*Title Page

Low-intensity pulsed ultrasound enhances the rate of lateral tooth movement and compensatory bone formation in rats.

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Author Contributions

Conceived and designed the experiments: CA, YNa. Performed the experiments: CA, AT. Analyzed the data: CA, NK, AT, YNo. Contributed reagent/materials/analysis tools: CA, NK. Performed the statistical analysis:YNo, Senior advisor: YNa, ET. Wrote the paper: CA, YNo, YNa, ET.

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Highlights

- LIPUS enhances osteoclastogenesis in the pressure zone of the alveolar bone during lateral tooth movement.
- LIPUS increases compensatory bone formation on the buccal surface of the alveolar bone during lateral tooth movement.
- LIPUS improves the rate of lateral tooth movement.

Low-intensity pulsed ultrasound enhances the rate of lateral tooth movement and compensatory bone formation in rats.

Running title: LIPUS effect on lateral tooth movement

Keywords

LIPUS; Orthodontic tooth movement; Osteoclastgenesis; 3D micro CT;

Bone histomorhometry

Abstract

Introduction: Because mechanical stimulation of the periodontal ligament (PDL) by low-intensity pulsed ultrasound (LIPUS) has been shown to increase the speed of bone remodeling, the aim of this study was to examine the effects of LIPUS stimulation on the rate of tooth movement and bone remodeling during lateral tooth movement. Methods: Twelve-week-old Wistar rats were divided into two groups. The LIPUS group received experimental tooth movement with LIPUS stimulation, and the tooth movement (TM) group had experimental tooth movement without LIPUS. For the LIPUS and TM groups, the upper right first molars were moved labially with fixed appliances. LIPUS exposure was placed in the region corresponding to the right maxillary first molar. Three days after tooth movement, tartrate-resistant acid phosphatase (TRAP) was examined. Fourteen days after tooth movement, the intermolar width, bone mineral content (BMC),

and bone volume fraction (BV/TV) were examined by micro computed tomography (micro-CT), and newly formed bone was measured histomorphometrically. Results: The number of TRAP-positive cells at the compressed region was obviously greater in the LIPUS group. The intermolar width was significantly greater in the LIPUS group than in the TM group. The alveolar bone around the maxillary first molar showed no differences in BMC or BV/TV between the LIPUS and TM groups. The LIPUS group exhibited a significantly greater amount of newly formed alveolar bone than the TM group. Conclusions: The present study provides evidence of the beneficial effects of LIPUS on the lateral tooth movement.

INTRODUCTION

Tissue remodeling surrounding tooth roots is essential to the rate of orthodontic tooth movement. Therefore, it is important to control the molecular mechanisms by which the behaviors of cells in the alveolar bone and periodontal ligament (PDL) are regulated. [1-3] The duration of orthodontic treatment is the primary concern for most patients and orthodontists. Unfortunately, long-term orthodontic treatment induces several disadvantages, such as a higher predisposition to dental caries, gingival recession, and root resorption. Consequently, much attention has been paid to find the possible remedies that increase the rate of tooth movement with the fewest possible

disadvantages.

To date, several novel modalities have been reported to accelerate orthodontic tooth movement.[4] Surgical modalities including corticotomy, dentoalveolar distraction and periodontal distraction is based on the principle that when the bone is irritated which causes increased osteoclastogenesis, the tooth moves faster.[5-7] Meanwhile, several non-surgical modalities have also been reported, such as low-level laser therapy, electromagnetic fields, and mechanical vibration.[4] In addition, mechanical stimulation of the PDL by low-intensity pulsed ultrasound (LIPUS) has been shown to increase the speed of bone remodeling; therefore, LIPUS is also considered as a non-surgical modality for accelerating tooth movement.[8] LIPUS has been proven to act by inducing osteoclastogenesis by stimulating the receptor activator of nuclear factor kappa-B(RANK)/RANK ligand (RANKL) pathway and activating signaling molecules such as MAPK.[9] Furthermore, the use of LIPUS is safe, but the very limited research-based evidence cannot support a solid conclusion that it accelerates orthodontic tooth movement.

Xue et al. revealed that LIPUS might promote alveolar bone remodeling via increasing the gene expression of the human growth factor/Runx2/bone morphogenetic protein 2 signaling pathway molecules, resulting in the rapid movement of teeth during

orthodontic treatment.[10] Recent study also showed that LIPUS enhanced the amount of tooth movement and the bone remodeling during orthodontic tooth movement in rat.[11] However, these results were only found in a mesial orthodontic tooth movement model. Although lateral tooth movement is frequently conducted in the clinical situation, the effect of LIPUS on lateral orthodontic tooth movement has not been fully examined. Thus, the aim of this study was to examine the effects of LIPUS stimulation on the rate of tooth movement and bone remodeling during lateral tooth movement.

MATERIAL and METHODS

All of the procedures described in this study were performed in accordance with the guidelines and regulations of the ///// University for Animal Research (29A038). A total of twenty-six 12-week-old male Wistar rats, weighing 320-350 g, were randomly divided into two groups. The LIPUS group received experimental tooth movement with LIPUS stimulation, and the tooth movement (TM) group had experimental tooth movement without LIPUS. Each animal was anesthetized with a mixture of three types of anesthetic agents at a dose of 2.5 ml/kg body weight.[12] The combination anesthetic was prepared with 0.15 mg/kg medetomidine (Domitol®; Nippon Zenyaku Kogyo Co., Ltd., Tokyo, Japan), 2 mg/kg midazolam (Dormicum®; Astellas Pharma Inc., Tokyo, Japan), and 2.5 mg/kg butorphanol (Vetorphale®; Meiji Seika Pharma Co., Ltd.). For the LIPUS

and TM groups, the upper right first molars were moved bucally with fixed appliances (Fig 1, a-b). The initial force magnitude was approximately 10 g.[13]

An ultrasound exposure machine (Osteotron D2, ITO Co., Tokyo, Japan) was employed in this study. This system was equipped with transducers with a circular surface area of 9.6 cm2. The sound head of this device had an average beam non-uniformity ratio (BNR) of 3.2-3.6:1 and an effective radiating area (ERA) of 90%. A pulsed ultrasound signal was transmitted at a frequency of 1 MHz (the pulse repetition frequency = 100 Hz), with an average spatial intensity of 30 mW/cm^2 and a pulse of 1:4 (2 ms on and 8 ms off). The stimulation protocol, used in this study consisted of a 20-min LIPUS stimulation repeated every day. The rats were kept in an immovable position under anesthesia, and the ultrasound transducer was placed in contact with one side of the face, in the region corresponding to the right maxillary first molar. The fur was shaved in the exposure region, and coupling gel was constantly in place in order to optimize penetration of the ultrasound waves into the tissues.

Three days after tooth movement, 4% paraformaldehyde in 0.1M phosphate-buffered saline (pH 7.4) was perfused for 15 min through the ascending aortae of ten rats from the experimental and control groups, respectively. After fixation, the maxillae were dissected and trimmed into small blocks containing the first molar, decalcified with

EDTA-Na (5.0%, pH 7.2, 4oC) solution containing 7.0% sucrose for 4 weeks, dehydrated with a graded ethanol series, and embedded in paraffin. The serial sections (7 μm) were cut perpendicular to the root axis. TRAP activity was examined in the sections, using a TRAP staining kit (Wako, Tokyo, Japan). An area measuring 700 × 2400 μm^2 was selected from the section for light microscopic examination (BZ-9000; Keyence, Osaka, Japan) according to previous study (Fig 4, a and b).[14] TRAP-positive multinucleated osteoclasts on the pressure zone of the upper first molar was counted on three sections for each specimen. The average of the three values was used in this study.

Sixteen rats served to measure tooth movement and to analyze the bone properties in micro-CT analysis. After 14 days of tooth movement, the micro-CT (The inspeXio SMX-225CT, SHIMADZU Co., Kyoto, Japan) images were taken. The tube voltage was set at 160kV and the current was constant at 70 μA. The resolution was set at 20 μm per voxel and 1024 × 1024 pixels. On the three-dimensional (3D) models, the distance between the distolingual cusps of the maxillary first molars was measured as the intermolar width (Fig 2, a). The Region of interest (ROI) was alveolar bone proper of the maxillary first molar (Fig 2, b-d).

Each ROI was measured with respect to bone mineral content (BMC; mg) and bone

volume fraction (BV/TV; %). Tissue volume (TV) was defined as the volume of tissue in the enlarged ROI. Bone volume (BV) was excluded of the teeth (Fig 2, d). The inter-molar width and bone parameters were measured by three-dimensional image-analysis software (TRI/3D-BON; Ratoc System Engineering, Tokyo, Japan).

Sixteen rats were intraperitoneally injected with 0.1 mL of calcein (1.6 mg/kg) solution one day before tooth movement and with xylenol orange (50 mg/kg) one day before the end of tooth movement as fluorochrome labels. At day 15, the animals were sacrificed under anesthesia with pentobarbital sodium at a fatal overdose of 50 mg/kg. After the micro-CT images were taken, the maxillae were dissected, cut in half along the sagittal plane, and immersed rapidly in liquid nitrogen. The frozen tissues were embedded with optical cutting temperature compound (Miles Inc., Torrance, CA, USA). Frozen blocks from the rats were frontally sectioned and 7-μm-thick serial sections were used for histomorphometric analysis. The sections were prepared according to Kawamoto's film method.[14] Newly formed bone was measured as the distance between the calcein and xylenol orange lines under a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan), which were visible as green and red marks, respectively, at 50 μm, 150 μm and 300 μm from the alveolar crest, the average of these three morphometric values was used for evaluation.

Statistical analysis

To determine the sample size of the experiments, power analysis was performed to detect statistically significant differences in each experiment between lateral tooth movement with or without LIPUS exposure during the experimental period was determined (α = 0.05 and β = 0.80). The power analysis was performed S Plus ver. 6.0 (NTT Data Tokyo, Japan).

Normality of variables were assessed by the Kolmogorov - Smirnov test separately by the control and LIPUS groups. According to the normality, t tests or Mann-Whitney U tests were used to detect the statistical significance between two groups.

For the assessment of reliability intermolar width measurements, samples were measured by two examiners under masking experimental conditions. Measurements were repeated after 2 weeks by the same examiner. A paired t test showed no significant differences between the 2 repeated measurements (p=0.689). And intraclass correlation coefficients evaluated by measured value (mean) was 0.967(95% CI: 0.912-0.989). P-value less than 0.05 was considered to be statistically significant. Analysis except for the power analysis were carried out by SPSS Statistics ver. 25.0 (IBM, Tokyo, Japan).

RESULTS

During the tooth movement, no significant differences in body weight were found between the two groups (Fig 3, a). Inter-molar width was significantly ($p<0.05$) greater in the LIPUS group than in the TM group (Fig 3, b and Table).

The number of TRAP-positive cells at the compressed region was clearly greater in the LIPUS group than in the control group (Fig 4, a and b). Furthermore, the number of osteoclasts was significantly increased by the LIPUS exposure (Fig 4, c and Table).

Fluorescence microscopy revealed bone labelling in the section. Sharp, bright calcein and xylenol orange labeling lines were observed in the alveolar bone on the periosteal sides in each group. The width between these two lines represented the formation of new bone during the experimental period. In the untreated control group without tooth movement, two lines were observed in the alveolar crest area and lower part of the alveolar bone (Fig 5, a). On the other hand, the TM group showed two parallel lines located along the periosteal bone surface in the alveolar bone, representing compensatory bone formation in response to the lateral tooth movement (Fig 5, b). The LIPUS group also showed the same observations, but the width between the lines was much greater than that in the TM group (Fig 5, c). The distance between the lines in the LIPUS group was significantly wider than that in the TM group (Fig 5, d and Table).

The alveolar bone labeling on the periodontal side showed quite different

characteristics. Double calcein and xylenol orange labelling was scattered in the alveolar bone on the periodontal side in the control group, but only a calcein labelling was recognized in the TM and LIPUS groups. The lack of green labelling suggests that the alveolar bone on the periodontal side was resorbed during the lateral tooth movement.

The micro-CT images showed no differences in the vertical height of the alveolar crest on the buccal side in the TM and LIPUS groups compared with the untreated control group without tooth movement (Fig 6, a-c). In addition, the alveolar bone surrounding the maxillary first molar was not significantly different in terms of the BMC or BV/TV values between the LIPUS and TM groups (Fig 6, d-e).

DISCUSSION

To the best of knowledge, this is the first attempt to investigate the effects of LIPUS on acceleration of tooth movement and compensatory bone formation during lateral tooth movement. Our results clearly showed that the oseteoclastgenesis in the pressure zone of the PDL was enhanced by LIPUS exposure (Fig 4, a-c). It is well known that RANKL is an important factor in osteoclastgenesis[16,17] and that it is expressed in the pressure side of the PDL during orthodontic tooth movement.[18] LIPUS induces osteoclastogenesis by upregulation of the RANK/RANKL pathway and signaling

molecules such as MAPK.[9] In addition, it has been shown that RANKL expression and the number of osteoclasts were increased by LIPUS stimulation in the pressure side of the PDL during orthodontic tooth movement in rats.[10] Thus, LIPUS might enhance induction of RANKL mediated osteoclastgenesis in the pressure side of the PDL during lateral tooth movement, consequentrly accelerating tooth movement.

Shimpo et al. have reported that alveolar bone is reactive to orthodontic stimuli, which induce periosteal bone formation in the palatal surface of the alveolar bone during lateral tooth movement.[19] The present results also demonstrated that compensatory bone formation was specifically observed in the buccal surface of alveolar bone during lateral tooth movement (Fig 5, b). However, the mechanism of this bone formation is unclear. Nonetheless, it is known that the orthodontic force on a tooth is considered to be a type of pathological stress loaded onto the PDL[20-26], and that it induces further bone remodeling.[26] In this context, this compensatory bone formation on the buccal surface may result from signals originating in the adjacent compressed periodontal tissues.

Another interesting finding in the present study was that the amount of compensatory bone formation was enhanced by LIPUS exposure (Fig 5, c). LIPUS has been reported to promote osteogenesis, protein synthesis, calcium uptake, and DNA synthesis in

various cells.[28] In addition, LIPUS stimulates ossification of the periosteal tissue.[29] Therefore, LIPUS stimulation might promote not only osteoclastgenesis but also ossification of the periosteum on the buccal side of alveolar bone, resulting in enhancement of compensatory bone formation during lateral tooth movement.

Xue et al. have shown that although there was no difference in the tissue reactions between with and without LIPUS stimulation in orthodontic tooth movement, the changes observed in tissue upon LIPUS stimulation were more extensive, resulting in the rapid movement of teeth during orthodontic treatment.[10] The present results revealed that although the micro-CT images of alveolar bone surrounding the molar root showed no differences in BMC and BV/TV values between the LIPUS and TM groups (Fig 6, d-e), the inter-molar width was significantly increased by LIPUS exposure (Fig 3, b), resulting

in accelerated lateral tooth movement. These results were similar to those reported previously.

Recent study showed that LIPUS not only accelerated orthodontic tooth movement but also reduced the orthodontic induced inflammatory root resorption in rat and [30] in dog. [31] In this context, LIPUS has beneficial effects for orthodontic tooth movement. In the future study, additional high-quality clinical research including tissue and cell biology is

required in order to estimate the efficacy of adjunctive interventions on orthodontic lateral tooth movement and their potential clinical use.

CONCLUSION

The present study provides evidence of the beneficial effects of LIPUS on lateral tooth movement. LIPUS accelerated not only tooth movement but also compensatory bone formation. However, additional high-quality clinical research is required in order to estimate the efficacy of adjunctive interventions on accelerating orthodontic tooth movement and their potential clinical use.

Acknowledgments

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Figure legends

Fig 1. (a) Illustration of the orthodontic appliance used in this study. The appliance consisted of a mesh band, 0.019 × 0.025-inch stainless steel wire, and 0.010-inch stainless steel wire. The parts were assembled with silver solder. (b) Image of the experimental tooth movement in a rat. The initial force magnitude was 10 g, moving the maxillary right first molar (M1) in the buccal direction (large arrow).

Fig 2. (a) Three-dimensional micro-CT image of the occlusal view of the maxilla. The intermolar width was measured between the distolingual cusp of the maxillary first molars (dashed line). R, right; B, buccal side; M, mesial side; L, lingual side; D, distal side; M1, first molar; M2, second molar; M3, third molar; bar = 3 mm. The white region shows the ROI of the maxillary first molar (a–c). There are five roots in the rat maxillary first molar. m, mesial root; mb, mesiobuccal root; ml, mesiolingual root; db, distobuccal root; dl, distolingual root; bar = 1 mm.

Fig 3. (a) Changes in body weight in the TM and LIPUS groups during the experiment. The body weights between the LIPUS group and the TM group

were not significantly different. (b) The intermolar width was significantly higher in the LIPUS group than in theTM group. Each column and vertical bar represent the mean ± standard deviation of eight preparations. *p < 0.05 by the *t* test.

Fig 4. TRAP staining results of the upper first molar in the TM group (a) and the LIPUS group (b). Representative photographs of both groups are shown. R, root; Bo, bone; B, buccal side; arrow, direction of tooth movement; black square, measurement area of 700 × 2400μm^2; bar = 300μm. (c) The number of osteoclasts on the alveolar bone surface adjacent to the root in the measurement area. The LIPUS group had significantly more osteoclasts than the TM group. Each column and vertical bar represent the mean ± standard deviation of five preparations. *p < 0.05 by the Mann-Whitney U-test.

Fig 5. Images of the buccal site of the alveolar crest in the control without tooth movement (a), TM (b), and LIPUS (c) groups. B, buccal; PDL, periodontal ligament; arrow, calcein line; arrow head, xylenol orange line; bar = 200 μm. (d) Comparisons of the amount of newly formed alveolar bone between the TM and LIPUS groups. The LIPUS group had a significantly greater amount of newly formed alveolar bone formation than the TM group. Each column

and vertical bar represent the mean ± standard deviation of eight preparations. *p < 0.05 by the *t* test.

Fig 6. Three-dimensional micro-CT frontal images cut at the mesiolingual and mesiobuccal root of the maxillary first molar of the control without tooth movement (a), TM (b), and LIPUS (c) groups. B, buccal side; mb, mesiobuccal root; ml, mesiolingual root; arrow, direction of orthodontic force; bar = 1 mm. BMC (d) and BV/TV (e) of the alveolar bone proper of the M1 in the TM and LIPUS groups. Each column and vertical bar represent the mean ± standard deviation of eight preparations.

Figure 1

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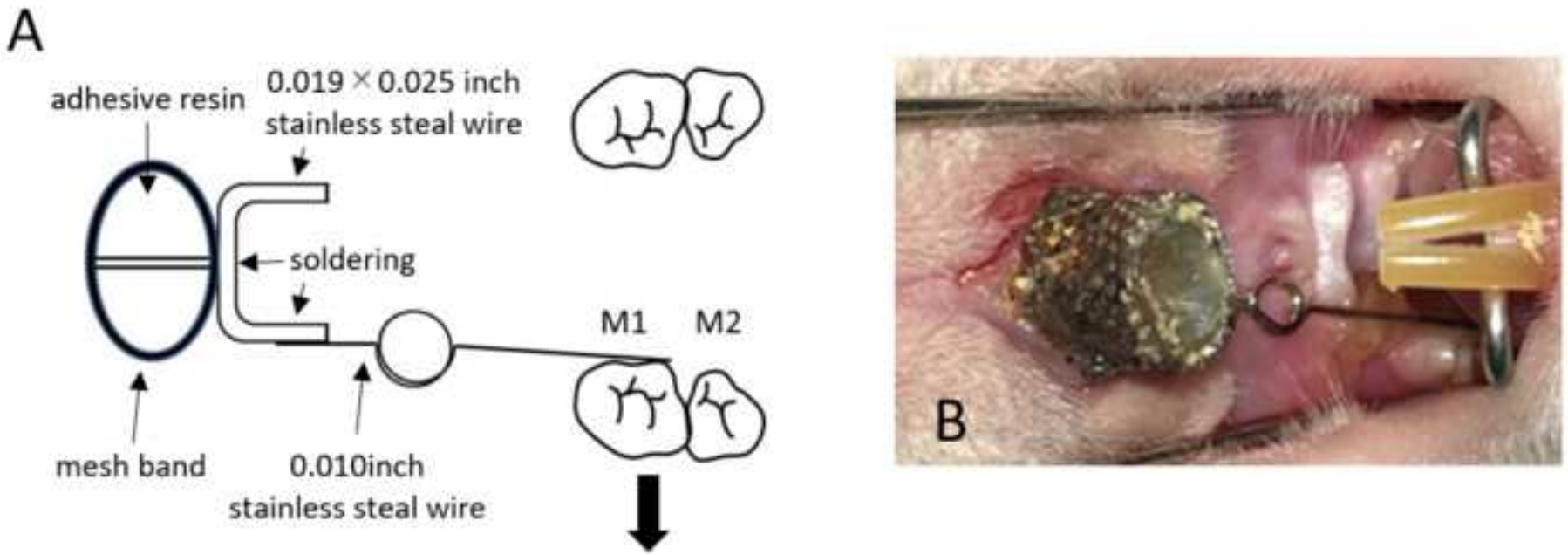

Figure 2

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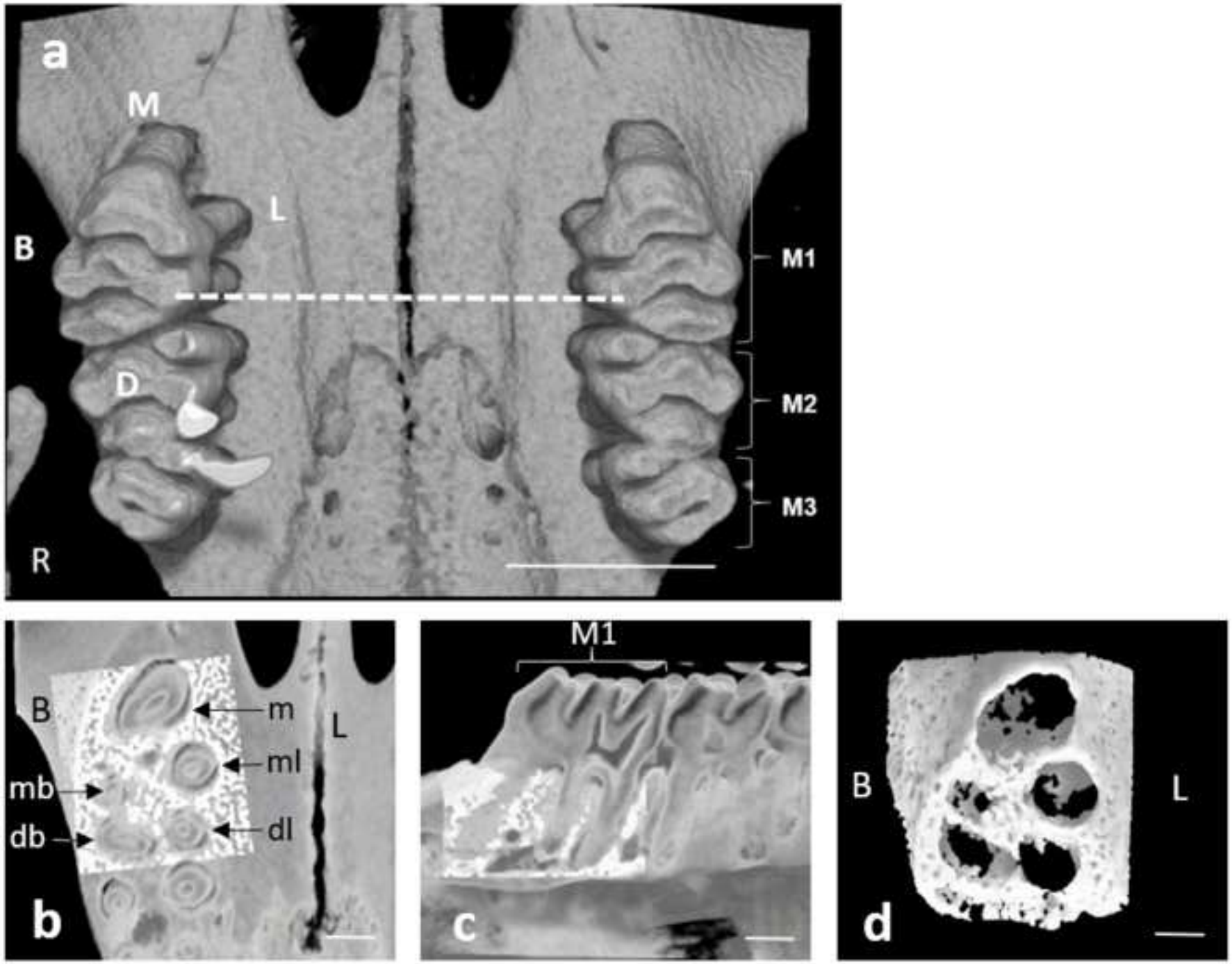

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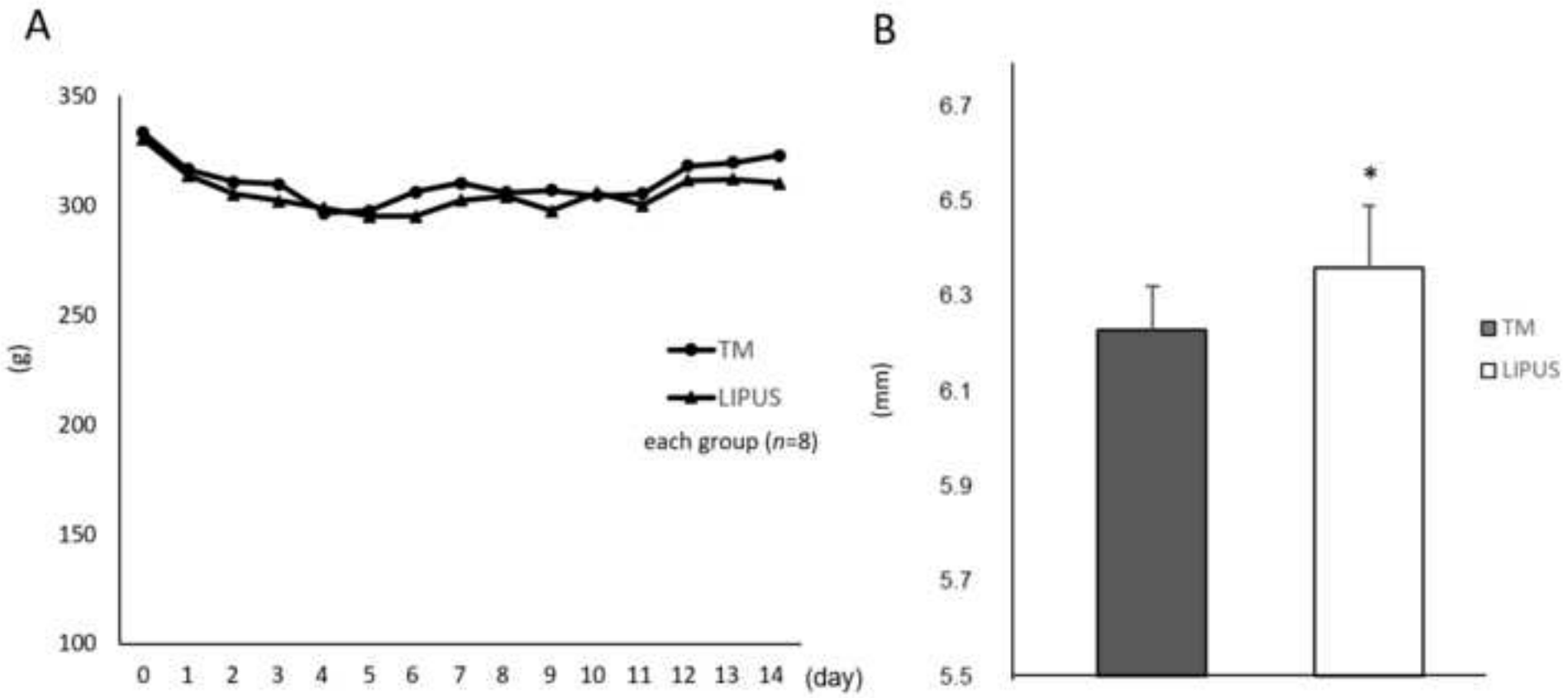

Figure 4

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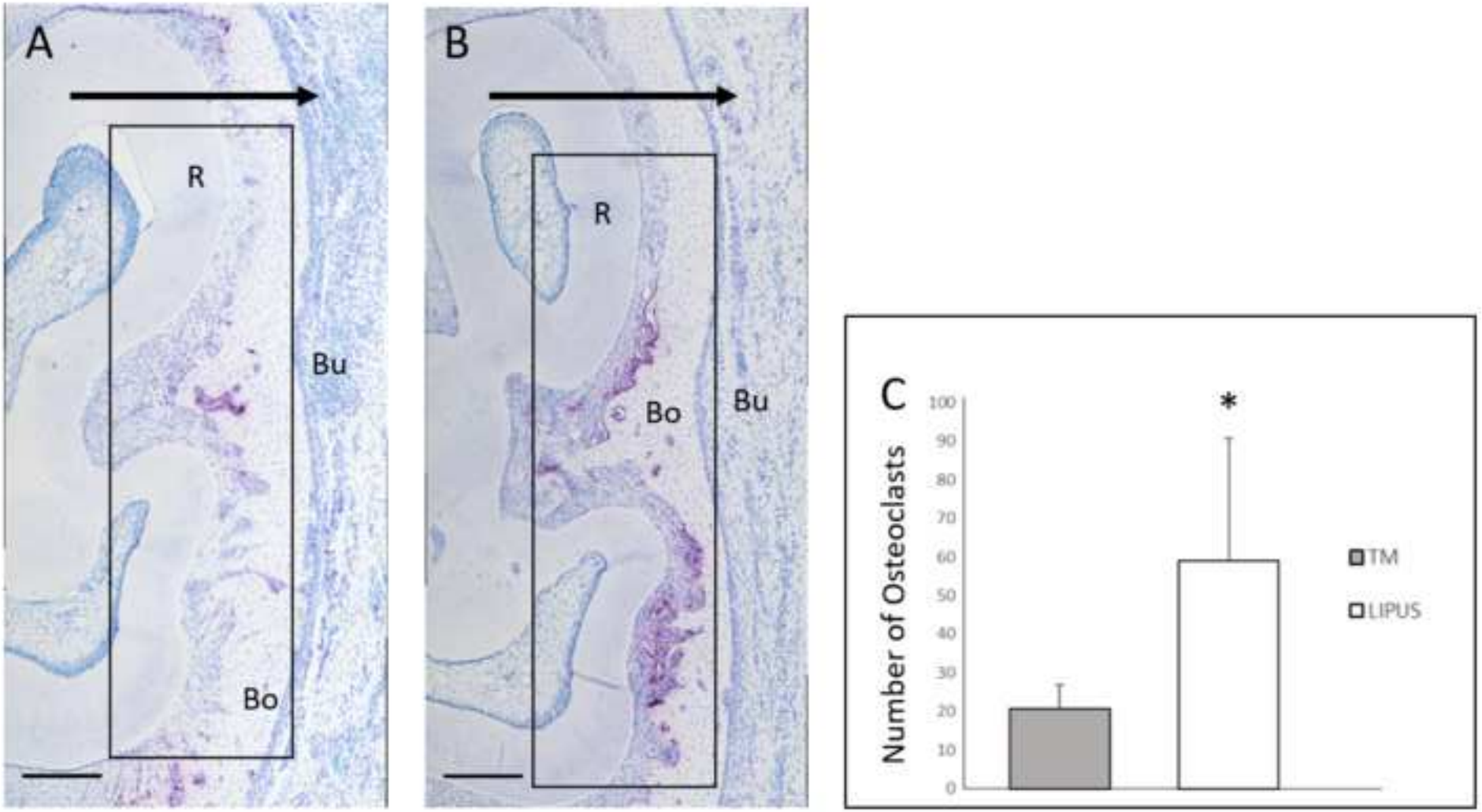

Figure 5

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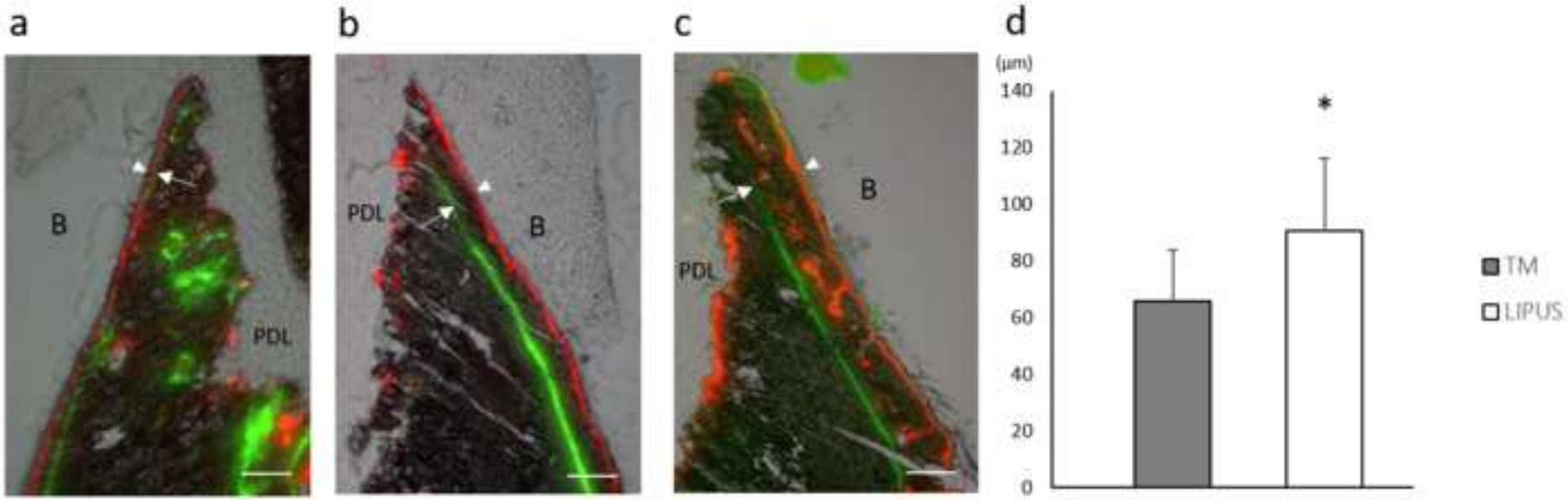

Figure 6

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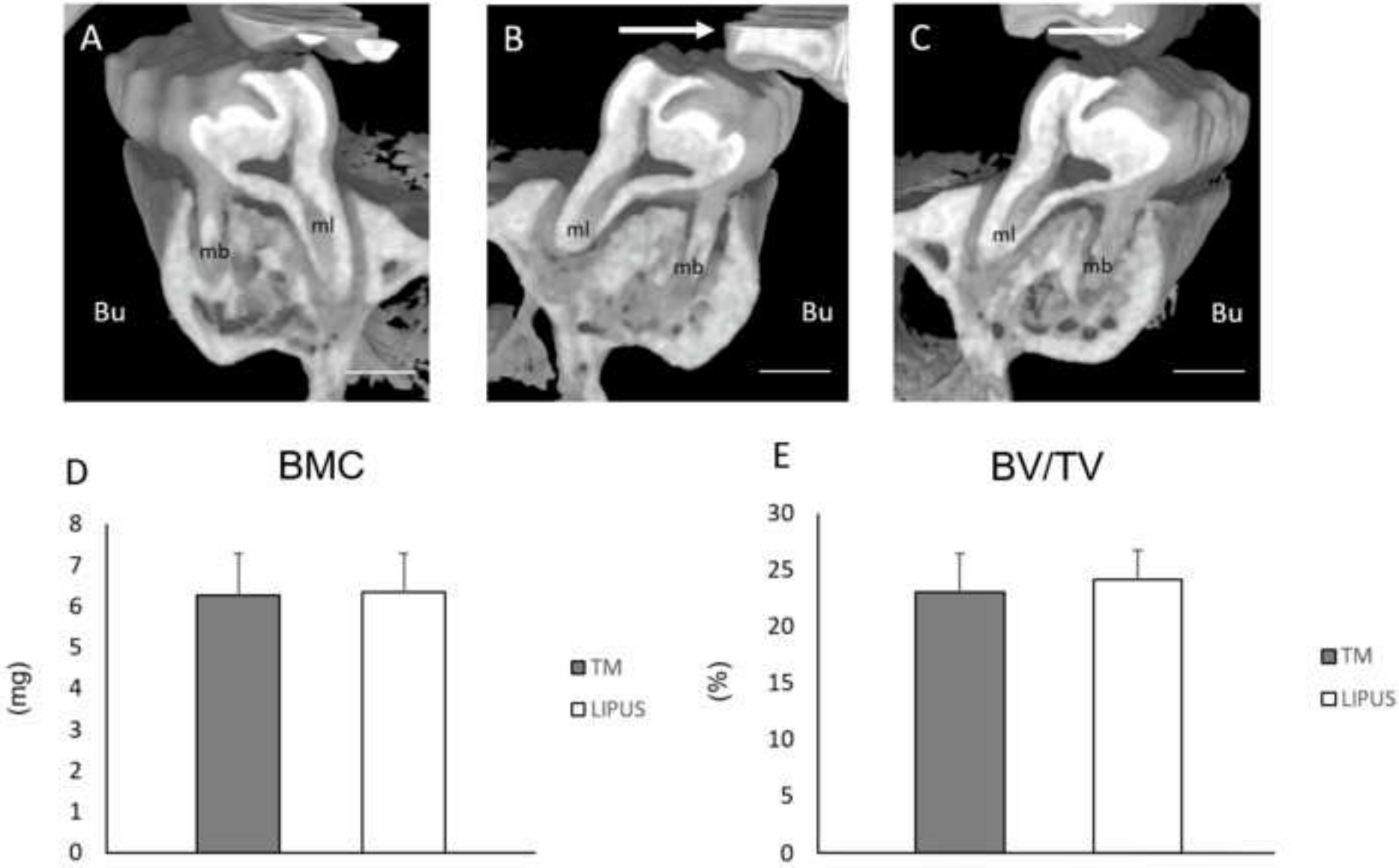

Table

Table Descriptive statistics of the data.

	Tooth Movement without LIPUS		Tooth Movement with LIPUS	
	Mean±S.D. (95% CI)	Median(25th-75th percentile)	Mean±S.D. (95% CI)	Median(25th-75th percentile)
Intermolar width (mm)	6.23 ± 0.09 (6.16 - 6.31)	6.24 (6.14 - 6.25)	6.36 ± 0.13 (6.15 - 6.55)	6.36 ± (6.26-6.49)
Number of Osteoclasts	20.73 ± 6.23 (13.00 - 28.46)	21.50 (20.33 - 25.00)	59.03 ± 31.66 (19.72 - 98.35)	69.25 ± (29.67-85.67)
Alveolar Bone Formation(mm)	65.90 ±18.00 (49.26 - 82.55)	77.50 (47.17 - 81.33)	90.74 ± 25.55 (67.10 - 114.37)	85.83 ± (75.50-99.50)
BMC (mg)	6.26 ± 1.03 (5.40 - 7.12)	6.67 (5.31 - 7.02)	6.35 ± 0.94 (5.56 - 7.14)	6.07 ± (5.66-7.29)