

**Original article: Histomorphometric analysis of overloading on the palatal tooth movement into the maxillary sinus**

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Author's contribution

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1. When mechanical stress was applied to molars, osteogenesis on the sinus wall was induced.
2. Root penetration into sinus and bone height reduction does not occur in palatal tooth movement.
3. Excessive loading will not accelerate tooth movement to the sinus but induce more root resorption.

## **Abstract**

**Objectives:** To evaluate the effect of overloading on the palatal movement of the maxillary molar.

**Materials and methods:** The maxillary first molar of male C57Bl/6 mice was moved palatally with 10-g or 30-g load for 14 days, and amount of tooth movement was longitudinally measured on micro-computed tomography images (each group, N=5). Bone remodeling around the molar root under the 30-g load was evaluated at days 3, 5, 7, and 14 after the starting of tooth movement using histomorphometry and immunodetection of bone-restricted Ifitm (interferon inducible transmembrane)-like protein, a novel marker of active bone formation (each group, N=5).

**Result:** In the 10-g load group, the amount of tooth movement increased dramatically between day 5 to day 7, and gradually increased thereafter. In 30-g load group, tooth movement at day 5 and day 7 was significantly lower than those in the 10-g load group; however, total tooth movement for 14 days was similar in both groups. Orthodontic load of 30-g stimulated bone formation on the sinus wall, but bone resorption on the periodontal ligament side was delayed because of the hyalinization, which means strong force application was not accelerate tooth movement. Moreover, some root resorption was induced under the excessive force application.

**Conclusion:** Root penetration into sinus and bone height reduction does not occur because new bone formation on the maxillary sinus is induced prior to bone resorption on the periodontal side, even though excessive orthodontic force is applied. However, excessive force is subject to induce root resorption.

## Introduction

Contemporary orthodontics can provide 3-dimensional tooth movement to treat various malocclusions. However, it is generally believed that orthodontic tooth movement can only be achieved when healthy periodontal tissues and sufficient bony support are present in the direction where the teeth will be moved. Therefore, tooth displacement in bone-deficient areas such as the maxillary sinus and the atrophic alveolar ridge is considered a major limitation in clinical orthodontics.<sup>1</sup>

As for the tooth movement through the maxillary sinus, Wehrbein et al.<sup>2,3</sup> pointed out the possibility of reducing the alveolar bone height and/or the root length in their dog experiment and human biopsy study. They suggested that the differentiation of osteoblasts required for compensatory subperiosteal bone apposition may be impaired by the structure and the specific metabolic condition of the mucosa in the maxillary sinus. In contrast to this common assumption, several reports have demonstrated that a tooth can be moved through the maxillary sinus while maintaining pulp vitality and bone support and exhibiting normal width of the periodontal ligament (PDL).<sup>4,5</sup>

These conflicting views should be seriously discussed in these days because of the emergence of implant-anchored orthodontics. In this decade, development of temporary skeletal anchorage devices (TADs) dramatically changed treatment strategies in clinical orthodontics. TADs provide absolute anchorage and enable tooth movement considered to be difficult by conventional mechanics.<sup>6,7</sup> Especially, absolute molar intrusion and group distalization of the dentition are considered as leading edge innovations.<sup>7-10</sup> These tooth

movements might increase the chance for root proximity or penetration into the maxillary sinus.

Recently, we evaluated aspect of tissue remodeling under palatal tooth movement using a mice experimental tooth movement (ETM) model, and revealed that the maxillary molar could be moved into the sinus safely owing to osteogenesis in the sinus induced prior to bone resorption on the periodontal ligament side.<sup>11</sup> On the contrary, amount of force application on teeth is suggested to be closely related with the safety tooth movement.<sup>12, 13</sup> Optimal force is affected by a lot of factors such as root morphology, type of tooth movement, the periodontal membrane area and alveolar bone in the tooth movement direction.<sup>13</sup> Clinicians have to be aware of a possibility to apply excessive orthodontic force on translatory tooth movement into the sinus unintentionally and know what happens after the overloading histologically. It is generally accepted in orthodontists that heavy forces produced significantly more root resorption than light forces or controls.<sup>14-16</sup> Therefore, there is a possibility that overloading might prevent the tooth movement and induce destruction of the periodontal tissues. However, few reports evaluate the effects of overloading in the palatal tooth movement which moves roots into the sinus, in detail.

In the present study, we have exploited a well-established ETM model to evaluate the effect of overloading on the palatal movement of the maxillary molar. Manner of tooth movement was evaluated by micro-computed tomography (CT), and histology of periodontal tissues on the compression area in the maxillary sinus using immunolocalized bone-restricted Ifitm-like protein (Bril), an

osteoblast-specific membrane protein associated with active bone formation.<sup>17</sup>

## **Material and methods**

### **Animal experimental procedures**

Thirty male C57Bl/6 mice (CLEA Japan, Tokyo, Japan) weighing  $25 \pm 5$  g were anesthetized with an intraperitoneal injection of 1 mg/g pentobarbital (Somnopenyl; Kyoritsu Seiyaku, Tokyo, Japan). Nickel-Titanium alloy wires, 0.012 and 0.016 inches in diameter, were fixed to the maxillary incisor by means of composite resin for orthodontic bonding (Beauty Orthobond; Shofu, Kyoto, Japan), to achieve loads of 10-g and 30-g, respectively, as shown in Fig. 1, and move the left maxillary first molar toward the palatal side.<sup>18</sup> Ten animals were served to measure tooth movement in micro-CT analysis, and other 20 were for histology. All appliances were retained until the time to sacrifice. The untreated contralateral tooth served as control. Mice were kept at a constant ambient temperature (22-24°C) under a constant day-night rhythm and fed on a powder diet ad libitum.

After 24 h of force application and before one day of sacrifice, mice for histology were intraperitoneally injected with 0.1 ml calcein (2.5 g/l) (Wako pure chemical industries, Tokyo, Japan) solution in 2% sodium hydrogen carbonate for fluorochrome labels. The experimental protocol described below was approved by the Ethical Committee of Tokushima University (Permit number: 11113).

### **Measurement of tooth movement**

At the starting of tooth movement (day 0) and 3, 5, 7, 11 and 14 days after force application (10-g or 30-g), micro-CT (LCT-200, Hitachi Aloka Medical, Tokyo, Japan) images of maxillae were taken under inhalation anesthesia with isoflurane (Forane, Abbott, Tokyo, Japan) and 100% oxygen. The tube voltage was set at 80 kV and the current was constant at 0.5 mA. Mice were scanned in a 48 mm wide specimen holder with a resolution of 24 x 24  $\mu\text{m}^2$  pixel size. The data were saved in Digital Imaging and Communications in Medicine (DICOM) format and the 3-dimensional reconstruction models were generated using a 3-dimensional visualization and simulation software (ZedView version 6.0, LEXI, Tokyo, Japan). On the 3-dimensional models, the distance between mesiolingual cusps of the maxillary first molars was measured (Fig. 2A, B). The inter-molar width before tooth movement was used as a baseline. Each group for this analysis consisted of five mice.

### **Tissue Processing**

At 3, 5, 7, and 14 days after the start of tooth movement, animals were anesthetized with 1 mg/g pentobarbital, and sacrificed by perfusion through the left ventricle with phosphate buffered saline (PBS, Mitsubishi chemical medience, Tokyo, Japan) for 30 sec, followed by a fixative solution consisting of 4% paraformaldehyde (Wako pure chemical industries, Tokyo, Japan) and 0.1% glutaraldehyde (Katayama chemical, Osaka, Japan) in 0.08 M sodium cacodylate (Wako pure chemical Industries) buffer containing 0.05% calcium chloride (Junsei chemical, Tokyo, Japan), pH 7.2, for 20 min. Maxillae were dissected, and specimens were immersed in the same fixative solution

overnight at 4°C and embedded into methyl methacrylate resin (Technovit 9100, Kulzer, Wehrheim, Germany). The resin blocks were sectioned at 5 µm thickness. Residual specimens were decalcified with solution consisting 5.4% formic acid (Kanto chemical, Tokyo, Japan) and 0.4 M containing trisodium citrate dehydrate (Wako pure chemical industries) for 7 days at 4°C. Decalcified samples were washed for 24 hrs in 0.1 M sodium cacodylate buffer, pH 7.2, processed for paraffin embedding, and sectioned at 5 µm thickness. For morphological observations, sections were stained with hematoxylin and eosin.

### **Immunohistochemistry**

To evaluate bone activity, we have immunolocalized an osteoblast-specific membrane protein, Bril.<sup>17</sup> Sections were deparaffinized with xylene, rehydrated through a decreasing ethanol series, and washed in distilled water. In order to avoid nonspecific sticking, sections were blocked with 0.01 M PBS, pH 7.2, containing 5% skim milk for 30 min at room temperature. After blocking, sections were incubated with an affinity-purified rabbit primary antibody raised against Bril (1:5000, 3 hrs, room temperature). Sections were washed with PBS containing 0.05% (v/v) Tween 20 (Bio-rad laboratories, Hercules, CA), pH 7.4 (0.01 M PBS-Tween 20), followed by treatment with the Dako Envision TM+ System, HRP-labeled polymer anti-rabbit kit (Dako, Glostrup, Denmark) as recommended by the manufacturer. Visualization was performed with 3, 3'-diaminobenzidine, and sections were counterstained with 0.5% methyl green (Dako).

## **Histomorphometry**

Histological examination focused on the bone and PDL surrounding the mesial root of the maxillary first molar at the palatal side. Each group for histomorphometry consisted of five mice. The area located about 100  $\mu\text{m}$  from the palate level was evaluated per animal. Newly formed bone was defined as the distance between two calcein lines under a fluorescence microscope (BZ-9000; KEYENCE, Osaka, Japan) (Fig. 3). The width of PDL, the total thickness of bone, and the newly formed bone were measured with an image-editing software (ImageJ, U. S. National Institutes of Health, Bethesda, MD) (Fig. 1C).

## **Statistical analysis**

Homoscedasticity of variance was confirmed by F test and unpaired-t test was used to compare the amount of tooth movement between the 10-g and 30-g load groups. The significance of differences in histomorphometric measurements was analyzed by one-way analysis of variance and the Tukey–Kramer test. Probability levels of  $P < 0.05$  were considered statistically significant.

## **Results**

### **Measurement of tooth movement**

Movement was observed immediately after the start of ETM. In the 10-g load group, the amount of tooth movement increased dramatically between day

5 to day 7, and gradually increased thereafter (Fig. 2C). On the other hand, tooth movement at day 5 and day 7 in the 30-g load group was significantly lower than those in the 10-g load group ( $P < 0.05$ ). Total tooth movement during 14 days was similar in both groups, that is approximately 65  $\mu\text{m}$  (Fig. 2C).

### **Bone response under 30-g load**

When 30-g load was applied, hyalinization of the PDL was observed and its width decreased significantly ( $23.7 \pm 3.5 \mu\text{m}$ , and  $33.3 \pm 4.2 \mu\text{m}$ ) at day 3 and day 5 as compared to the contralateral side, but increased thereafter (Figs. 4A, B, 5A). Bone formation was evident on the maxillary sinus from day 3 to day 7 (Figs. 4A-C, 5B). At day 7, bone resorption by osteoclasts had started on the PDL side (Fig. 4C). As a result, the thickness of the sinus wall was temporarily increased from day 5 to day 7 (Fig. 5C). At day 14, there was still evidence of bone formation on the PDL side, and odontoclasts were observed on the surface of the root at the compression side (Fig. 4D). New bone formation on the sinus wall was gradually induced (Fig. 5C).

At days 3, 5, and 7, Bril was immunodetected in active osteoblasts located on the surface of the maxillary sinus (Figs. 4F-H). At day 14, Bril expression was also spread over the surface of bone on the PDL side, which resembled the one seen in control side (Figs. 4I, J), confirming the histological observations.

## Discussion

In the previous study, we found that when mechanical stress was appropriately applied to molars, osteogenesis on the sinus wall was induced ahead of bone resorption on the PDL side, and the bone thickness of the sinus wall was consistent throughout the period of palatal tooth movement.<sup>11</sup> In the present study; however, strong force application stimulated bone formation on the sinus wall but bone resorption on the PDL side was delayed. The resulting temporary increase in total thickness of the sinus wall essentially indicates that strong force application will not accelerate tooth movement. Moreover, some root resorption was induced under the excessive force application.

We have used Bril as a marker of bone formation. Bril is a novel osteoblast-specific membrane protein which was identified from a UMR106 rat osteosarcoma cell line library.<sup>12</sup> Screening of cell lines showed high expression of Bril in osteoblasts during onset of matrix mineralization, and the protein was immunodetected in developing bones.<sup>19</sup> Recent studies revealed that mutations in Bril cause type V osteogenesis imperfecta, which evidence the close relationship between Bril and osteogenesis.<sup>20, 21</sup> The specific expression of Bril at active bone formation sites in the present study also demonstrates its usefulness for evaluation of osteogenesis.

There is no agreement in the literature regarding the amount of optimal load for ETM in mice. Pavlin et al.<sup>22</sup> concluded that an average orthodontic force of 10-12-g provided a predictable mesial tipping movement of the first molar. Taddei et al.<sup>23</sup> suggested that 35-g was the ideal force for mesial molar movement in mice with no side effects. As for a palatal tooth movement, Sakai et

al.<sup>18</sup> moved the maxillary first molar in mice with a 10-g load and observed no hyalinized tissue nor root resorption. In our previous study, under a 10-g load, bone formation on the surface of the maxillary sinus was evoked by transduction of mechanical stress applied to a tooth.<sup>11</sup> As osteogenesis on the sinus wall was induced ahead of bone resorption on the PDL side, the sinus maintains its bone thickness during palatal tooth movement. No hyalinization of the PDL or accentuated root resorption was observed. These results suggested that a 10-g load was appropriate for our palatal tooth movement model. Based on these studies, we have applied 10-g and 30-g loads in the present study to evaluate the effect of overloading on the tooth movement toward the sinus.

In histological observations, hyalinization of the PDL was readily evident under the 30-g load. An orthodontic force of 30-g is regarded as overloading in this mouse ETM model, since this degenerative change is caused by excessive mechanical stress to the periodontal tissue.<sup>24</sup> Histomorphometric analysis aimed at identifying suitable orthodontic forces in an ETM model has been more often carried out in rats than in mice. In a rat ETM model for mesial movement of the maxillary first molar, King et al.<sup>25</sup> indicated that a loading of 30-40-g was suitable and Brudvik and Rygh<sup>26</sup> reported hyalinization and root resorption of molar roots under 70-75-g initial orthodontic load. Considering the size of the mouse dentition, we believe our results are in agreement with the findings from the rat model even though the molar was moved palatally.

The tooth movement in the 30-g load group was significantly suppressed at days 5 and 7, compared with that in the 10-g load group. These results suggest that optimal load is important for efficient tooth movement to the

maxillary sinus. Hyalinization inhibits osteoclast accumulation on the compression side thereby undermining bone resorption and prolonging tooth movement.<sup>27</sup> Von Böhl et al.<sup>28</sup> showed that induction of hyalinization was associated with applied force levels, and also suggested that the development and removal of necrotic tissues was a continuous process during tooth displacement.

New bone formation on the sinus wall was remarkably induced under the 30-g load. When a tooth is moved toward the sinus, the Schneiderian membrane is stimulated by mechanotransduction, which transmits through PDL and the sinus wall, and new bone formation is induced.<sup>11, 29</sup> This phenomenon is considered to be similar to reactive bone formation on the periosteum under mechanical stress.<sup>30</sup> Churches and Howlett<sup>31</sup> reported that the amount of bone formation was proportional to the force applied. However, bone resorption on the PDL side was delayed because of PDL hyalinization. As a result, the thickness of sinus wall was temporarily increased from day 5 to day 7 after ETM under the 30-g load. Interestingly, the total distance of palatal molar movement during 14 days was similar in 10- and 30-g load groups. These results indicate that overloading does not accelerate palatal tooth movement.

In the present study, odontoclasts and their resorption lacunae were observed on molar roots under the 30-g load at 14 days after ETM. Orthodontic overloading tended to induce undesirable root resorption during tooth movement.<sup>12-16, 22, 26</sup> Wehrbein et al.<sup>3</sup> reported in their observation of human autopsy that root resorption occurred in the basal cortical bone of the maxillary sinus after translatory tooth movements. It might be possible that this results

from overloading caused by orthodontic appliances.

Orthodontic tooth movements such as intrusion or passing through the roots into the maxillary sinus, become possible when TADs are used as absolute anchorage. The present study provides histological evidence that the sinus wall is a dynamic structure that responds favorably to mechanical stress, alleviating concerns about tooth movement into the sinus under the adequate orthodontic force. We believe that such safety should ultimately contribute to expanding the limits of orthodontic treatment.

## **Conclusion**

Root penetration into sinus and bone height reduction does not occur in our ETM mouse model because new bone formation on the maxillary sinus is induced ahead of bone resorption on the PDL side, even though excessive orthodontic force is applied. However, excessive force is subject to induce root resorption.

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## Figure legends

Fig. 1: An experimental tooth movement (ETM) model. A superelastic Nickel–Titanium alloy wire (w) was fixed at the maxillary incisors (i) (before activation, A). For palatal tooth movement, the end of wire was moved to buccal side of the right first molar (m) (after activation, B). Black arrow indicated direction of tooth movement. Hematoxylin–eosin staining of the maxillary first molar at day 3 after the start of ETM (C), and enlarged view of the boxed area (D). The white arrow indicates the direction of ETM. The region located about 100  $\mu\text{m}$  (white dotted line) under the palate bone (white solid line) was morphometrically analyzed (C). Width of periodontal ligament (PDL) (black solid line with two-way arrow), total thickness of the sinus wall (black dotted line with two-way arrow) and newly-formed bone on the sinus (white arrows) were measured (D). d dentin, p dental pulp, b-ab buccal alveolar bone, p-ab palatal alveolar bone, MS maxillary sinus.

Fig. 2: Measurement of tooth movement. Micro-computed tomography (CT) image of the maxillae in control group (A) and at day 14 after the start of ETM (B). As the result of continuous force, the left maxillary first molar (red dotted line) was tipped toward the palatal side (red solid line), and the distance between mesiolingual cusps was reduced (yellow arrows). The amount of tooth movement during the ETM (C). Tooth movement increased gradually in both groups, but the amount in the 30-g group was significantly smaller than that in the 10-g group at days 5 and 7 after ETM ( $P < 0.05$ ).

Fig. 3: Fluorochrome bond label of ETM at day 7. Calcein bone labels were shown as green lines (A) and hard tissues were visualized in blue (B). Arrowheads indicate the start of newly bone formation and arrows point out the end of bone formation (C). d dentin, ab alveolar bone, MS maxillary sinus, PDL periodontal ligament.

Fig. 4: Bone remodeling in the 30-g loaded group. Hematoxylin–eosin staining (A-E) and immunolabeling for Bril (F-J). At days 3 and 5 after the ETM, hyalinization of the periodontal ligament (PDL) was observed and its width decreased greatly (white arrows). White arrowheads point the reversal line. Expression of Bril was noted in bone in the maxillary sinus side (black arrowheads) and on the PDL side (black arrows). Root resorption by odontoclasts was observed at day 14. ab alveolar bone, d dentin.

Fig. 5: Linear measurements of the periodontal tissue during the ETM. The PDL width decreased at day 3 but gradually increased thereafter as compared to the contralateral side (A). The amount of newly formed bone on the sinus wall increased significantly between the day 3 and day 5 (B). As a result, the total thickness of the sinus wall temporarily increased at day 5 and day 7 (C). \*P<0.05.

**Figure 1**  
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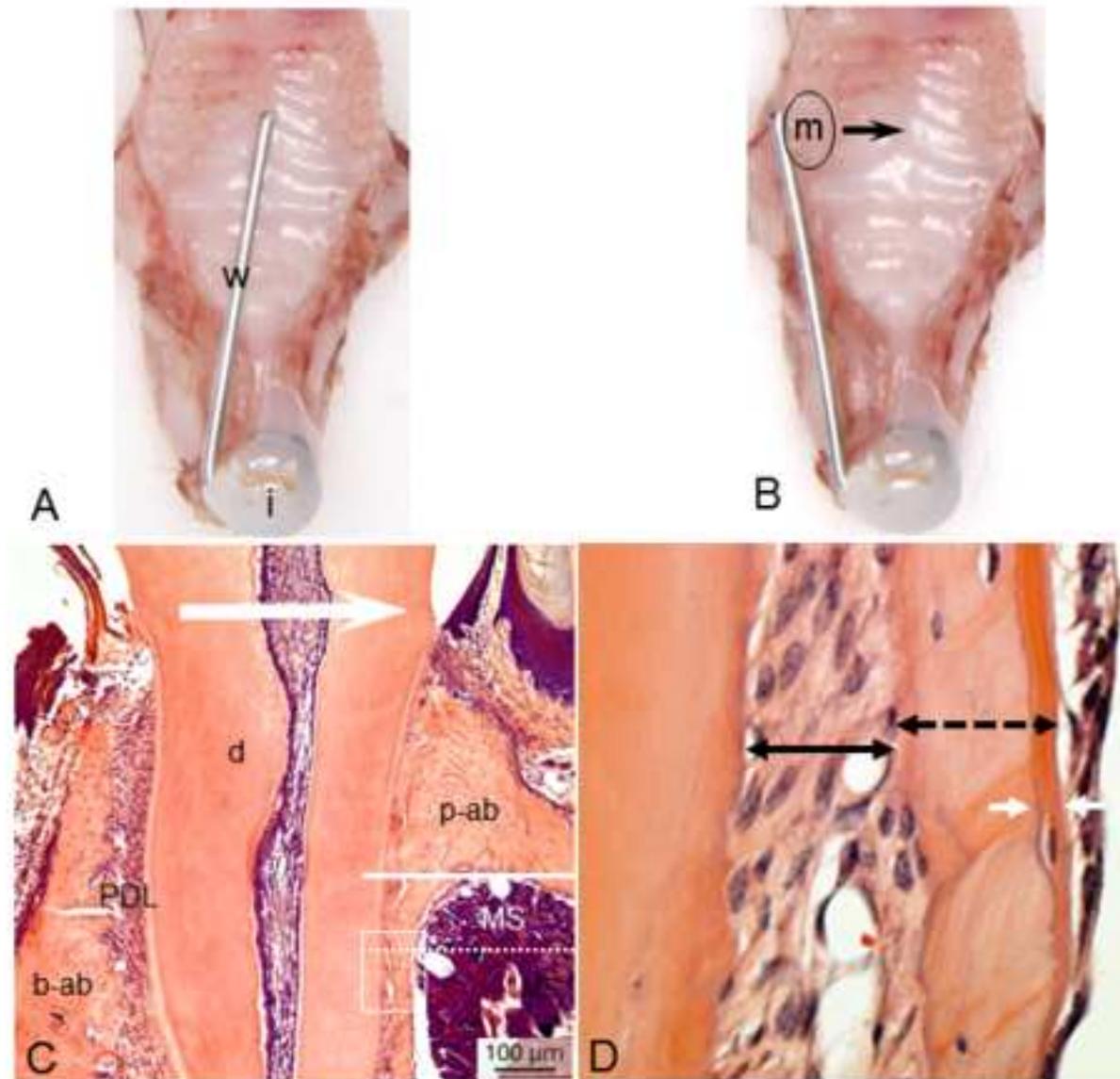
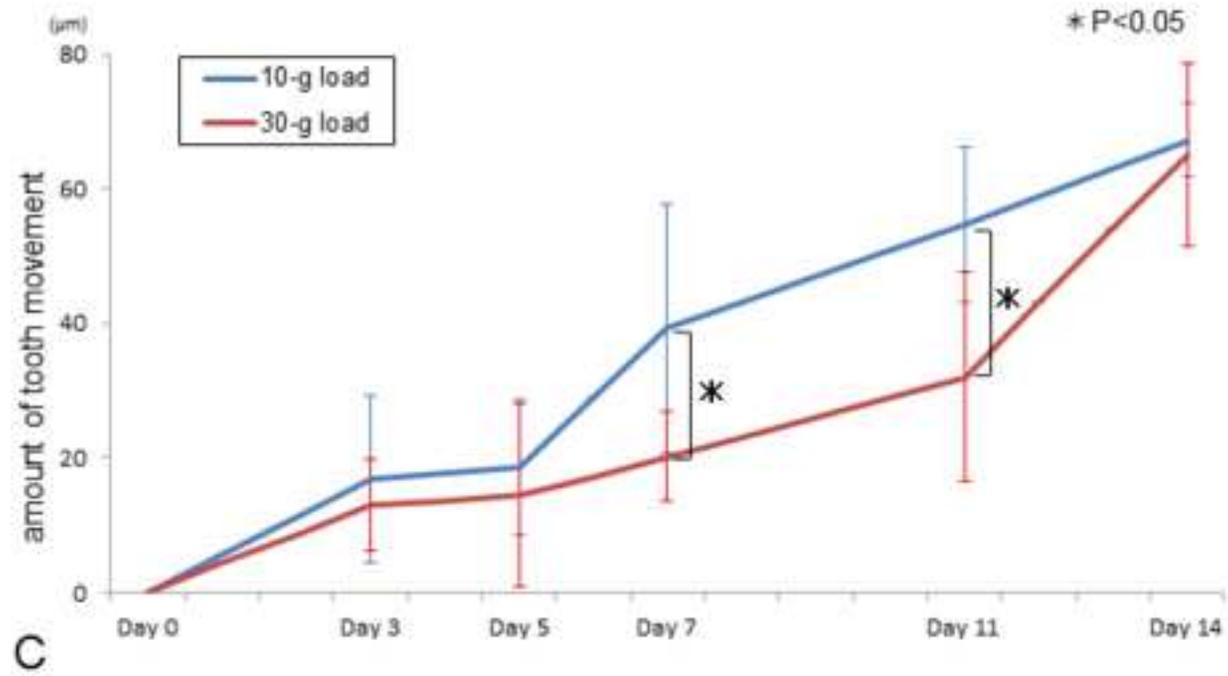
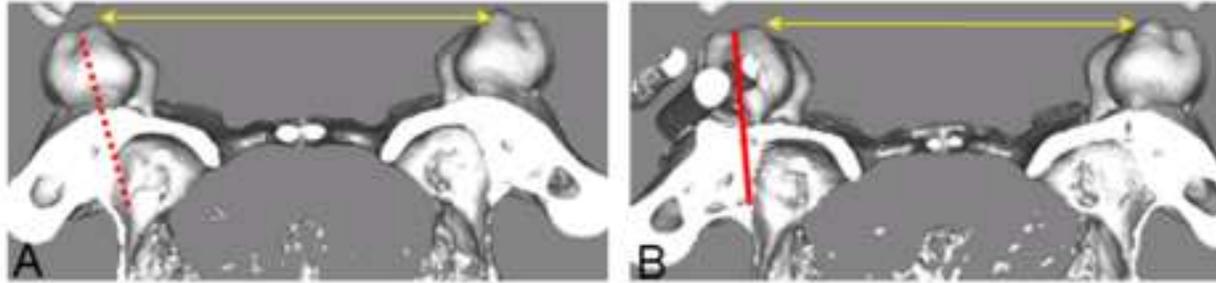
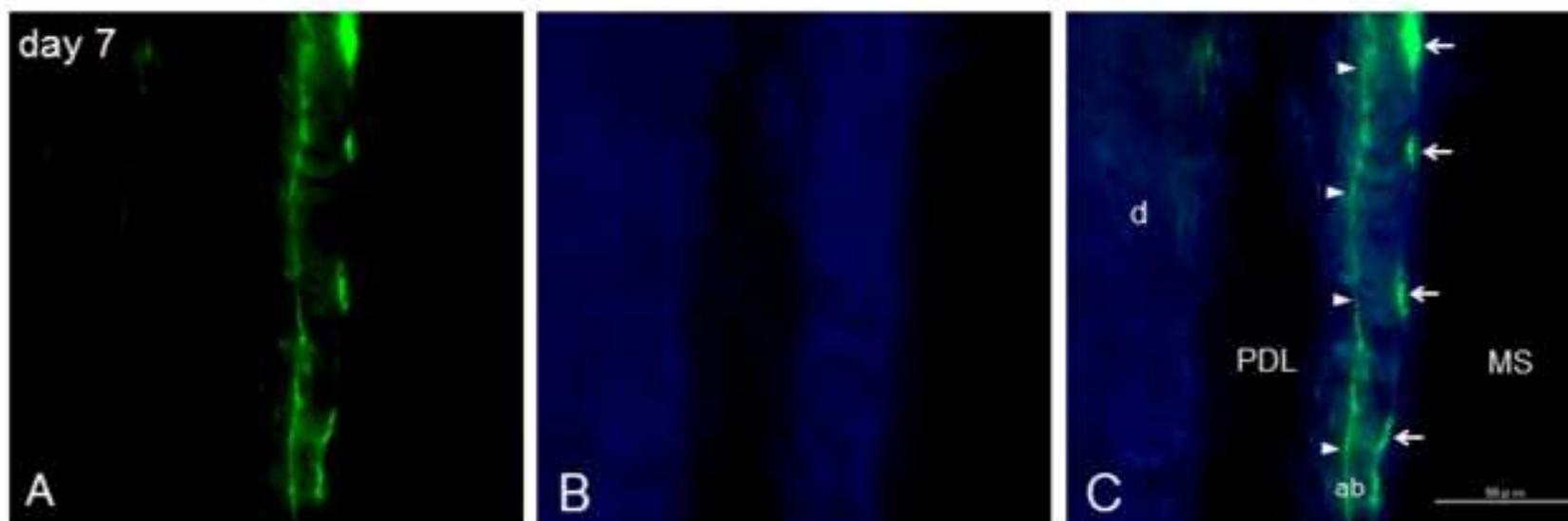


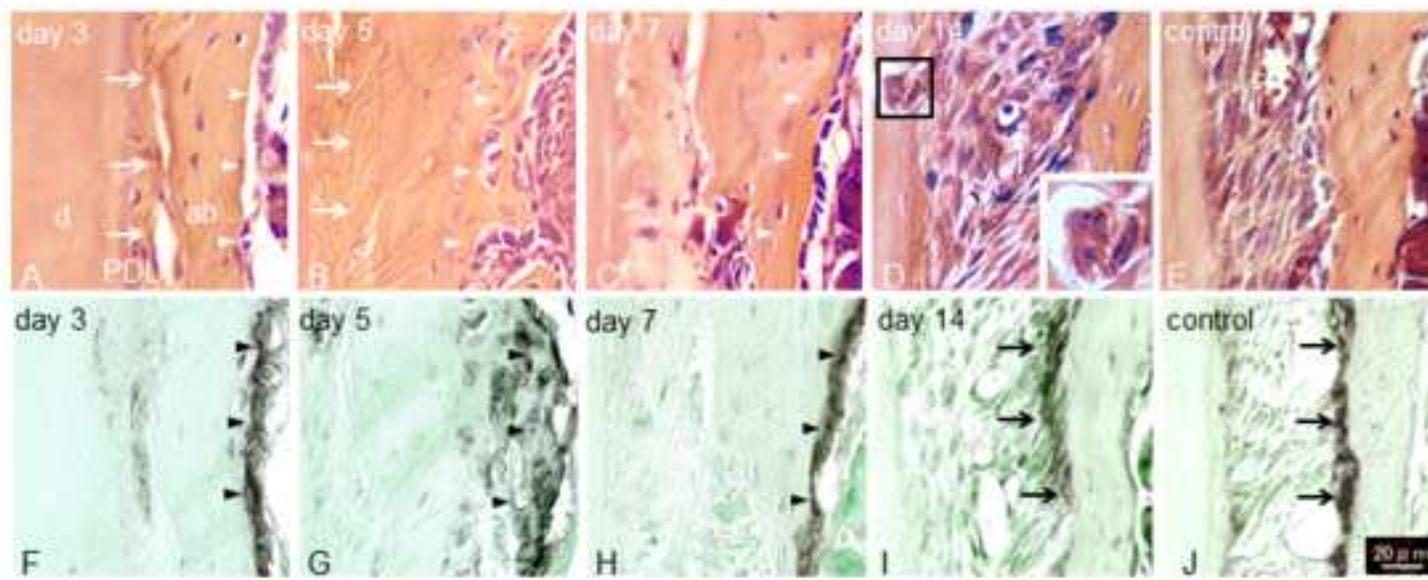
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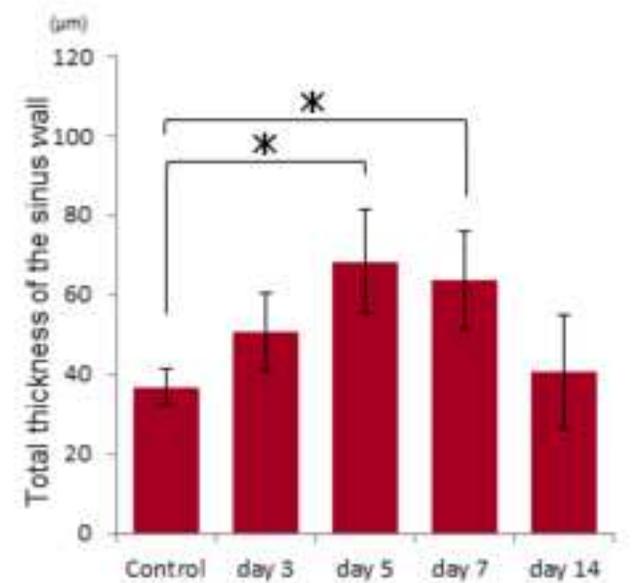
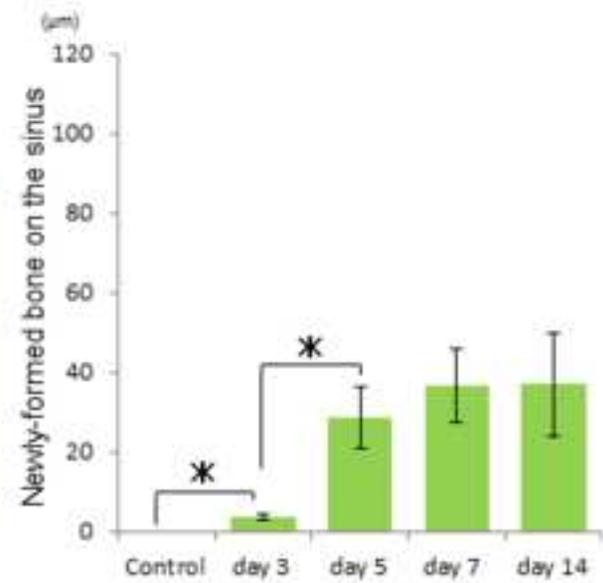
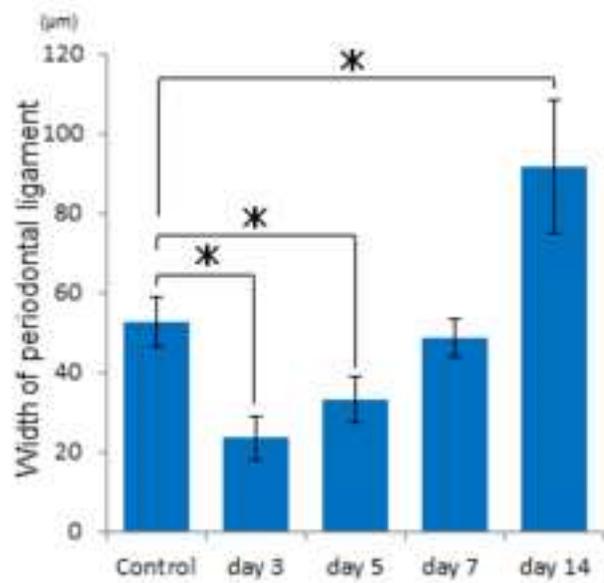
**Figure 3**  
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**Figure 4**  
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**Figure 5**  
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\* P<0.05