Effect of the number of micro-osteoperforations on the rate of tooth movement and periodontal response in mice

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Effect of the number of micro-osteoperforations on the rate of tooth movement and periodontal response in mice

Directed by Professor Jung Yul Cha

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ABSTRACT

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(Directed by Professor **Jung Yul Cha**)

This study sought to evaluate the biological effects of the number of micro-osteoperforations (MOPs) on the rate of tooth speed and the potential risk for root resorption in a mouse model.

Male CD1 mice (n=36) were divided into 4 groups based on the number of MOPs, as follows: Sham group (Non-orthodontic tooth movement (OTM)); Control (OTM only group); 2MOPs (2 holes) and OTM group; and 4MOPs (4 holes) and OTM group. A force of 25 g was applied from the maxillary right first molar to the maxillary incisors using a superelastic nickel-titanium (NiTi) closed-coil spring. The tooth movement distance was measured using a Hyperscope. Micro-computed tomography (micro-

CT) was performed to evaluate bone mineral density (BMD), bone volume (BV), bone volume fraction (BVF), trabecular number (Tb. N), and volume of root resorption (VRR) and area of root resorption (ARR) on the root surface. Furthermore, the number of osteoclasts was thereafter assessed using tartrate-resistant acid phosphatase (TRAP) staining at 14 days of consolidation.

The 4MOPs group showed significantly increased (p <0.05) speed of tooth movement compared with the Control (OTM), and significantly decreased BMD, BV, BVF and Tb. N (p <0.05). The number of osteoclasts in the 4MOPs group (74 ± 13) was significantly higher than in the Control (OTM) group (59 ± 8). The VRR of the Control (OTM) group (0.00049 ± 0.00027 mm^3) was less than that of the 4MOPs group (0.00061 ± 0.00021 mm^3).

In this mouse model, the 4MOPs group had effective acceleration of the rate of tooth movement by increased bone turnover, as evidenced by an increased number of osteoclasts and a decrease in bone density compared with the Control (OTM) and 2MOPs groups for 14 days. However, this did not affect the volume or area of root resorption compared to the 2MOPs and 4MOPs groups.

Key words: Micro-osteoperforation, orthodontic tooth movement, micro-CT, root resorption

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I. INTRODUCTION

In the last decade, various comparative studies have used minimally invasive methods to accelerate and assess orthodontic tooth movement (OTM) during treatment, as well as to confirm biological responses (Sugimori et al. 2018). According to clinical reports, orthodontic tooth movement was accelerated by 1.75 fold (Kundi, Alam, and Shaheed 2020; Sivarajan et al. 2020), or the duration of treatment is reduced by 57 % (Alikhani et al. 2013; Alkebsi et al. 2018; Attri et al. 2018). Reducing the duration of orthodontic treatment may minimize unwanted side effects. For example, this increases the risk of complications such as dental decalcification (Richter et al. 2011), gingivitis with alveolar bone

loss (Ristic et al. 2007), pulpal response and root resorption (Grunheid, Morbach, and Zentner 2007; Weltman et al. 2010).

As mentioned, less invasive methods are more widely used in clinical practice, because patients do not report strong pain with these methods (Alikhani et al. 2015; Shahabee et al. 2020). Despite augmenting tooth movement and patient discomfort, these treatments have had limited clinical use due to many negative local and systemic side effects, such as hyperalgesia (Yamaguchi and Kasai 2005), bone loss and osteoporosis, delayed wound healing, and root resorption (Krishnan 2005; Sekhavat et al. 2002; Long et al. 2013) (Kalemaj, Debernard, and Buti 2015). Invasive methods include traditional surgical methods, including different types of corticotomies (Kole 1959; Wilcko et al. 2001; Wilcko et al. 2009), and alveolar distractions (Terbish et al. 2015), which have been shown to stimulate localized inflammatory responses and thereby increase the regional acceleratory phenomenon (RAP).

In 2009, Kim et al (Kim, Park, and Kang 2009; Kim et al. 2009), introduced the corticision procedure in an effort to develop a minimally invasive method to induce an RAP effect without incurring a flap reflection. With the RAP, given severe stimuli, abscopic involvement can occur, meaning that accelerations of ongoing tissue turnover and perfusion can occur in the contralateral regions of the body (Frost 1983).

Recently, a new technique has been developed to accelerate tooth movement using micro-osteoperforations (MOPs), stimulating the regeneration of the alveolar bone during minimal surgical trauma (Alikhani et al. 2013). This technique was developed based on previous animal studies showing that small and shallow perforations in alveolar bone increased the expression of inflammatory markers, as well as the rate of tooth movement by 1.3 – 2.3 fold, without the need for bone grafting, or suturing (Cheung et al. 2016; Librizzi et al. 2017; Baloul et al. 2011; Dutra et al. 2018).

However, these clinical and animal studies have shown side effects, such as root resorption (Shahabee et al. 2019; Chan et al. 2018). Therefore, there is a lack of evidence available, and the use of

MOPs can be recommended after weighing the benefits against the risk and side effects. On the contrary, some authors have found that minimally invasive interventions do not cause increased root resorption for at least two weeks (Cheung et al. 2016; Kurohama et al. 2017). In other words, all methods have differed in terms of size, force, whether they were with a flap or flapless, and number of holes. Thus, there remains no standard protocol to adapt and practice MOPs. Moreover, there remains a paucity of reports on the side effects of MOPs. Important questions as such remain to be answered in order to best adapt the method (with regard to whether the protocol should be flap or flapless, the hole size, and clarifying the hole number) and assess side effects based on biological evidence.

Our null hypotheses are the following:

1. No significant differences exist in terms of the rate of tooth movement by increasing MOP procedure compared with the no MOP (Control, OTM) group
2. No significant differences exist in terms of bone parameters by increasing MOP procedure compared with the no MOP (Control, OTM) group
3. No significant differences exist in terms of root resorption by increasing MOP procedure compared with the no MOP (Control, OTM) group

The aim of this study was to evaluate the rate of tooth movement and the risk for root resorption from micro-osteoperforations combined with an applied orthodontic force in mice.

II. MATERIALS AND METHODS

1. Study design and subjects

Thirty-six 8-week-old male CD1 mice weighing 35 - 40 g were used in this study. All animal sections, preparations, and surgical protocols were conducted according to the Association for Assessment and Accreditation of Laboratory Animal Care international (AAALAC) guidelines and approved by the Institutional Animal Care and Use Committee, Yonsei Medical Centre, Seoul, Korea (Approval No. 2018-0052). The animal laboratory was set to 22 °C and 50 % humidity with a 12-hour light-dark cycle for the experiments. Two mice were kept per cage. A regular soft diet (Transgenic Dough Diet™, Product #S3472, BioServ) was provided during the experimental period. Signs of infection or prolonged inflammation were not observed.

The sample size was determined with data from a pilot study and various other studies. The mice were randomly divided into 4 groups (12 mice *per* group):

1. Sham group (Non - orthodontic tooth movement (OTM) left side) group
2. Control (OTM) only group
3. 2Micro-osteoperforations (2MOPs) and OTM group
4. 4Micro-osteoperforations (4MOPs) and OTM group of the palatal cortical plate group.

The experimental protocol is shown in Figure 1. B, C, D, E. The contralateral (left) side was as the unloaded control for each animal. The application of orthodontic force and surgical procedure were performed only on day 0, and the OTM was performed for 14 days. The health status, body weight, and appliance of the mice were evaluated daily, and no significant differences were observed among the groups. From each group, all mice (12) were measured for tooth movement and bone parametric data, and underwent root resorption analyses used for micro-CT, and histomorphometric analyses.

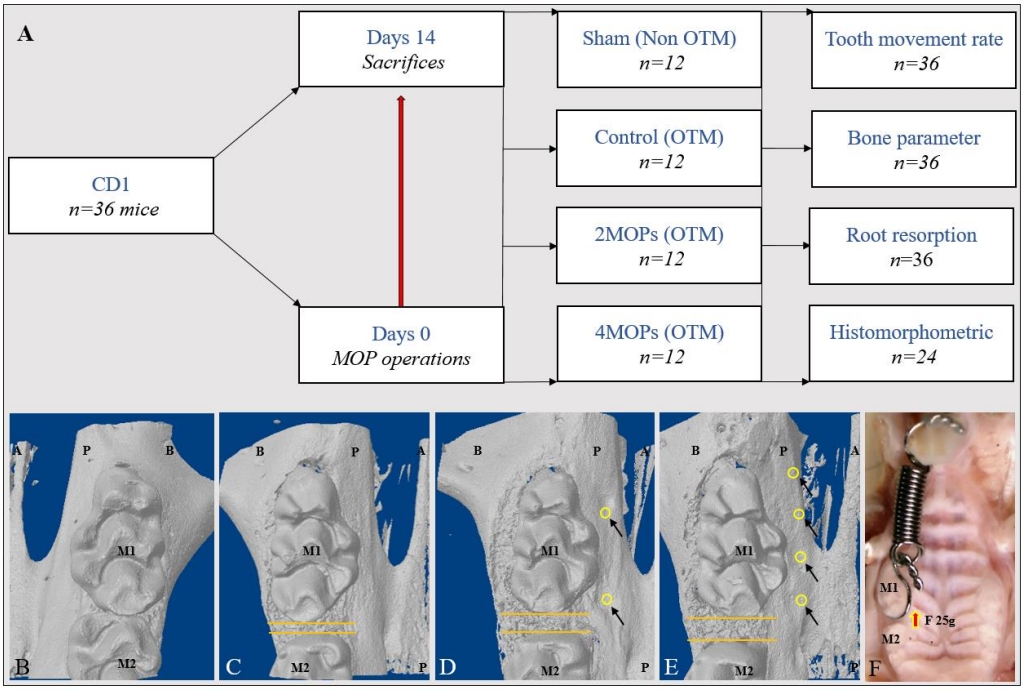

Figure 1. Study design and timetable. (A) Flow chart included baseline observation, where performed, 14 days of active orthodontic tooth movement was applied after MOP application and number of animals studied, and methods used for the all mice. 3D volumetric model of maxillary bone of mice for MOPs, and yellow dots are placed intra-radicular space on palatal alveolar bone around the maxillary first molar on only the right side. (B) Sham (Non-OTM) left side; (C) Control (OTM) only; (D) 2MOPs and OTM group; and 2 holes were placed, 1st drilling positioned fourth rugae which was mesial to the between mesial and palatal root. 2nd drilling positioned sixth rugae which was mesial to the between disto-buccal and palatal root. (E) 4MOPs and OTM group; and 4 holes were placed, 1^{s} drilling positioned palatal third rugae which was mesial to the mesial root's mesial aspect, 2nd drilling positioned fourth rugae which was mesial to the between mesial and palatal root. 3rd drilling positioned fifth rugae which was mesial to the palatal root. 4th drilling positioned sixth rugae which was mesial to the between disto-buccal and palatal root. holes distance was created 0.33 mm. (F) Appliance design indicating placement of the super-elastic nickel-titanium (NiTi) closed-coil spring from the maxillary first molar to the central incisors by F 25 g.

2. Surgical procedures

All animals were irradiated according to the experimental conditions under general anesthesia with ketamine-xylazin (0.10 ml/10 g). A 0.009-inch stainless steel ligature (GAC international, Bohemia, NY) was placed around the contact between the first and second right maxillary molars and securely ligated. A second 0.009-inch stainless steel ligature was then ligated to the maxillary incisors, and orthodontic force was applied using a super-elastic NiTi closed-coil spring with 25 g of force (EW, JISCOP, Korea) and attached to this ligature. After the ligature had been tied and cut, a self-etching primer and light cure adhesive composite resin (Transbond Plus; 3M Unitek, Monrovia, California, USA) were applied to the buccal and lingual surface of the maxillary incisors to prevent the slipping of the ligature wire holding the super-elastic NiTi closed-coil spring. Finally, the mandibular incisors were trimmed, and a collar was worn to prevent appliance breakage, after which the animals were returned to their cages.

Mice in the MOP group received two and four shallow perforations, after which drilling was performed, fully immersed into the bone for every perforation. These perforations were created using a ¼ round bur (Komet, Germany) with a low-speed hand piece. Body weight and appliance stability were checked daily, and mice were sacrificed 14 days after consolidation.

3. Dissection and tissue preparation

On day 14, the mice were sacrificed by carbon dioxide asphyxiation, and mandibles were removed and cleansed of soft tissue, and the maxilla was hemisected and fixed in 10 %, pH 7.4 neutral buffered formaldehyde (Duksan Pure Chemicals Co., ltd., Ansan-city, Kyungkido, Korea) solution for 24 hour at 4 °C. For micro-CT imaging, the maxilla from all mice were dehydrated in graded 70 % ethanol. Next, they were split along the hemi-maxillae, after which samples were fixed and decalcified in 7.4

pH, 15 %, ethylenediaminetetraacetic acid (EDTA) for 6 - 7 weeks and then processed for standard paraffin embedding. Sections that were four microns thick were mounted on the SP 1600 microtome (SP 1600 microtome, Leica DFC 290, Leica, Nussloch, Germany). The sections were sliced parallel to the sagittal plane of the upper molars, and the level of the sections from the start of the mesial and disto-buccal root to the end of mesial and disto-buccal roots was assessed by counting the number of serially sliced sections.

4. Assessment of the rate of tooth movement

Tooth movement distance was measured using a Hyperscope (Olympus Stereo zoom microscope SZ61, Tokyo, Japan). The micro-CT images on day 14 immediately after sacrifice were superimposed using the maxillary right second molar, the third molar, and the surrounding alveolar bone reference structures. The following two parameters were used to measure tooth movement: (1) the images were taken at a 0.67X and 1.5X magnification with a 2190X1640-pixel size (DIXI Imaging Solution, v.2.8). The amount of tooth movement was calculated as the average of two reference lines, which corresponded to the central cusp and palatal groove of the maxillary first and second molars measured at the inter-proximal heights of the contour between the most mesial point of the second molar crown and the most distal point of the first molar crown (Figure 4, A.); (2) the change in tooth inclination, which is the angle of tooth inclination of the maxillary right (experimental) and left (Non OTM) side was measured in a sagittal section. Reference lines were second, third molar line and distal root vertical line. Method was determined following previously reported (Verna, Dalstra, and Melsen 2000).

5. Micro-computed tomography (micro-CT) analysis

In vivo three-dimensional (3D) images were taken for each sample using high-resolution micro-CT (SkyScan 1173; BRUKER-MICROCT, Kartuizersweg 3B 2550 Kontich, Belgium) at a voltage of 90 kVp and a current of 88 μA with an X-ray source and a 5.33 μm pixel size. Scanning data were reconstructed using NreconVer 1.6 (Recons v.1.6.10.4, Bruker).

The micro-CT scans were taken on day 14 after all subjects were sacrificed. Adjusted micro-CT images were used to measure the mineralized tissue surrounding the maxillary first molar. The bone parameters were analyzed by CTAn (CTAn v.1.17.7.1, Skyscan, Bruker) to estimate alveolar bone changes. Root resorption was also assessed. Serial transverse scan images were taken at a resolution of 18 μm. Two-dimensional and three-dimensional images were reconstructed with the Data Viewer (DataViewer v.1.5.4.0 Skyscan, Bruker), CTvox (CT vox v.3.3.0, Bruker) program.

Next, ROIs were defined in the dataset delineating the trabecular area from the samples using CTAn. The ROI was selected as the intra-radicular space of the first molar between the roots (Figure 2. A, B). A square shape (450X450X450 μm) was selected, and the height of 60 % of the length of the disto-buccal root was determined. The distance between the ROI and the mesial surface of disto-buccal root was 100 μm. The threshold value for binary image was determined from 255 to 66 through all specimens, providing an accurate representation of the alveolar bone image within micro-CT images. Bone mineral density (BMD; g/cm^3), bone volume fraction (BVF; %), bone volume (BV; mm^3), trabecular thickness (Tb. Th; mm), trabecular number (Tb. N; 1/mm), and trabecular separation (Tb. Sp; mm), volume of root resorption (VRR; mm^3) and area of root resorption (ARR; mm^2) were analyzed accordingly (Table 2 and 3). Bone data were calibrated to BMD values using a hydroxyapatite phantom. The phantom was scanned using the same protocol as for the bone data.

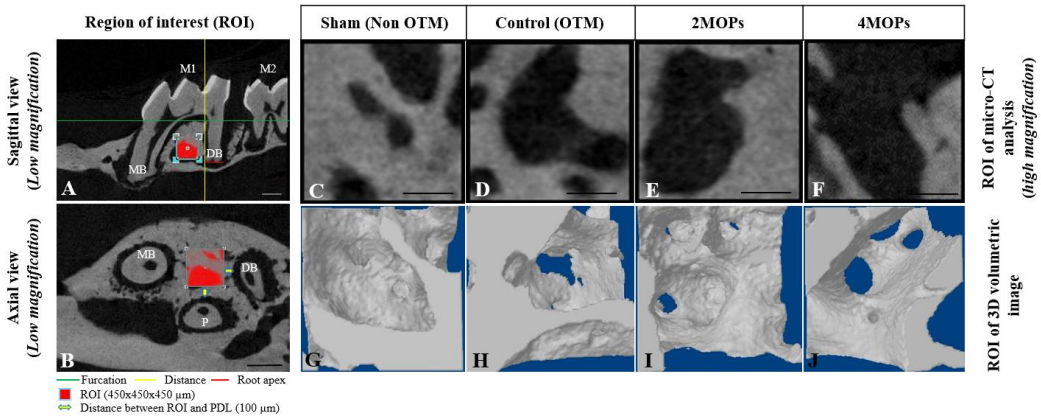

Figure 2. Micro-CT images depicting the ROI. (A) and (B) Micro-CT image and the ROI for bone parameter measurements (shaded) (Scale bar: 100 μm; 50 μm); (MB, mesio-buccal; DB, disto-buccal; P, palatal roots). (C) – (F) Micro-CT (Scale bar: 20 μm) and (G) – (J) 3D volumetric images of alveolar bone microstructures in selected ROI in the three groups at 14 days.

6. Root resorption analysis

Volumetric quantification of the root volume of the first molar was performed following a previously established protocol (Crowther et al. 2017). Given the variability and size of the molar roots, it was decided that only the distal root of the maxillary first molar would be sufficient to ensure consistent recording of the location of the root resorption crater. When orthodontic forces were applied to the maxillary first molar, the produced moment would cause the tipping of the molar along with its center of resistance. This situation was simulated by finite element modeling, reporting that mesial movement led to tensile and compressive strains in the middle third of the PDL on the mesial side of the distal root (Huang et al. 2016). We therefore focused our analysis on this region in this study. The apical section of the molar root was also too porous. Therefore, in order to ensure a clear differentiation between normal tooth anatomy and root resorption craters. For the VRR measurement, ROIs were selected at the disto-buccal root of the first from the furcation to 1/3 of apex (460 μm).

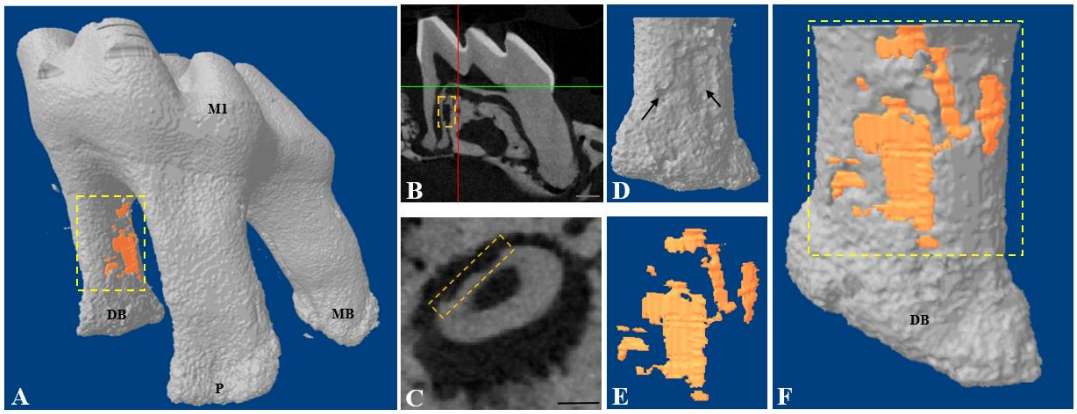

Figure 3. Manipulation of the three-dimensional image of the first maxillary mice molar. (A) Maxillary right first molar, and the area defined by the yellow box is the segment of the root that was analyzed for root resorption. (B) Locating the sagittal section at which the crater; (Scale bar: 100 μm). (C) View of the axial section at which the crater; (Scale bar: 50 μm). (D) Extracting the craters of resorption. (E) Isolating the craters of resorption. (F) Distal root mesial side with crater (M1, first molar; DB, disto-buccal; P, palatal; MB, mesio-buccal).

For the purpose of computing the volume and area of root resorption, images were viewed in axial slices and a “mask” was created for the volumetric analysis of the crater images (Figure 3, C). Mask creation started with locating the axial section at which the crater began. Next, the outline of the crater was traced, a segmentation tool was used following the internal contours of the crater, and the external margin of the crater was an estimate of the continuation of the convexity of the root surfaces. If the mask ceased to resemble the resorption crater, a new outline was redrawn. Subsequently, crater masks of each tooth were accumulated in a single segmentation. Finally, the software calculated the volume and area of the segmented mask in voxels which represented the volume of root resorption.

7. Histological and histomorphometric analysis

To quantity osteoclast activity and relate it to the bone resorption changes, histomorphometric analyses were performed on all groups. Hemi-maxillae were obtained and stained with routine hematoxylin and eosin (HE) and tartrate-resistant acid phosphatase (TRAP), using an acid phosphatase leukocyte kit (Sigma Chemical, St Louis, Mo) according to the manufacturer's instructions. Stained sections were scanned on a Microscope (Leica, DM 2500 LED, Wetzlar, Germany) at 20X/0.40 PH1 magnification. Active osteoclasts were defined as TRAP-positive, multinucleated cells with more than 3 nuclei, touching the bone surface (Murphy et al. 2014). The ROI of the disto-buccal root of the maxillary first molar was divided into mesial and distal halves, and osteoclasts in the mesial half were counted number and area of TRAP-positive cells (View Point Light). The exact area for osteoclast quantification included a rectangular box of 125.6 μm^2 that was placed on the compression side of the alveolar bone, including the cementum, periodontal ligament and alveolar bone. This ROI started from the tap of the alveolar bone. The size of the ROI was determined in the same way as the analysis of bone parameters and VRR, at 60 % of the length of the disto-buccal root. The values from 3 sections were then averaged for each mouse, and the means from 8 animals in each experimental group were used to conduct statistical tests. All the measurements were collected by the same researcher and repeated two times. Then mean value was calculated and recorded as the final value.

8. Statistical analysis

The Kruskal-Wallis test and post-hoc test (Mann-Whitney *U*-test) were used to determine the statistical significance of the intergroup comparisons of the rate of tooth movement, bone parameters, TRAP-positive cells, and root resorption. The significance level was set at $p = 0.05$. All statistical analyses were performed using SPSS software (version 25; IBM Co., Armonk, NY, USA).

III. RESULTS

Animal data

Forty-five male CD1 mice were used in this study. Among these, two mice died during general anesthesia, three mice were eliminated from the study as their appliances failed during the experimental period, and four mice were eliminated from the study because they experienced severe weight loss. As such, a total of 36 mice were left at the completion of the study. During the decalcification period, four specimens in each group failed due to the rapid solution. Histomorphometric examinations were based on eight datasets for each group. All mice that were caged together had the same soft diet (BioServ, US).

1. Comparison of the rate of tooth movement

The experimental phase for each study group was 14 days. We examined the amount of tooth movement and tooth movement inclination that occurred in the Sham group (Non OTM); Control group (OTM), orthodontic force only group; 2MOPs and OTM group; and 4MOPs and OTM group. All data were reported as means and standard deviations for each parameter. The distance of the orthodontic tooth movement in the 4MOPs group, of 0.38 mm (SD 0.08), was increase with the Control (OTM) group, at 0.29 mm (SD 0.06) (p <0.05); that of the 2MOPs group was 0.31 mm (SD 0.07), and showed no significant difference to the other groups (Table 1) (p >0.05). Tooth movement inclination in the 4MOPs group was 86.02 ° (SD 7.22), showing significant differences compared with the Control (OTM) group, at 92.67 ° (SD 4.57). Finally, that of the Sham (Non-OTM) group was 0.08 mm (SD 0.02), showing significant differences between the groups (p <0.05). The tooth movement inclination was 98.88 ° (SD 4.83), with significant differences between the 2MOPs and 4MOPs groups (p <0.05).

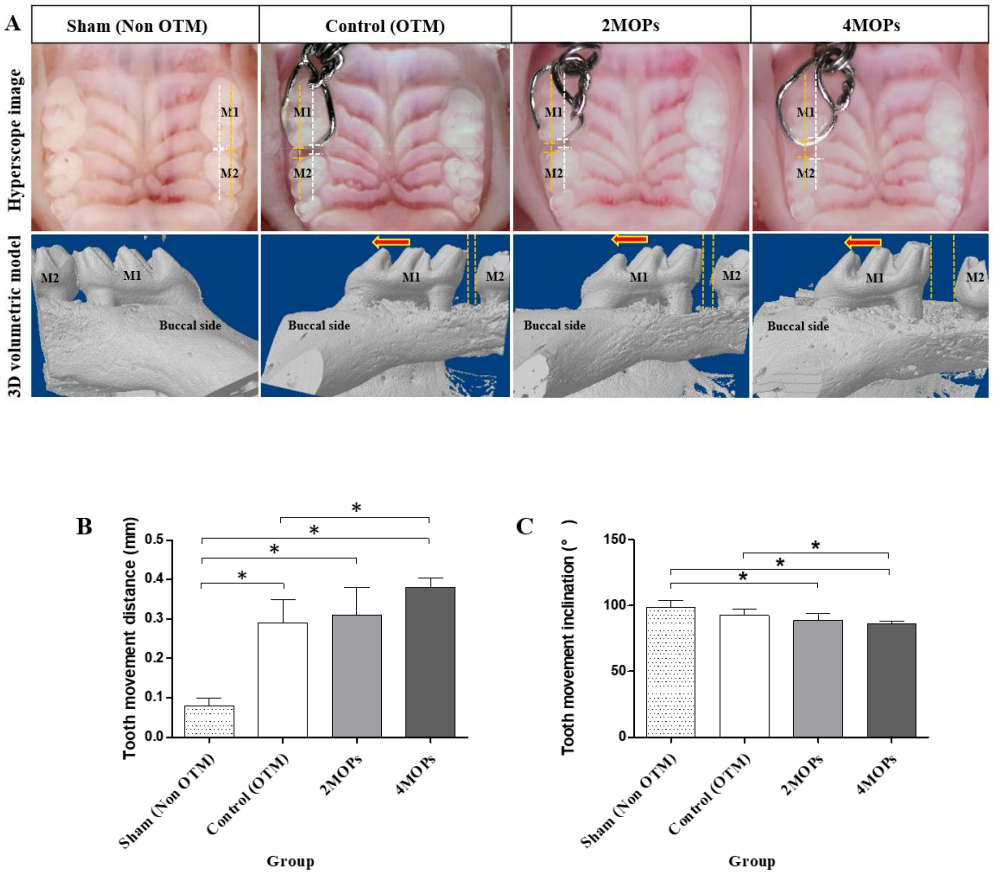

Figure 4. Tooth movement distance and inclination measurement. (A) Representative Hyperscope image indicating the measurement of the tooth movement between the first and second molars at 1.5X magnification. 3D volumetric model (*buccal side*) of maxillary first molar of mice showed comparison of tooth movement between the groups after 14 days. (Sham group (Non force); Control (OTM), orthodontic force only; 2MOPs and OTM; 4MOPs and OTM). (B) The rate of orthodontic tooth movement in the 4MOPs group was 1.31-fold increase compared with the Control (OTM) group. (C) Tooth movement inclination significantly decreased 12.86 ° (SD 2.39) in the 4MOPs group compared with the Control (OTM) group. Further, statistically significant differences in Sham (Non OTM) group were found between the 2MOPs and 4MOPs groups, respectively (p <0.05).

Table 1. Comparison of the tooth movement distance and inclination among groups

	Group																				
	Sham (Non OTM) (n=12)					**Control (OTM) (n=12)**					**2MOPs (n=12)**					**4MOPs (n=12)**					
	Mean	SD	Med	Max	Min	**Mean**	SD	Med	Max	Min	**Mean**	SD	Med	Max	Min	**Mean**	SD	Med	Max	Min	***p* value**
Tooth movement distance *(mm)*	0.08	0.02	0.07	0.13	0.04	0.29	0.06	0.29	0.40	0.18	0.31	0.07	0.30	0.41	0.19	0.38	0.08	0.35	0.55	0.28	**0.043***
Tooth movement inclination *(°)*	98.88	4.83	99.60	107.53	90.21	92.67	4.57	93.19	98.80	80.75	88.74	5.73	88.45	96.56	77.34	86.02	7.22	87.76	95.23	69.86	**0.027***

Data are presented a Non-parametric test using Mann-Whitney U test. Group comparisons were tested with an independent *t*-test; Sham group (Non - orthodontic tooth movement (OTM) left side) group; Control (OTM) only group; 2Micro-osteoperforations (2MOPs) and OTM group; 4Micro-osteoperforations (4MOPs) and OTM group of the palatal cortical plate group; SD, standard deviation; Med, median; Max, maximum; Min, minimum. *Significant difference between the Control (OTM) and 4MOPs groups.

2. Comparison of the micro-computed tomography (micro-CT)

Micro-CT analyses were performed on hemi-maxillae of all animals for 14 days. The serial images were used for the quantitative analysis of alveolar bone changes occurring in the region ROI on the maxillary first molar (Figure 2). Parameters studied included changes in the bone volume and trabecular region (Table 2). According to the micro-CT volumetric analyses, the BMD, BV, BVF significantly decreased in the 4MOPs group 0.55 mg/cm^3 (SD 0.05), 0.03 mm^3 (SD 0.03), 35.27 % (SD 4.31) compared to the Control (OTM) group 0.69 mg/cm^3 (SD 0.02), 0.04 mm^3 (SD 0.002), 49.59 % (SD 2.77) and Sham (Non OTM) group 0.71 mg/cm^3 (SD 0.03), 0.04 mm^3 (SD 0.03), 52.39% (SD 3.33) (p <0.05). Regarding the changes in Tb. Th, Tb. Sp were found significant differences between the 4MOPs group and Sham (Non OTM) group, which were 0.09 mm (SD 0.005), 0.20 mm (SD 0.01) and 0.11 mm (SD 0.006), 0.15mm (SD 0.01). The 4MOPs group 3.78 1/mm (SD 0.25) also revealed significant differences in Tb. N, compared with Control (OTM) 4.71 1/mm (SD 0.20) group (p <0.05). Further, the 2MOPs group showed no significant differences between groups (p >0.05).

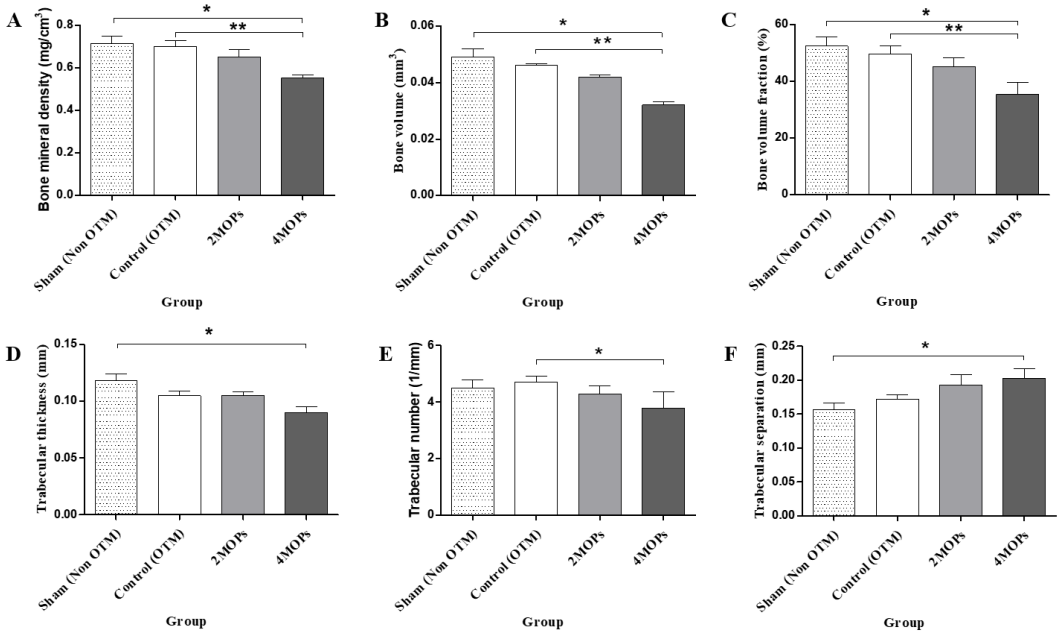

Figure 5. Comparison of the micro-CT analysis of bone quality. The asterisk indicates differences in in bone parametric, according to Mann-Whitney U test, at p <0.05. (A), (B), (C) and (E) Regarding the changes in BMD, BV, BVF and Tb. N, respectively p <0.05, showing significant decrease in the 4MOPs group 20.2 %, 25 %, 28.6 % and 19.74 % compared to the Control (OTM) group.

Table 2. Comparison of volumetric analysis in micro-CT among groups

	Group																				
	Sham (Non OTM) (n=12)					**Control (OTM) (n=12)**					**2MOP (n=12)**					**4MOP (n=12)**					
	Mean	SD	Med	Max	Min	**Mean**	SD	Med	Max	Min	**Mean**	SD	Med	Max	Min	**Mean**	SD	Med	Max	Min	***p value***
BMD *(mg/cm3)*	0.71	0.03	0.74	0.89	0.48	0.69	0.02	0.69	0.87	0.56	0.65	0.03	0.61	0.87	0.51	0.55	0.05	0.50	0.94	0.29	**0.035***
BV *(mm3)*	0.04	0.003	0.05	0.06	0.03	0.04	0.002	0.04	0.06	0.03	0.04	0.002	0.04	0.05	0.03	0.03	0.003	0.02	0.06	0.01	**0.037***
BVF *(%)*	52.39	3.33	53.60	75.15	38.19	49.59	2.77	49.55	67.05	34.70	45.11	3.03	43.75	62.29	32.51	35.27	4.31	30.72	65.85	16.34	**0.027***
Tb. Th *(mm)*	0.11	0.006	0.12	0.16	0.07	0.10	0.003	0.09	0.13	0.08	0.10	0.003	0.10	0.12	0.08	0.09	0.005	0.08	0.11	0.06	**0.081**
Tb. N *(1/mm)*	4.50	0.29	4.55	6.28	3.11	4.71	0.20	4.82	6.22	3.58	4.29	0.27	4.03	6.35	3.06	3.78	0.25	3.67	5.49	2.63	**0.037***
Tb. Sp *(mm)*	0.15	0.01	0.15	0.25	0.10	0.17	0.007	0.16	0.21	0.14	0.19	0.01	0.19	0.28	0.10	0.20	0.01	0.19	0.28	0.11	**0.171**

Data are presented a Non-parametric test using Mann-Whitney U test. Group comparisons were tested with an independent *t*-test; Sham group (Non - orthodontic tooth movement (OTM) left side) group; Control (OTM) only group; 2Micro-osteoperforations (2MOPs) and OTM group; 4Micro-osteoperforations (4MOPs) and OTM group of the palatal cortical plate group; SD, standard deviation; Med, median; Max, maximum; Min, minimum; BMD, bone mineral density; BV, bone volume; BVF, bone volume fraction; Tb. Th, trabecular thickness; Tb. N, trabecular number; Tb. Sp, trabecular separation.

3. Comparison of the root resorption

The effect of the number of MOPs on the rate of tooth movement on the root surface was studied by a micro-CT volumetric analysis. The distal roots were covered with a thick cementum with a rough and irregular surface that occasionally contained resorption craters. These wide, shallow, and deep resorption craters were scattered mainly on the cervical middle and mesial portions of the distal roots. Descriptive statistics regarding the VRR and ARR for all groups shown in Table 3. Considering the total VRR, perforations in the 4MOPs group averaged 0.00061 mm^3 (SD 0.00021), while those in the 2MOP group were 0.00058 mm^3 (SD 0.00035), and those in the Control (OTM) group averaged 0.00049 mm^3 (SD 0.00027). In terms of measured ARR, that of the 4MOPs group averaged 0.14 mm^2 (SD 0.05), the 2MOPs group averaged 0.13 mm^2 (SD 0.07), and the Control (OTM) group averaged 0.12 mm^2 (SD 0.06). These results were not statistically significant (p >0.05). The Sham (Non OTM) group's values of 0.00003 mm^3 (SD 0.00003) and 0.009 mm^2 (SD 0.007) were significantly lower than those of other groups (p <0.05) in the VRR and ARR.

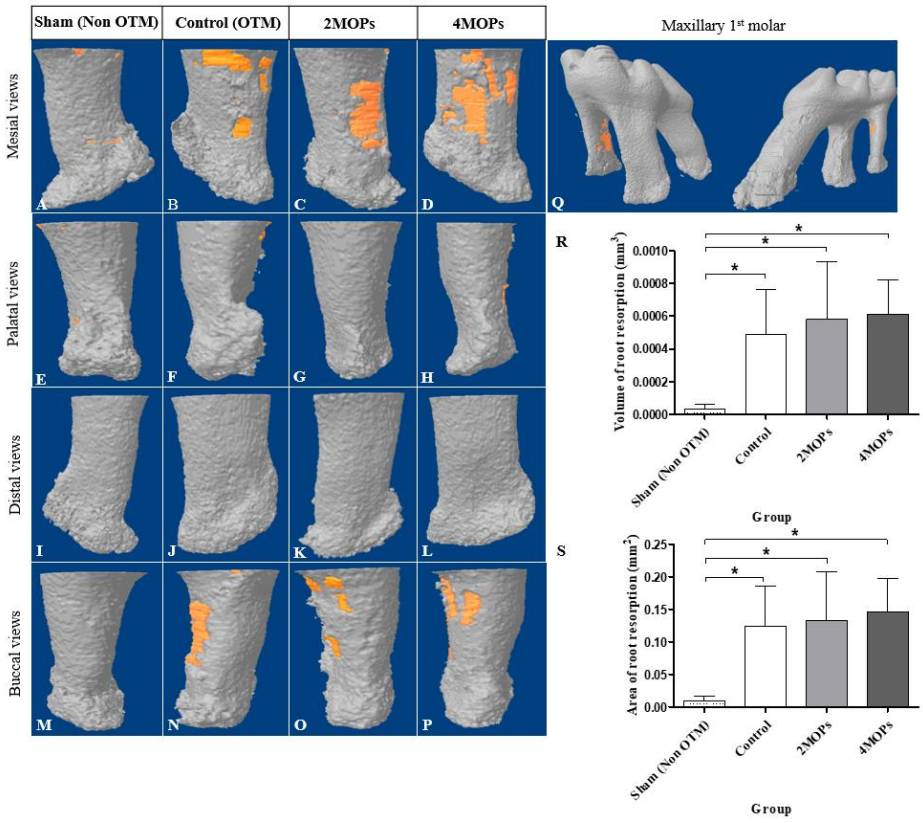

Figure 6. Representative micro-CT images of first molar and distal root surfaces. Evaluation of root resorption area by micro-CT volumetric analysis on the pressure side of the mesial side of the disto-buccal root of the first molar during orthodontic tooth movement. Grey area, non-resorption; orange area, resorption (crater). (A) (E) (I) (M) Sham (Non OTM) group; (B) (F) (J) (N) Control (OTM) group; (C) (G) (K) (O) 2MOPs group; (D) (H) (L) (P) 4MOPs; mesial, palatal, distal, buccal surface. (Q) Maxillary first molar of the mouse. (R) and (S) Box graph show the volumetric analysis of the distal root surface of VRR; mm^3 and ARR; mm^2. In general, 4MOPs group were resulted in greater VRR and ARR compared with the 2MOPs and Control (OTM) groups, which were 5.17 %, 24.5 % and 9.02 %, 17 %, respectively ($p > 0.05$). However, Sham (Non-OTM) group was significantly than those of other groups ($p < 0.05$).

Table 3. Comparison of root resorption among groups

	Group																				
	Sham (Non OTM) (n=12)					**Control (OTM) (n=12)**					**2MOP (n=12)**					**4MOP (n=12)**					
	Mean	SD	Med	Max	Min	**Mean**	SD	Med	Max	Min	**Mean**	SD	Med	Max	Min	**Mean**	SD	Med	Max	Min	***p value***
VRR ***(mm³)***	0.00003	0.00003	0.00003	0.00010	0.00000	0.00049	0.00027	0.00042	0.00104	0.00012	0.00058	0.00035	0.00048	0.00128	0.00020	0.00061	0.00021	0.00061	0.00101	0.00031	**0.393**
ARR ***(mm²)***	0.009	0.007	0.005	0.190	0.001	0.124	0.061	0.108	0.256	0.038	0.133	0.074	0.117	0.294	0.004	0.145	0.050	0.152	0.211	0.049	**0.476**

Data are presented a Non-parametric test using Mann-Whitney U test. Group comparisons were tested with an independent *t*-test; Sham group (Non - orthodontic tooth movement (OTM) left side) group; Control (OTM) only group; 2Micro-osteoperforations (2MOPs) and OTM group; 4Micro-osteoperforations (4MOPs) and OTM group of the palatal cortical plate group; SD, standard deviation; Med, median; Max, maximum; Min, minimum; VRR, volume of root resorption; ARR, area of root resorption.

4. Comparison of the histomorphometry

To identify osteoblasts and osteoclasts histologically, HE and TRAP staining was performed on sagittal histological sections of the disto-buccal root. Figure 7 summarizes the histology of the TRAP staining Sham (Non OTM); Control (OTM); 2MOPs and 4MOPs groups. TRAP activity was observed on the alveolar bone, periodontal ligament, and root surface at the mesial region of the periodontium, while no activity was noted in the distal region. The number and area of TRAP-positive osteoclasts were statistically significant increased (p <0.05) in the 4MOPs group 74 (SD 13); 3089.36 μm (SD 270.01) compared with the Control (OTM) group 59 (SD 8); 2540.02 μm (SD 299.07). The 2MOPs group 67 (SD 8) and 2632.37 μm (SD 456.95) showed no difference between groups in terms of the number and area of TRAP-positive osteoclasts (p >0.05). Further, the Sham (Non OTM) group 4 (SD 5) and 107.73 μm (SD 114.57) was significantly lower than those of other groups (p <0.05).

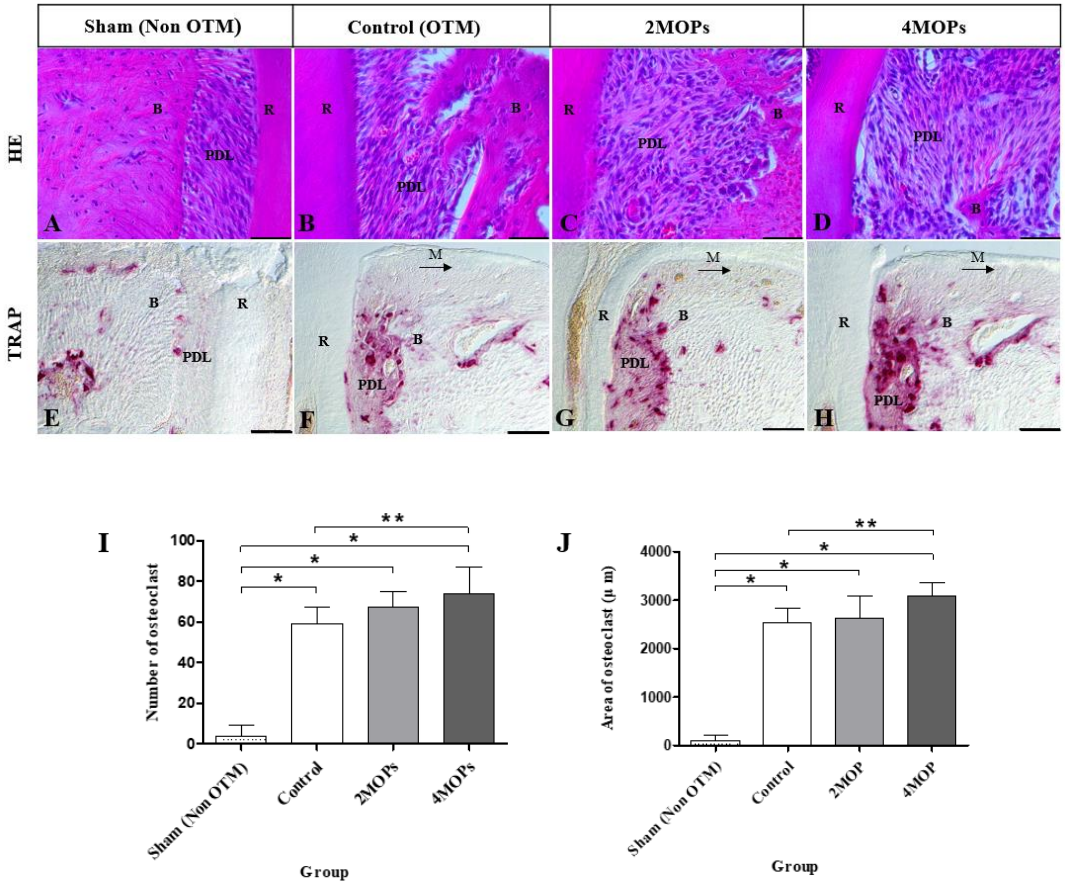

Figure 7. Evaluation of HE and TRAP staining. Histologic images used for histomorphometric analysis. 8 mice used in each group. (A) and (E) Sham (Non OTM) group; (B) and (F) Control (OTM) group; (C) and (G) 2MOPs group; (D) and (H) 4MOPs group. The black arrows indicate direction of orthodontic force; (M, mesial side, B, alveolar bone, PDL, periodontal ligament, R, root) (Magnification: X40; Scale bar: 50 μm). (E) to (H) Area of TRAP positive cells on the pressure side of the distobuccal root of the first molar during orthodontic tooth movement. (I) and (J) 4MOPs group 25.4 % and 21.6 % greater number and area of osteoclasts compared with Control (OTM) group (p <0.05). Further, statistically significant differences in Sham (Non OTM) group were compared those of other groups (p <0.05).

Table 4. Comparison of TRAP-positive cells among groups

	Group																				
	Sham (Non OTM) (n=8)					**Control (OTM) (n=8)**					**2MOP (n=8)**					**4MOP (n=8)**					
	Mean	SD	Med	Max	Min	**Mean**	SD	Med	Max	Min	**Mean**	SD	Med	Max	Min	**Mean**	SD	Med	Max	Min	***p value***
Osteoclast number	4	5	3	14	1	59	8	56	71	47	67	8	69	78	56	74	13	75	95	53	**0.036***
Osteoclast surface *(μm)*	107.7	114.5	89.7	338.1	2.08	2540.0	299.0	2561.0	2997.0	2071.1	2632.3	456.9	2541.9	3553.6	2125.6	3089.3	270.0	3121.0	3544.1	2750.5	**0.012***

Data are presented a Non-parametric test using Mann-Whitney U test. Group comparisons were tested with an independent *t*-test; Sham group (Non - orthodontic tooth movement (OTM) left side) group; Control (OTM) only group; 2Micro-osteoperforations (2MOPs) and OTM group; 4Micro-osteoperforations (4MOPs) and OTM group of the palatal cortical plate group; SD, standard deviation; Med, median; Max, maximum; Min, minimum. *Significant difference between the Control (OTM) and 4MOPs groups.

IV. DISCUSSION

In this study, the rate of tooth movement differed depending on the number of MOPs (Sham (Non OTM); Control (OTM); 2MOPs; 4MOPs) of the alveolar bone. The 4MOPs group had a 1.31 times faster rate of tooth movement compared with the Control (OTM) group after 14 days of consolidation (Table 1). This characteristic of the 4MOPs group resulted in significantly faster tooth movement, which increased the number of MOPs required to induce a RAP. These findings parallel those of other authors (Teixeira et al. 2010; Cheung et al. 2016; Chang et al. 2019) who found 1.35 – 2.13 times faster rates of tooth movement in a 3 - 5 MOP group than a control group. From previous studies, it was concluded that the small number of (<2) MOPs of a force-level magnitude, presence of a flap, and age of the subjects had no impact on the rate of orthodontic tooth movement (Murphy et al. 2014; Teixeira et al. 2010; Ren et al. 2003; Alikhani et al. 2015). In this study, proved that the 2MOPs group had no significant difference compared to the Control (OTM) and 4MOP groups in terms of the rate of OTM, in with decreasing bone parameters and osteoclast activity. However, another study reported that a 10 MOPs group experienced significantly increased tooth movement during the initial phase, and continuously accelerated tooth movement until 42 days (Baloul et al. 2011). Taking all of these findings into consideration, we speculated that the number of MOPs had a major effect on the rate of tooth movement, and could sharply induce RAP in the initial phase of orthodontic tooth movement.

OTM primarily depends on the quantity and quality of bone. Although a few studies have examined bone parameters after MOP, none has evaluated the between bone and trabecular changes (Tb. N) of alveolar bone. In the present study, the BMD, BV, BVF and Tb. N were significantly decreased in the 4MOPs group compared with the Control (OTM) group (Table 2). Bone density can be reduced solely by induction of tooth movement (Chang et al. 2012), but MOP procedures can also increase bone turnover on the MOP side, underlying the regulatory processes that initiate accelerated tooth movement. Our results confirmed those of previous studies (Baloul et al. 2011; Chang et al. 2019) that showed that alveolar bone density was decreased significantly with alveolar decortication. Thus, the fact that the

number of MOPs can reduce bone mineralization may indicate a highly active bone catabolism of tooth movement.

Although some researchers have previously proven which MOP method is the most effective in accelerating tooth movement with increased bone turnover, these techniques were challenged for their invasiveness (Dutra et al. 2018). However, one of the adverse effects of tooth movement is root resorption (Shahabee et al. 2020). Based on this evidence, therefore, one of our null hypotheses was that the number of MOP did not induce root resorption in orthodontic tooth movement compared with the Control (OTM) group. In order to test this hypothesis, we used quantitative micro-CT for the volumetric analysis, associated with volume and area of the distal root. Four groups were used to investigate the response of VRR and ARR in an experimental animal model. The results suggested that different groups with different numbers of MOPs presented more volume and area of root resorption than the Control (OTM) group, but this difference was not statistically different (Table 3). Similarly, some studies have suggested that histological and micro-CT volumetric analysis of the root resorption of the distal root was severe, but with no statistically differences with the MOP side. On the other hand, enhanced alveolar bone turnover associated with an increase in osteoclast activity has been found to induce the root resorption process (Cheung et al. 2016; Kurohama et al. 2017). This result suggested that 4MOP might induce a considerable amount of inflammatory mediators around the periodontal tissue and increase alveolar bone turnover; however, this inflammatory reaction on the surface of the cortical bone might not cause an obvious increase in root resorption.

The osteoclast activity and alveolar bone resorption were evaluated with the examination of TRAP staining. After 14 days of consolidation, there were active osteoclast cells and alveolar bone resorption within the compression (mesial) side of distal root of the maxillary first molar in the 4MOPs group. TRAP activity was significantly higher in the 4MOPs (25.4%) group compared with the Control (OTM) group. Meanwhile, the 2MOPs group did not show a significant increase of TRAP activity compared with the Control (OTM) and 4MOPs (Figure 7. F, H) groups. This finding reflected vigorous osteoclast

activity and bone resorption in alveolar bone with 4MOPs. The results of the TRAP analysis after MOP coincide with the results of previous animal studies (Teixeira et al. 2010; Cheung et al. 2016; Dutra et al. 2018). They found that TRAP activity was significantly increased along with osteoclast number with MOP (12.9 % - 55 %). It was reported that 3 - 5 MOPs induced more osteoclast activity with a continuous stimulation of osteoclast proliferation and maturation until 28 days of orthodontic tooth movement.

This study had a few limitations. First, we only measured tooth movement for a short time. And did not evaluate the long term effects of MOP. Additionally, a limitation in our study design was the fact that used a mouse (CD1) model, which is genetically similar to the rat. Our model could not allow wide space during tooth movement due to the failure of appliances. We need a different species of animal. In addition, there are morphologic and physiologic differences between rat and human alveolar bone. The alveolar bone more in rats is denser and exhibits no osteon remodeling (secondary remodeling). Its bone plates are devoid of marrow spaces. Humans have more osteoid tissue along the alveolar bone surface (Reitan and Kvam 1971). Despite this limitation, our study shows genetically associated outcomes related to MOP and root resorption.

Nevertheless, this is in-vivo study helped us to understand the effect of number of MOP on the surrounding alveolar bone. As already mentioned, MOP is one of the surgical interventions to accelerate tooth movement. However, one may argue that the number of MOPs and side effects of root resorption on tooth movement were not clearly understood. This study was designed to monitor and evaluate this possibility. We tried to define the maximum and minimum number of perforations (2 and 4) for the acceleration of tooth movement.

Surgical interventions that were performed in animal studies differed in terms of the damage they caused to surrounding periodontal tissue; invasive procedures are difficult to be accepted in daily practice. In the present study, demonstrated that 4MOPs successfully accelerated tooth movement without any significant root resorption compared with other groups. Therefore, our animal study found

the optimal amount of damage incurred in the alveolar bone to ensure efficient tooth movement without causing significant damage to the root surface. However, there is still concern about the clinical application of the present results because humans and mice show different potential with regard to bone remodeling and tissue regeneration.

Our future studies will focus on understanding the signaling pathways associated with the 4MOP group and in-vitro gene expression of the micro-osteoperforated osteoclasts and cementoblasts, while considering the longer term of effects of MOPs. Further studies are required to assess the frequency of MOPs with optimally effective side effects.

V. CONCLUSION

This study sought to evaluate the biological effects of the number of MOPs on the rate of tooth movement and the potential risk for root resorption in a mouse model. Thirty-six male CD1 mice were used in this study for 14 days. The mice were divided into 4 groups (12 mice *per* group): Sham group (Non OTM); Control (OTM) only group; 2MOPs and OTM group; and 4MOPs and OTM of the palatal cortical plate group. In each group, tooth movement distance was measured, and bone parameters, amount of root resorption, and osteoclast activity were analyzed using micro-CT and histological slides. The results were as follows:

1. The 4MOPs group had a significantly accelerated the rate of tooth movement.
2. The 4MOPs groups had a significantly reduced bone density and trabecular number.
3. The 4MOPs group had significantly increased number of TRAP-positive cells.
4. Both the 2MOPs and 4MOPs groups had no differences in terms of the volume and area of root resorption compared to the Control (OTM) group.

In this mouse model, 4 MOPs could effectively accelerate the rate of tooth movement by an increased bone turnover, as evidenced by an increase in osteoclast quantity and a decrease in bone density compared with the Control (OTM) and 2MOPs groups for 14 days. However, it did not affect the volume and area of root resorption compared to the 2MOPs and 4MOPs groups.

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국문요약

마우스에서 미세골천공시술에 대한 치아 이동속도와 치주적인 반응

지도교수 차정열

연세대학교 대학원 치의학과

Tselmuun Erdenebat

본 연구의 목적은 마우스 모델에서 미세골천공시술(Micro-osteoperforation, MOP)이 치근흡수 및 교정적인 치아이동에 미치는 영향을 분석하는 것이다.

36 마리의 수컷 CD1 마우스를 MOP 수에 따라 다음과 4 개의 군으로 분류하였다: 가짜군; 대조군(교정적인 치아이동, OTM) 군; 2MOPs (2 홀) 군; 4MOPs (4 홀) 군. 니켈-티타늄 스프링을 사용하여 상악 우측 제 1 대구치에서 상악 절치에 25 g 의 교정력을 가하였다. 14 일 간 교정력을 부여한 후 치아 이동 거리를 측정하였고 주변치조골을 미세 컴퓨터 단층 촬영(micro-computed tomography, Micro-CT)하여 골밀도(bone mineral density, BMD), 골부피(bone volume, BV), 골부분율 (bone volume fraction, BVF), 골소주수(trabecular number, Tb.N) 및 치근흡수량 (volume of root resorption, VRR)과 치근흡수면적 (area of root resorption,

ARR)을 측정하였다. 또한 파골 세포의 수도 tartrate-resistant acid phosphatase (TRAP) activity assay 를 사용하여 평가하였다.

4MOPs 군이 대조군 (OTM) 군과 보다 치아이동 속도가 유의미하게 높았으며 BMD, BV, BVF 및 Tb.N 값이 유의하게 낮았다 (p <0.05). 4MOPs 군 (74 ± 13) 의 파골 세포 수는 대조군 (OTM) 군이 (59 ± 8)보다 유의하게 더 높게 나타났다. 4MOPs 군이 VRR 값은 (0.00061 ± 0.00021 mm^3) 대조군 (OTM) (0.00049 ± 0.00027 mm^3) 군과 높았으나 유의한 차이는 없었다 (p >0.05).

본 연구의 마우스 모델에서는 4MOPs 군에서 대조군과 2MOPs 군에 비해 골교체율이 증가함으로서 효과적으로 치아 이동 속도가 증가하였다. 이는 실험 14 일 뒤에 4MOP 군에서 파골세포 수가 증가하고 골밀도가 감소한 결과가 뒷받침해준다. 하지만 대조군과 실험군을 비교하였을 때 MOP 는 치근 흡수의 부피나 면적에는 영향을 미치지 않았다.

주요어: 미세골천공, 교정적인 치아이동, 마이크로 씨티, 치근흡수