modulation effect of nicotine on bone metabolism, considering the similarity between root resorption and bone resorption. This result is also consistent with another finding that the RANKL expression, which has been demonstrated to be associated with root resorption,<sup>22</sup> in the nicotine-treated group was elevated and more than the other two groups.

Previous studies have indicated that nicotine plays an important role in the secretion of bone-resorbing cytokines and the process of bone metabolism. Hapidin et al.<sup>17</sup> reported that nicotine was detrimental to bone by causing an increase in the serum bone-resorbing cytokines IL-1 and IL-6. In a human in vitro study, nicotine was found to upregulate the expression of IL-6 and tumor necrosis factor (TNF)- $\alpha$ .<sup>23</sup> Those factors can stimulate RANKL expression and are important for the induction and the further process of root resorption in the rat.<sup>24,25</sup> These findings suggest that the effect of nicotine on root resorption may act via various cytokines and signal pathways and further verify our hypothesis that the aggravated root resorption caused by nicotine is related to the elevated expression of RANKL. However, the role that nicotine is playing in this biologic process of root resorption is still incompletely understood, and whether other doses of nicotine or longer exposure time to nicotine may influence the process of root resorption during OTM should be further investigated.

## CONCLUSIONS

- In an in vivo rat model of OTM, significantly more root resorption occurred under nicotine exposure related to promoting odontoclastogenesis and elevated expression of RANKL.
- It indicated that orthodontists should inform their smoking patients of the potential risks of root resorption and advise them to quit smoking.

## ACKNOWLEDGMENTS

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